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Progress on musculoskeletal disorders and stem cell therapies

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Progress on musculoskeletal disorders and stem cell therapies

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Table of contents

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Editorial: Progress on musculoskeletal disorders and stem cell therapies

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Editorial on the Research Topic
Progress on musculoskeletal disorders and stem cell therapies

Accompanying the aging of the population worldwide, growing concern emerges about the health and wellbeing of elderly with chronic diseases. Musculoskeletal disorders, the degenerative diseases of the musculoskeletal system, including bone, tendon, skeletal muscle, cartilage and intervertebral disc, are the leading contributors to years lived with disability worldwide, affecting people of all ages but the prevalence peaks in aged population. Musculoskeletal disorders compromise the function of bone and joints, and require extensive care and long recovery time. Musculoskeletal disorders have negative impact on the health quality of life, causing mental and physical stress to people as well as loss of work hours. Consequently, musculoskeletal disorders cause a heavy burden on patients and the whole society.

The underlying molecular mechanisms have not been fully elucidated for musculoskeletal disorders. Understanding the key factors and signaling involved in this process may greatly facilitate targeted repair and future drug development. In this Research Topic, Xiongfeng et al. reported a novel phantom-less quantitative computed tomography system, which can predict osteoporosis with relatively high accuracy and precision utilizing low-dose chest computed tomography obtained for COVID-19 screening. Zhang et al. summarized the role of adipokine signaling in osteoarthritis, which is a degenerative disease of cartilage.

Stem cells, due to their high self renewal capacity and differentiation potential, have been highlighted as a promising cell tool for tissue regeneration and engineering in musculoskeletal disorders. Mesenchymal stem cells (MSCs) (Lv et al., 2012; Lv et al., 2014) as the progenitors for mesenchymal lineages have been intensively investigated for musculoskeletal tissue regeneration (Leung et al., 2014; Chen et al., 2022; Wang et al., 2022). Other types of stem cells, such as tissue-specific progenitor cells (Lyu et al., 2019) and induced pluripotent stem cells, also received attention for their potential for tissue regeneration. In this Research Topic, Kragl et al. investigated the role of HSD11B1 in the differentiation of mesenchymal stem cells (MSCs) into adipocytes and osteoblasts, and found that HSD11B1 could increase the cortisol expression of MSCs and switch MSCs from osteogenic to adipogenic differentiation. Wang et al. discussed the recent advances in stem

cell therapies for rotator cuff injuries, including bone marrow-derived MSCs, adipose-derived stem cells, tendon-derived stem cells, umbilical cord-derived MSCs, subacromial bursa-derived cells, and urine-derived stem cells. Campbell et al. discussed the ideal stem cell population for cartilage regeneration, including endogenous stem cells from cartilage, stem cell-rich dental pulp, or the adolescent growth plate, as well as MSCs from bone marrow, adipose tissue or umbilical cord, *etc.*

Stem cell-derived exosomes have independently attracted research attention for its role in targeting musculoskeletal disorders. Here, Ma et al. summarized the action mechanism of stem cell exosomes on aseptic loosening of joint prostheses, with the effects including augmenting angiogenesis, enhancing osteogenesis, suppressing osteoclast activity, and regulating immune cells and cytokines. Yuan et al. discussed the current status of exosome-based therapeutic strategy in temporomandibular joint osteoarthritis treatment, and compared the exosomes from MSCs, chondrocytes, synoviocytes, subchondral osteocytes, adipose tissue and other tissues in the expression of cell surface receptors, different contents and biological effects. They also discussed future opportunities and challenges of exosome-based treatment in temporomandibular joint osteoarthritis.

The microenvironment surrounding stem cells, including acidity, oxygen level, nutrient supply, osmolarity, etc., has non-negligible influence on the biological behavior and ultimate fate of stem cells (Huang et al., 2020). Manipulation of the local microenvironment may further enhance the regenerative effect of stem cells and benefit musculoskeletal tissue repair (Lyu, 2022). In this Research Topic, Chu et al. summarized the impact of microenvironment in stem cell-based regeneration of intervertebral disc (IVD). They discussed the recent advances on the presence of endogenous stem cells in the IVD, reviewed the impact of the microenvironment similar to IVD on the characteristics and function of MSC, summarized the current progress of IVD graft substitutes, and updated the current use of MSC transplantation for IVD diseases. Wang et al. found that the resistance of human nucleus pulposus-derived MSCs to severe acidity environment can be enhanced by Sa12b, a wasp peptide that can inhibit acid-sensitive ion channels, as demonstrated by reduced cell apoptosis, enhanced cell proliferation, chondrocyte marker expression, and stemness marker expression.

In addition to stem cell therapies, other therapies, either using various reagents or biomaterials, physiological stimulation or genetic modification, are also in the scope to regenerate damaged musculoskeletal tissue. In this Research Topic, Lin et al. reviewed the arthroscopic application of radiofrequency to treat articular cartilage lesions. They reviewed the history of radiofrequency and its application in orthopedic arthroscopy, and the underlying mechanism for the repair, in addition to the controlling factors, such as power and temperature, in ensuring the safety and effectiveness of radiofrequency therapy. Xu et al. reported the application of cell-free fat extract to prevents tail suspension–induced bone loss by inhibiting osteocyte apoptosis. Liang et al. discussed the recent progress in gene targeted therapeutic strategies in Duchenne Muscular Dystrophy.

In summary, this Research Topic highlights recent advances on the research of musculoskeletal disorders, including the degenerative mechanisms and developments of various repair strategies, including stem cell related therapies, biomaterials and others. We hope that this Research Topic will add new strength to the scientific community and contribute to future collaborations among research groups across the world.

Author contributions

F-JL drafted the manuscript. SP and JL revised the manuscript. All authors approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
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The Arthroscopic Application of Radiofrequency in Treatment of Articular Cartilage Lesions

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Articular cartilage lesion is a common disease to be treated by arthroscopic surgery. It will eventually progress to osteoarthritis without proper management, which can affect patients' work and daily life seriously. Although mechanical debridement and laser have been used clinically for its treatment, due to their respective drawbacks, radiofrequency has drawn increasing attention from clinicians as a new technique with more advantages. However, the safety and efficacy of radiofrequency have also been questioned. In this article, the scope of application of radiofrequency was reviewed following an introduction of its development history and mechanism, and the methods to ensure the safety and effectiveness of radiofrequency through power and temperature control were summarized.

Keywords: cartilage lesions, radiofrequency, articular cartilage, thermal energy, chondroplasty

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INTRODUCTION

Articular cartilage (AC), a kind of hyaline cartilage covering the articular surface, is a viscoelastic connective tissue composed of chondrocytes and the extracellular matrix (ECM), while ECM is mainly composed of collagen, aggregated proteoglycan and water (Maly et al., 2021). In normal cartilage, the interaction between water, collagen and proteoglycan-rich matrix constitutes the compressive properties and tensile strength of AC. In addition, AC also has excellent wear resistance and lubrication ability (Dutcheshen et al., 2012; Deng et al., 2018).

While participation in sports and daily physical activities, acute impact or chronic injury accumulated by repeated impact of the joint may damage the articular surface and cause AC lesions (Zhang et al., 2019). For example, an earlier study reported full-thickness focal cartilage defects in more than 1/3 of 931 athletes (Flanigan et al., 2010). The cartilage defects play a vital role in cartilage degeneration. Without effective treatment, such degeneration will eventually lead to osteoarthritis (OA) that can affect the patients' work and daily life seriously and represent a significant health and economic burden on society (Mahmoudian et al., 2021). In a recent epidemiological study, OA occurred in 54.4 million (22.7%) adults, and 43.5% of the patients

Abbreviations: AC: articular cartilage; bRFE: bipolar radiofrequency energy; ECM: extracellular matrix; ICRS: International Cartilage Repair Society; IL: interleukin; MMP: matrix metalloproteinase; mRFE: monopolar radiofrequency energy; OA: osteoarthritis; OARSI: Osteoarthritis Research Society International; PAGCL: post-arthroscopic glenohumeral chondrolysis.

reported negative impact on their life due to limitation of motion (Barbour et al., 2017). Unfortunately, cartilage has a limited self-repair ability, due to lack of blood vessels, nerves, and lymph tissue. Therefore, the treatment of articular cartilage lesions is still a great challenge in clinical practice (Buckwalter and Mankin, 1998; Ganguly et al., 2010; Huang et al., 2021; Zhou et al., 2021).

Chondroplasty is an effective surgical option for the treatment of AC lesions (Barber and Iwasko, 2006; Gowd et al., 2019). This technique debrides the fibroblast cartilage in the joint to form a smooth and stable articular surface to avoid further degeneration caused by delamination, fragmentation and fibrillation of the injured AC. Chondroplasty includes mechanical debridement, laser, and radiofrequency chondroplasty (Khan and Dillingham, 2002; Rocco et al., 2016). Traditionally, mechanical debridement has been used to debride and smooth the damaged articular surface, but this method usually removes adjacent normal cartilage while treating focal lesions. In addition, it cannot completely smooth the surface of cartilage, which will cause fibrillation on the cartilage surface, leading to further degeneration (Turner et al., 1998; Spahn et al., 2016). As a thermal energy technology, laser has also been applied to treat AC lesions, but it has raised a great concern from clinicians due to safety and cost considerations (Shellock and Shields, 2000; Wienecke and Lobenhoffer, 2003; Cook et al., 2004; Ganguly et al., 2010). In view of the respective deficiencies of the two techniques aforementioned, radiofrequency chondroplasty has recently become a new hotspot as a safer and more effective option (Khan and Dillingham, 2002). In this article, the development history, parameter control, biological research, clinical research, indications, and complications of radiofrequency were comprehensively reviewed.

MANUSCRIPT FORMATTING

History of Radiofrequency

The Origin and Development of Radiofrequency

In 1891, d'Arsonval et al. reported that radiofrequency waves could increase the temperature of local tissue when passed through, making this thermal energy feasible to be used for clinical application (D'Arsonval, 1891). At the beginning of the 20th century, Cushing et al. used the bovie electric knife to assist in intra-cranial tumor resection, which was a technique using the thermal energy generated by radiofrequency waves to stanch bleeding and remove abnormal tissues (Cushing and Bovie, 1928). In 1975, radiofrequency was applied to treat chronic pain in unilateral limbs (Pawl, 1975). Subsequently, it has been widely used in neurology, oncology, cardiology and other fields (Pawl, 1975; Dickson and Calderwood, 1980; Huang et al., 1987).

Application of Radiofrequency in Orthopedic Arthroscopy

Due to the numerous side effects of laser chondroplasty, there was an urgent need to find an alternative method for the treatment of AC lesions (Whipple et al., 1985; Miller et al., 1989; Lane et al., 1997). The application of radiofrequency under arthroscopy played a significant role in the development of orthopedic surgery. In 1986, Schosheim et al. applied radiofrequency under arthroscopy for the first time to perform meniscectomy on rabbit knee joints (Schosheim and Caspari, 1986). The authors found that radiofrequency treatment caused less damage to soft tissues. Turner et al. compared 6 histological variables of the cartilage treated by mechanical debridement and radiofrequency, and reported that the radiofrequency group achieved more favorable results in all the variables, indicating a superior performance of radiofrequency to mechanical debridement for AC lesions (Turner et al., 1998). Since then, an increasing number of studies have supported that radiofrequency is an effective treatment for AC lesions (Allen et al., 2006; Uthamanthil et al., 2006; Edwards et al., 2007). In recent years, there has been a proliferation of relevant clinical studies. However, while gaining more experience, there are still many problems and controversies to be addressed.

Mechanism of Radiofrequency

Types and Grades of Cartilage Lesions

Acute impact or chronic injury of the joint may both damage the articular surface, cause cartilage degeneration, and lead to biomechanical and histological changes of the cartilage, such as joint pain, dysfunction and joint effusion (Buckwalter, 2002). Cartilage lesions can be divided into three types depending on the depth of the damage, namely, partial-thickness defects, full-thickness defects and osteochondral defects. In partial- or full-thickness defects, the damage is completely confined to the cartilaginous tissue and does not penetrate the subchondral bone (Nukavarapu and Dorcemus, 2013). A commonly-used method for classifying cartilage lesions was described by Outerbridge et al. (Slattery and Kweon, 2018). According to the classification of cartilage lesions observed under arthroscopy by the improved Outerbridge system, Grade I lesions refer to softened cartilage surface only, which usually does not need to be treated; Grade II or III lesions refer to a partial-thickness defect with fissures of the cartilage with a diameter less than (II) or more than (III) 0.5 inches in diameter, which can be treated by mechanical debridement and radiofrequency; Grade IV lesions refer to subchondral bone exposure, which cannot be effectively treated by radiofrequency, mechanical debridement and other interventions, and needs to take further measures, such as autologous chondrocyte implantation, osteochondral allograft transplantation and so on (Osti et al., 2010). However, the Outerbridge grading system does not take into account the depth of the lesion. On the contrary, the classification system of the International Cartilage Repair Society (ICRS) mainly focuses on the lesion depth (Brittberg and Winalski, 2003). The ICRS Grade I lesions are only superficial, such as soft indentation or superficial fissures and cracks. The ICRS Grade II lesions have extended to less than half of the cartilage depth, while the ICRS Grade III lesions have extended to half or more of the cartilage depth but not yet into the subchondral bone. The ICRS Grade IV lesions are osteochondral lesions. In addition, some other classification systems focus on histopathology, such as the Osteoarthritis Research Society International (OARSI) system (Pritzker et al., 2006). The OARSI system was classified as 1) uneven but intact chondral surface (OARSI Grade I), 2) surface discontinuity (OARSI Grade II), 3) vertical fissures (OARSI Grade III), 4) erosion (OARSI Grade IV), 5) denudation of the cartilage

(OARSI Grade V), or 6) deformation and osteophytes formation of the joint (OARSI Grade VI).

Biomechanical and Histological Changes

Radiofrequency can debride the damaged cartilage surface to create a smooth cartilage surface and improve the mechanical integrity and function of the treated cartilage (Dutcheshen et al., 2012). Through biomechanical modification of the cartilage, the function of the joint can be restored, the degeneration process can be delayed, and the patients' function and quality of life can be improved. Cook et al. demonstrated that no significant difference in compression stiffness between radiofrequency-treated and untreated AC (Cook et al., 2004). Thus, they believed that radiofrequency had no adverse effect on the biomechanical properties of AC. However, it was obviously not sufficient to judge the biomechanical properties of cartilage only by testing its stiffness. On the other hand, due to the local high temperature caused by the probe and the cartilage defects in the treatment area, the use of radiofrequency might cause secondary damage to the border of the chondromalacic and non-chondromalacic area (Cook et al., 2004). The cartilage defects in the treated area made it impossible to contribute to the bearing capacity of nearby areas. The heat energy would induce the denaturation of collagen, make the collagen shrink, and reduce the immediate and short-term stiffness (Lopez et al., 1998; Edwards et al., 2002a; Uthamanthil et al., 2006). Based on a fiber-reinforced biphasic cartilage model, Dutcheshen et al. inferred that the collagen fibril modulus of the treated cartilage decreased, which might reduce the instantaneous or short-term stiffness (resistance to shear and tension) of AC while increasing the matrix modulus (Dutcheshen et al., 2012). This was beneficial to the restoration of the long-term stiffness (load-bearing capacity) of cartilage.

Laboratory evidence showed that the use of radiofrequency chondroplasty could not only improve mechanical stability but also reduce the release of inflammatory mediators, which might benefit from the reduction of permeability of the cartilage due to the annealing effect of bipolar radiofrequency energy (bRFE) (Uthamanthil et al., 2006; Dutcheshen et al., 2012). Although the decrease in permeability had a potentially positive effect (i.e., it reduced the release of inflammatory mediators), it affected the exchange of nutrients at the same time. Cook et al. pointed out that the matrix metalloproteinase (MMP)-13 immunoreactivity increased after the bRFE therapy, and the long-term increase of the MMP-13 activity in ECM might lead to a negative balance between ECM synthesis and degradation, which would further accelerate the progression of OA (Cook et al., 2004). MMP-1 can cleave native triple-helical collagens at a single bond, whereas MMP-2 plays a non-specific role in the degradation of fibrillar collagens, basement membrane components, and matrix molecules such as fibronectin. Yasura et al. observed that the secretion of MMP-1 and MMP-2 in AC decreased after the use of monopolar radiofrequency energy (mRFE), which was helpful in preventing the further degeneration of AC (Yasura et al., 2006). Enochson et al. revealed that the expressions of interleukin (IL)-6 and IL-8 in the cartilage were up-regulated after the radiofrequency treatment (Enochson et al., 2012). IL-6 played a role in promoting and inhibiting the chondrocyte proliferation. In addition, it had been proven that IL-6 and IL-8 contributed to proliferation of mesenchymal stem cell which would differentiate into chondrocytes (**Figure 1**).

Monopolar and Bipolar Radiofrequency Energy

Radiofrequency equipment includes mRFE (**Figure 2**) and bRFE (**Figure 3**), which differ in work mode and temperature distribution (Khan and Dillingham, 2002; Uribe, 2002).

In mRFE, the current from the box reaches the target tissue through the probe in contact with the tissue and then is derived from the return negative plate connected to the patient. Subsequently, the current goes back to the box to form a complete circuit. Because the target tissue has a higher resistance than the rest of the circuit, heat is generated in the target tissue. The system requires the probe to be in contact with the target tissue during the process of treatment, and the energy is directly transmitted to the target tissue, thereby causing an effect similar to electrical damage (Khan and Dillingham, 2002; Ganguly et al., 2010).

Compared with the working mode of mRFE that generates heat, the mode of bRFE is ablation (Good et al., 2009). The bRFE plasma system is a low-temperature ablation technique, which does not directly act on the tissue, but applies a voltage to the conductive fluid between the electrode and the target tissue to generate a plasma field (Wienecke and Lobenhoffer, 2003; Voloshin et al., 2007). Through the impact of charged particles in the plasma field with the target tissue, the molecular chain of the target tissue is broken, thus playing the role of tissue cutting and removal.

The two types of radiofrequency differ greatly not only in work mode, but also in treatment results. Lu et al. reported that significant chondrocyte death occurred after treatment with both mRFE and bRFE, and the depth of death was higher in the bRFE group than in the mRFE group (Lu et al., 2001; Lu et al., 2002a). Edwards et al. explored the temperature distribution of mRFE and bRFE in different depths of cartilage matrix, and the results showed that bRFE led to a higher temperature and more significant chondrocyte death than mRFE (Edwards et al., 2002a; Edwards et al., 2002b). Caffey et al. studied 5 commercially available radiofrequency instruments in human cartilage under simulated surgical conditions, and the results showed that both mRFE and bRFE caused significant cell death in the cartilage under minimal setting conditions (Caffey et al., 2005).

Many studies have suggested that radiofrequency can lead to remarkable chondrocyte death, especially in bRFE, relative to mRFE. However, the cartilage surface becomes smooth after receiving radiofrequency treatment, providing AC with resistance to wearing and tearing, which can delay the progress of cartilage degeneration. Meanwhile, cartilage has a certain self-repair capability (Caplan et al., 1997). Therefore, whether radiofrequency is suitable for treating cartilage lesions in terms of safety and practicability still requires further *in vivo* experiments to validate.

Control Factors of Radiofrequency

The effectiveness of the radiofrequency therapy can be affected by several variables, including the choice of treatment device,

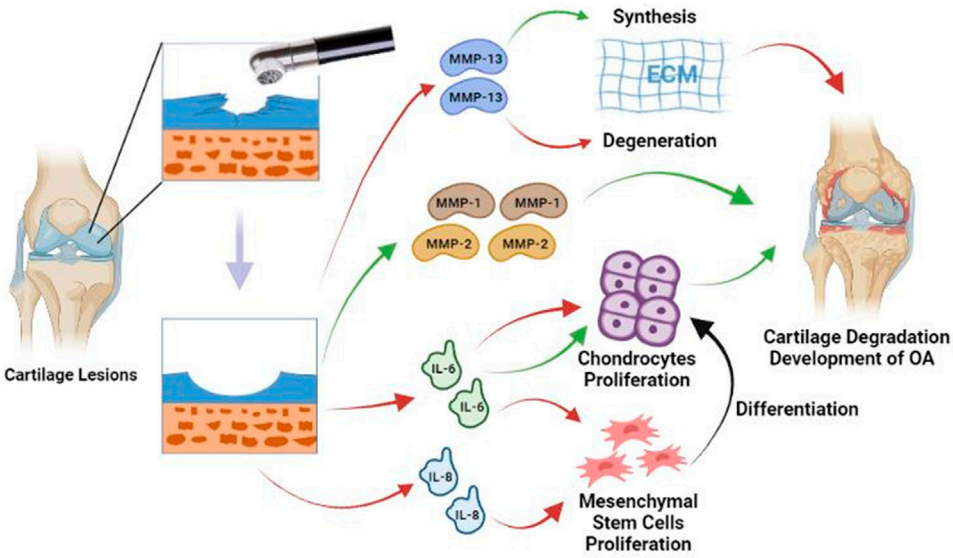

FIGURE 1 | Radiofrequency debrides the damaged cartilage surface to create a smooth cartilage surface. After treatment, MMP-13 tissue immunoreactivity increases, which may lead to a negative shift in the balance between synthesis and degradation of the ECM. IL-6 plays a role in promoting and inhibiting the chondrocyte proliferation. IL-6 and IL-8 promote the proliferation of mesenchymal stem cell. Mesenchymal stem cells can differentiate into chondrocytes and enhance cartilage repair. Secretion of MMP-1 and MMP-2 is significantly reduced, which may help prevent further degradation. Red lines = Stimulating; Green lines = Suppressing; Black line = Differentiation.

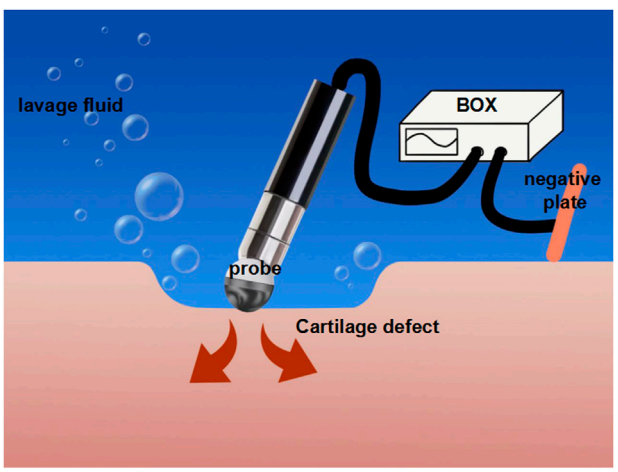

FIGURE 2 | The mechanism of monopolar radiofrequency energy (mRFE).

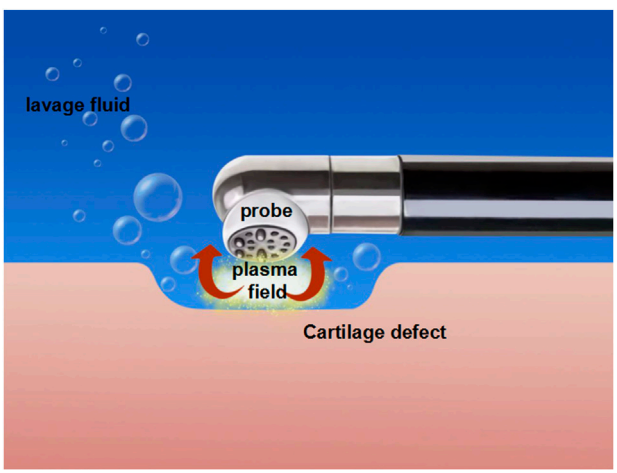

FIGURE 3 | The mechanism of bipolar radiofrequency energy (bRFE).

exposure time, power setting, and temperature control and speed of the lavage fluid flow (**Table 1**).

Time

Clinicians are prone to premature termination of the treatment due to limited arthroscopic magnification when using radiofrequency to treat cartilage lesions, resulting in a less smooth cartilage surface after treatment. Therefore, it is necessary to accurately control the use time of radiofrequency.

Lu et al. performed mRFE and bRFE treatments on fresh osteochondral sections for 5–40s (Lu et al., 2002a). The electron microscope observation showed that the AC surface became smooth with the color gradually turning to yellow after 15 s of radiofrequency treatment in both groups. Excessive treatment time resulted in a significant increase in the depth of chondrocyte death. Similar result was obtained by Peng et al. (Peng et al., 2020). They noted that the glycosaminoglycan content was negatively correlated with exposure time, suggesting that radiofrequency treatment was also negatively correlated with

TABLE 1 | Influence of radiofrequency time, power setting, temperature control and lavage fluid on articular cartilage.

Factor	Radiofrequency type	Specimen	Main result	Ref
Time				
5–40s	mRFE、bRFE	42 fresh human knees	after radiofrequency treatment for 15s, the knee surface began to become smooth	Lu et al. (2002a)
0–50s	bRFE	6 fresh bovine knees	At least 20 s of radiofrequency treatment is needed to smooth the surface of the cartilage	Peng et al. (2020)
B1 = continuous treatment, 1 pass B2 = continuous treatment, 2 passes	bRFE	36 fresh tibial plateau of pigs	The cartilage becomes smooth in B2 mode	Huber et al. (2020)
Power setting	—	—	—	—
Setting 2 vs setting 7; coagulation vs ablation	bRFE	12 fresh porcine knees	Thermal radiation damage can be reduced by ablation mode at high power setting	Wang et al. (2012)
20W–110W		9 healthy adult bovine patellae	The lowest ablation mode setting (60W) resulted in the minimum depth of chondrocyte	Lotto et al. (2006)
20W、40W、60W	bRFE	Paired patellae from 11 horses	Radiofrequency use above 20W is harmful to chondrocytes	Ryan et al. (2003)
50W vs 110W	mRFE	13 healthy viable adult bovine patellae	Tissue effect of cartilage is minimal under 50W power and 25 μm probe	Mitchell et al. (2006)
Temperature control	—	—	—	—
45°C、50°C、55°C	N/R	Specimens of arthritic and nonarthritic femoral articular cartilage	At 50°C, the cartilage recovered to a certain degree of thermal stress 1 week after treatment	Kaplan et al. (2003a)
37–65°C	N/R	318 fullthickness cartilage explants from sheep	The death of chondrocytes increased rapidly when the temperature exceeded 50–55°C	Voss et al. (2006)
Control probe distance	bRFE	N/A	The temperature decreases with increasing distance	Kaplan et al. (2003b)
Control the lavage fluid temperature	mRFE	16 fresh human knees	Thermal chondroplasty with 37°C lavage fluid resulted in less depth of chondrocyte death and produced smoother surfaces	Lu et al. (2002b)
Control the irrigation flow of the lavage fluid	bRFE	6 cadavermens	Avoid temperatures above 50°C by using a high irrigation flow	Ahrens et al. (2018)

N/A not applicable, N/R not reported.

exposure time and had time-dependent damage to the metabolic chondrocyte vitality. Huber et al. compared different modes of radiofrequency therapy. In order to treat a 1 cm^2 cartilage defect area, one continuous pattern, at least 17 s, is required to smooth the articular cartilage (Huber et al., 2020). The cartilage surface only appeared to become smooth after two continuous radiofrequency treatments. With this continuous treatment pattern extending for a long time, the chondrocyte death could reach up to 95%.

These results indicate that radiofrequency needs to be used for more than 15 s in order to smooth the AC surface during clinical treatment. On the contrary, the exposure time should be controlled as short as possible to reduce the effect on the chondrocyte vitality. Due to different specimens used and obvious variation in the thickness of cartilage in different studies, it is difficult to reach a consensus on this issue.

Power Setting

Wang et al. showed that high-power settings in the ablation mode could reduce the thermal radiation injury and was therefore more suitable for the treatment of cartilage lesions (Wang et al., 2012). Lotto et al. discovered that a continuous "char-like layer" was observed on the cartilage surface when the power setting was greater than 60W, which significantly reduced the depth of cell death (Lotto et al., 2006). As the power increased, the current increased significantly. A higher electrical current correlated with increased cell death, even though "char-like layer" appeared in all groups with power higher than 60W. Therefore, they concluded that chondrocytes had the minimum death depth when the cartilage surface was treated at 60W. In a study on the safety of radiofrequency chondroplasty, Ryan et al. found that the cell survival rate of the 40W and 60W groups decreased to 81 and 73%, respectively (Ryan et al., 2003). However, the local peak temperature of the articular surface cartilage was lower than 50°C, at which the chondrocyte vitality might be able to restore (Kaplan et al., 2003a; Voss et al., 2006). Mitchell et al. proposed that in addition to power setting, the selection of probe was also an important factor. Compared with the 110W power and 125 μm probe, the 50W power setting and 25 μm probe had the least influence on the tissue (Mitchell et al., 2006).

In general, different types and manufacturers of radiofrequency will lead to different conclusions. In addition, the experiments mentioned above are only *in vitro* studies or zero-time studies after treatment. Long-term *in vivo* studies are expected to simulate the actual clinical conditions in the future. Since all the manufacturers have provided guidelines for the use of radiofrequency, clinicians can follow these guidelines in clinical practice.

TABLE 2 | Indications: Application of radiofrequency in arthroscopy.

Radiofrequency type	Sample size	Subject or therapeutic site	Grade of cartilage lesions	Follow-up time	Main result	Ref
Knee arthroscopy						
bRFE	25	Patellofemoral joint or tibiofemoral joint	Grade III lesions (23/25)	10.4 ± 9.6 months	3 (12%) lesions continued to progress; Defects were partially or completely filled in more than 50% of patients	Voloshin et al. (2007)
bRFE	4	Femoral trochlea, medial condyle, or patella	Grade III lesions	N/R	The articular cartilage defects become smooth after radiofrequency treatment	Voloshin et al. (2005)
bRFE	824	Medial femoral condyle, patella and the trochlea	The mean lesion size was 358 mm^2	129 days	The improvement in the total WOMAC and KOOS scores after treatment with bRFE	Gharaibeh et al. (2018)
mRFE + MD	28	Femoral condyle	Outerbridge Grade III lesion 1.5–3.0 cm in diameter	12 and 24 months	Both pain and functional outcomes were significantly improved	Barber and Iwasko, (2006)
mRFE + MD	15	Medial femoral condyles and lateral femoral condyles	7 Grade II lesions and 8 Grade III lesions	19 months	The score of IKDC was significantly improved	Kang et al. (2008)
Shoulder arthroscopy	—	—	—	—	—	—
N/R	88	Patients with PAGCL	N/A	N/R	41 (45%) patients with PAGCL had surgeries involving radiofrequency devices	Solomon et al. (2009)
Wrist arthroscopy	—	—	—	—	—	—
mRFE、bRFE	14	14 cadaver arms	N/A	N/A	Peak temperature in the lunate fossa almost reached 70°C even under continuous irrigation	Huber et al. (2015)
(radiofrequency shrinkage)	4	Scapholunate ligament	scapholunate ligament injuries	4.8 years	Patients had significant improvements in pain and satisfaction with outcomes	Jang et al. (2014)
N/R	6	Patients with complications	N/A	N/A	Six cases of complications from use of radiofrequency at wrist arthroscopy were reported	Giddins et al. (2020)
Hip arthroscopy	—	—	—	—	—	—
bRFE + microfracture	1	Acetabulum	N/R	4 months	The authors suggest that the cause of chondrolysis in the patient may have been caused by radiofrequency	Más et al. (2015)
N/R	1	Acetabulum	N/R	1 month	The use of radiofrequency during labral excision may have been responsible for the subsequent chondrolysis	Rehan-Ul-Ha et al. (2010)
N/R	3	human hip cadaveric specimens	N/A	N/A	Five-second-interval pulsed lavage is effective in keeping the hip temperature below 50 °C	McCormick et al. (2013)
Ankle arthroscopy	—	—	—	—	—	—
bRFE (Ankle debridement)	30	Patients with ankle impingement syndrome	N/A	21.5 months	Meislin, AOFAS and VAS scores were significantly improved compared with preoperative score	Han et al. (2014)
N/R (cystectomy)	7	Symptomatic cystic lesions of the talus	N/A	1 year	The postoperative functional scores of the patients were significantly improved and no complications developed	Zhu et al. (2019)
bRFE	6	Cadaver ankle specimens	N/A	N/A	Use high irrigation flow to avoid temperatures exceeding 50°C/122°F	Ahrens et al. (2018)

N/A not applicable, N/R not reported.

Temperature Control

It has been reported in previous studies that radiofrequency chondroplasty can cause AC to be subjected to destructive heat stress, which will further lead to chondrocyte death. Therefore, it is necessary to explore whether there is a temperature "safe zone" when using radiofrequency, at which it will not cause the death of chondrocytes. Kaplan et al. placed cartilage specimens in water baths at 45°C, 50°C and 55°C respectively for up to 3 min and evaluated the chondrocyte vitality immediately and 1 week after treatment (Kaplan et al., 2003a). The chondrocyte vitality was restored 1 week after treatment in the 50°C group, but not in the 55°C group. Voss et al. also confirmed that there was a strong relationship between temperature increase and chondrocyte death (Voss et al., 2006). The chondrocytes were more likely to restore their vitality after thermal injury when the temperature was less than 50°C (Mitchell et al., 2006). Therefore, the local temperature should also be properly controlled to ensure the safety of AC while achieving the desired clinical results.

In another study by Kaplan et al., the temperature was controlled by changing the distance between the bRFE probe

and the cartilage surface (Kaplan et al., 2003b). Lu et al. noted that the use of higher temperature lavage fluid could shorten the time required to reach the preset temperature at the beginning of treatment, thus prolonging the effective treatment time (Lu et al., 2002b). In order to prevent the radiofrequency temperature from exceeding 50°C, Ahrens et al. used a higher lavage fluid flow in arthroscopic surgery to reduce the temperature (Ahrens et al., 2018). This method had also been mentioned and confirmed by Good et al. (Good et al., 2009).

In clinical practice, attention should be paid to controlling the local temperature of radiofrequency to prevent the environment of cartilage from exceeding 55°C for a long time. The radiofrequency temperature can be controlled by increasing the lavage fluid flow, shortening the working time of radiofrequency, controlling the distance of the radiofrequency probe, and increasing the initial temperature of the lavage fluid.

Arthroscopic Application of Radiofrequency

In the arthroscopic application of radiofrequency for the treatment of cartilage lesions, the scope of application and indications of this technique are worthy of attention. The types of patients included in the related clinical studies and the procedures in which the radiofrequency system was used were presented in **Table 2**.

Knee Arthroscopy

Partial-thickness cartilage defects can be detected in more than 60% of the cases that received knee arthroscopy, with Outerbridge Grade III lesions of the patella being the dominant one (Curl et al., 1997). Voloshin et al. investigated 15 patients who underwent knee arthroscopic radiofrequency chondroplasty (a total of 25 cartilage lesions, including 11 patellofemoral lesions and 14 tibiofemoral lesions, of which 23 were Outerbridge Grade III lesions) (Voloshin et al., 2007). At the second-look, significant reductions in the mean extent of the lesions compared with the preoperative status were observed, and partial or complete filling of the cartilage defects in 56% of the patients and continued progression of cartilage degeneration in only 3 cases (12%) were detected. Similar results were reported in another case report by Voloshin et al. (Voloshin et al., 2005). Gharaibeh et al. retrospectively reviewed 824 patients who underwent bRFE for the treatment of cartilage lesions, where the most common lesion involved was the medial femoral condyle (27%), followed by patella (21%) and pulley (9%), with a lesion area ranging 7–820 mm^2 (mean 358 mm^2) (Gharaibeh et al., 2018). Neither postoperative complications nor reoperation in patients were found to be directly related to the use of bRFE. Barber et al. treated 28 patients with Outerbridge Grade III lesions of the femoral condyle (1.5–3.0 cm in diameter) using radiofrequency combined with mechanical debridement (Barber and Iwasko, 2006). Similarly, the follow-up study of Kang et al. included patients with Outerbridge Grade II (7 cases) or III (8 cases) lesions of the medial or lateral condyle of the femur, who were also treated by radiofrequency combined with mechanical debridement (Kang et al., 2008). The clinical results of these patients were significantly improved compared with their preoperative status (Spahn et al., 2016).

Consistently, in several earlier clinical studies, the patients treated by radiofrequency chondroplasty were also classified as Outerbridge Grade II or III lesions. Most cartilage lesions were on the medial condyle of the femur, followed by lateral condyle, patella and trochlea, while the lesion area was generally less than 800 mm^2. A majority of the patients included under this standard had achieved good clinical results, with a low incidence of complications. The detailed indication for the use of radiofrequency in knee arthroscopy needs further research and more long-term follow-up studies to confirm.

Shoulder Arthroscopy

Radiofrequency has long been used in other arthroscopies as well, and its earliest application was in shoulder arthroscopy to reduce periarticular soft tissue laxity and treat shoulder instability. However, in a review by Solomon et al., it was found that most published studies on the relationship between post-arthroscopic glenohumeral chondrolysis (PAGCL) and surgical factors focused on radiofrequency (Solomon et al., 2009). Around 45% of the patients with PAGCL were treated by radiofrequency during surgery. Hence, the side effects of radiofrequency have discouraged its application in shoulder arthroscopy (McFarland et al., 2002).

Hip Arthroscopy

There are few studies on the use of radiofrequency in the treatment of cartilage lesions in hip arthroscopy, and in the relevant studies, radiofrequency was mostly used in iliopsoas release, ligament debridement, treatment for osteoid osteoma and so on (Suarez-Ahedo et al., 2015). In two case reports on the use of radiofrequency to treat acetabular cartilage lesions, both patients developed chondrolysis within 6 months after surgery (Rehan-Ul-Ha et al., 2010; Más et al., 2015). Although there is no direct evidence indicating the cause of chondrolysis by radiofrequency, it has been mentioned in previous reports that the local high temperature produced by radiofrequency led to the death of chondrocytes and even destruction of the entire cartilage layer. Thus, the authors believed that this could be a side effect of radiofrequency.

Wrist Arthroscopy

With the rapid development of the radiofrequency technology, small radiofrequency probes have been created to be used in the wrist and ankle joints, mainly for "thermal shrinkage" and "joint capsule shrinkage" treatment of small joint lesions (Huber et al., 2013; Han et al., 2014; Huber et al., 2015; Leclercq and Mathoulin, 2016; Giddins et al., 2020). Jang et al. executed radiofrequency shrinkage in 4 patients with scaphoid ligament injury and achieved significant improvement during a follow-up of 4.8 years (Jang et al., 2014). Zhu et al. reported 7 cases of excision of lesions by radiofrequency in ankle arthroscopy with no complications complained during the follow-up (Zhu et al., 2019).

Summary of Radiofrequency Applications in Arthroscopy

Although some laboratory studies had shown that the shoulder temperature could be effectively reduced under the condition of continuous lavage fluid flow, it might still exceed 50°C in a short time interval of limited flow, causing thermal damage to the

cartilage (Lu et al., Arthroscopy, 2005, 21, 592–596; Good et al., J Bone Joint Surg Am, 2009, 91, 429–434; Zoric et al., J Bone Joint Surg Am, 2009, 91, 2,448–2,454). For the hip joint, McCormick et al. proposed that 5s-interval pulse irrigation was effective in maintaining the intra-articular temperature below 50°C (McCormick et al., Arthroscopy, 2013, 29, 336–342). For the wrist and ankle joints, the local temperature of cartilage lesions could often exceed 50°C due to a small size and varying cartilage thickness (Huber et al., J Hand Surg Am, 2016, 41, 1,080–1,086). It was also reported that the lavage fluid could not effectively dissipate heat, resulting in PAGCL, chondrolysis, distal radioulnar joint and local skin necrosis (Curtin and Friebe, Orthopedics, 2014, 37, e746–e749). Hence, treatment of cartilage lesions by radiofrequency is rarely seen in other than knee arthroscopy, and the use of "thermal shrinkage" and "joint capsule shrinkage" techniques to ablate the diseased tissue requires particular attention to controlling the local temperature, so as to prevent the occurrence of complications.

Radiofrequency Compared With Other Treatments

Radiofrequency Versus Mechanical Debridement

In 1998, Turner et al. reported that, compared with mechanical debridement, less histological changes and less destruction of cartilage were observed in the treatment with bRFE (Turner et al., 1998). Uthamanthil et al. showed that the postoperative cartilage thickness and stiffness were significantly higher in the mRFE group than in the mechanical debridement group (Uthamanthil et al., 2006). Allen et al. used bRFE and mechanical debridement to treat meniscus injury and cartilage lesions respectively (Allen et al., 2006). The results showed no difference between bRFE and mechanical debridement in terms of the effect on chondrocyte vitality. However, compared with the "tearing" approach of mechanical debridement, radiofrequency could treat cartilage lesions more precisely, create a smoother cartilage surface, and avoid damage to the articular surface.

The earliest prospective clinical study using radiofrequency for the treatment of cartilage lesions was conducted by Owens et al. (Owens et al., 2002). They included 39 patients with Outerbridge Grade II or III lesions of the patella to compare radiofrequency chondroplasty with mechanical debridement chondroplasty. The patients were evaluated before and after surgery based on the Fulkerson-Shea Patellofemoral Joint Evaluation Score, and the results showed that radiofrequency chondroplasty achieved better clinical outcomes. Clearing the cartilage lesions by radiofrequency cannot regenerate the original tissue, but is effective in relieving symptoms and delaying the progression of cartilage degeneration. This encouraging clinical effect can be explained by the microscopic observations that radiofrequency chondroplasty removes fibrotic cartilage, which is the source of chemical and mechanical irritation in the joint.

Although several studies have reported short-term and medium-term follow-up of radiofrequency treatment of AC lesions, long-term follow-up has not been reported yet. Recently, Spahn et al. published the 1-year, 4-years and 10-years follow-up results of bRFE versus mechanical debridement, respectively (Spahn et al., 2008; Spahn et al., 2010; Spahn et al., 2016). It was found that the subjective Knee injury and Osteoarthritis Outcome Score in the radiofrequency group was better than that in the control group. Although the Tegner score of the two groups was at the same level in the 10-years follow-up results, the radiofrequency group reached a higher level of exercise in the 1- and 4-years follow-up results. The medial joint space narrowed in both groups during the follow-up period, and was narrowing obviously faster in the mechanical debridement group. The results of long-term clinical follow-up showed that radiofrequency chondroplasty achieved a better subjective effect. The level of vitality at the 10-years follow-up was lower than that before operation, which might be explained by the increase in age and the progression of OA. Although neither of the two treatment methods can completely prevent the progression of OA, radiofrequency chondroplasty is able to delay the progression of OA more effectively.

Radiofrequency Versus Microfracture

Microfracture has also been used to treat cartilage lesions, relieve knee pain, and restore knee function. Techniques for repairing cartilage injuries by microfracture have been reported in the existing literature (Cerynik et al., 2009). Osti et al. applied radiofrequency and microfracture to treat postoperative Outerbridge Grade I-II lesions and Outerbridge Grade III-IV lesions, respectively (Osti et al., 2010). At the 2-years and 5-years postoperative follow-up, the findings suggested that microfracture achieved similar results to RF in terms of short-term functional improvement; however, microfracture also failed to prevent the continued progression of OA.

Radiofrequency Chondroplasty: A Cost-Effective Technology

Cartilage lesions can eventually progress to OA, which has a enormous impact on the social economy and health (Mahmoudian et al., 2021). Early chondroplasty for cartilage lesions avoids the need for chondrocyte implantation or arthroplasty if the disease progresses further, which potentially decrease the socio-economic burden. In a study on the analysis of the economic benefits of comparing radiofrequency and mechanical debridement, the results showed that radiofrequency chondroplasty resulted in a total cost saving of more than 3000 USD per patient over a 4-years follow-up period. This was attributed to the better efficacy and lower revision rate of radiofrequency (Adeyemi et al., 2020). Moreover, radiofrequency is also considered superior to mechanical debridement in terms of operation time and contributes to the reduction in bleeding, which is facilitated by the coagulation effect of radiofrequency on the small vessels in the adjacent tissue (Camillieri et al., 2001; Cetik et al., 2009). For doctors and patients, this is clearly the treatment of choice.

Safety and Efficacy

Thermal Damage

It has been mentioned above that the use of radiofrequency may expose the surrounding healthy cartilage to heat stress and affect

the chondrocyte vitality, but when the temperature is controlled at 50°C, the chondrocyte vitality will be able to restore to a certain extent after 1 week (Kaplan et al., 2003a). In order to avoid the thermal damage caused by radiofrequency, it is necessary to set appropriate working conditions. Firstly, the probe with a chondroprotective design can be used, and the power should be set appropriately according to the manufacturer's guideline before the surgery (Huang et al., 2014). Secondly, the use time and probe distance must be precisely controlled during the surgery, because the damage of radiofrequency on the cartilage is time-dependent and the increase of probe distance can significantly reduce local temperature (Kaplan et al., 2003b). Last but not least, appropriate initial temperature and sufficient lavage fluid flow are required to ensure that radiofrequency can reach the working temperature more quickly and maintain an appropriate temperature environment in the joint (Lu et al., 2002b; Ahrens et al., 2018).

Osteonecrosis

The local high temperature generated by thermal energy equipment will not only affect the chondrocyte vitality, but also cause deeper bone damage. As mentioned earlier, Lu et al. revealed that bRFE penetrated deeper into the cartilage than mRFE, and hence the energy might also penetrate deeper into the subchondral bone and lead to osteonecrosis (Lu et al., 2001). It has been reported that subchondral bone necrosis occurs after the use of laser and radiofrequency thermal energy equipment in arthroscopic meniscectomy (Muscolo et al., 1996; Rozbruch et al., 1996; Encalada and Richmond, 2004; Cetik et al., 2009). Mehmet et al. found that the addition of radiofrequency chondroplasty to meniscectomy did not increase the incidence of osteonecrosis (Türker et al., 2015). Therefore, the occurrence of osteonecrosis following the use of thermal energy equipment during meniscectomy may be attributed to the increase of tibiofemoral contact pressure due to the decrease of weight-bearing area after meniscectomy, which may lead to subchondral bone microfracture and synovial fluid leakage in the bone, thereby resulting in osteonecrosis (Encalada and Richmond, 2004). In comparison, the use of radiofrequency equipment will not cause osteonecrosis. Similarly, what is desired is to guarantee that radiofrequency is used in a relatively safe environment, which can be achieved by taking the same steps as preventing thermal damage to ensure that local temperature does not exceed 50°C.

Is Radiofrequency Chondroplasty Really Safe?

After over 10 years of clinical application, accumulated evidence has supported the effectiveness of radiofrequency in the treatment of cartilage injury, which appears to be superior to mechanical debridement. This includes the long-term follow-up study conducted by Spahn et al. for 10 years. Recently, Koller et al. put forward an opposite point of view (Koller et al., 2020). They planned to use the magnetic resonance imaging T2 Mapping technique to quantitatively evaluate the efficacy of radiofrequency chondroplasty in patients with Outerbridge Grade II lesions of the patella 1 year after surgery. However, the trial was terminated prematurely because the T2 Mapping quantitative analysis of 5 patients showed postoperative cartilage lesions. Koller et al. believed that radiofrequency was often combined with meniscus repair in treating cartilage lesions, so the improvement in the postoperative functional score might be due to meniscus repair. T2 Mapping is a new and sensitive MR technique, which can provide information about the interaction between extracellular matrix and cartilage water molecules, and better reflect the cartilage recovery of postoperative patients (Banjar et al., 2021). In particular, specific techniques for glycosaminoglycan (GAG) assessment, such as delayed gadolinium-enhanced MRI has also shown utility in detecting AC damage (Link et al., 2017). T2 Mapping has been widely used for the assessment of cartilage activity as a non-invasive technique, and MRI techniques such as delayed gadolinium-enhanced MRI are also promising. Future studies on the efficacy of radiofrequency will need to make greater use of these techniques.

FUTURE OUTLOOK

Radiofrequency has been proposed as an effective method for the treatment of cartilage lesions. However, since the local high temperature produced by radiofrequency will affect the chondrocyte vitality, it is necessary to control the use time, power setting and temperature adjustment precisely, so as to obtain a sufficiently smooth surface of the cartilage while avoiding the side effects of radiofrequency. In terms of clinical application, although a large number of clinical studies (even including 10-years follow-up studies) have proven the effectiveness and safety of radiofrequency, there is still a lack of follow-up studies to evaluate the cartilage growth at the biochemical level. Therefore, more effort is needed to include other indicators such as T2 Mapping, which can be used to evaluate the status of cartilage *in vivo*, to further confirm the safety of radiofrequency.

CONCLUSIONS

Cartilage lesion is a disease of AC loss caused by acute injury or repeated injury, which will eventually develop into OA without proper management. As an alternative to mechanical debridement and laser for the treatment of cartilage lesions, radiofrequency has achieved encouraging results in the past 2 decades. However, there is insufficient evidence to support its indications and safety. At present, we believe that, when radiofrequency is used to treat cartilage lesions, it is necessary to control its indications, mainly for Outerbridge Grade II or III lesions of the knee cartilage, and the range of lesions should generally not exceed 800 mm^2. The use of radiofrequency in such cases seems to be safe with fewer complications.

AUTHOR CONTRIBUTIONS

ZD contributed to the conception and design of this review article. CL and ZD performed searches, analyses, and interpretations. CL and ZD drafted the paper. JX, WZ, WL,

KC and YZ substantially revised the paper. ZD and WZ gave final approval of the version to be submitted.

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Sa12b Improves Biological Activity of Human Degenerative Nucleus Pulposus Mesenchymal Stem Cells in a Severe Acid Environment by Inhibiting Acid-Sensitive Ion Channels

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Sa12b is a wasp peptide that can inhibit acid-sensitive ion channels (ASICs). The biological effects of nucleus pulposus mesenchymal stem cells (NP-MSCs) have not been investigated. Therefore, this study investigated the effect of Sa12b on the biological activity of NP-MSCs through ASICs in the acidic environment of intervertebral disc degeneration (IVDD). In this study, NP-MSCs were isolated from the nucleus pulposus (NP) in patients who underwent lumbar disc herniation surgery, identified by flow cytometry and tertiary differentiation, and cultured *in vitro* in an acidic environment model of IVDD with a pH of 6.2. Proliferation, and apoptosis were observed after different Sa12b concentrations were added to P2 generation NP-MSCs. The Ca^{2+} influx was detected using flow cytometry and laser confocal scanning microscopy, and qPCR was used to detect the relative expression of stem cell–associated genes (Oct4, Nanog, Jag1, and Notch1), the relative expression of extracellular matrix (ECM)–associated genes (collagen II, aggrecan, and SOX-9), and the relative expression of genes encoding ASICs (ASIC1, ASIC2, ASIC3, and ASIC4). Western blotting was used to detect the protein expression of collagen II and aggrecan in different treatment groups. Cells isolated and cultured from normal NP were spindle-shaped and adherent, and they exhibited expansion *in vitro*. Flow cytometry results showed that the cells exhibited high expression of CD73 (98.1%), CD90 (97.5%), and CD105 (98.3%) and low expression of HLA-DR (0.93%), CD34 (2.63%), and CD45 (0.33%). The cells differentiated into osteoblasts, adipocytes, and chondrocytes. According to the International Society for Cellular Therapy criteria, the isolated and cultured cells were NP-MSCs. With an increase in Sa12b concentration, the cell proliferation rate of NP-MSCs increased, and the apoptosis rate decreased significantly, reaching the optimal level when the concentration of Sa12b was 8 μg/μl. When the Sa12b concentration was 8 μg/μl and contained the ASIC non-specific inhibitor amiloride, the Ca^{2+} influx was the lowest, followed by that when the Sa12b concentration was 8 μg/μl. The Ca^{2+} influx was the highest in the untreated control group. qPCR results showed that as the concentration of Sa12b increased, the relative expression of Oct4, Nanog, Jag1, Notch1, collagen II,

aggrecan, and SOX-9 increased, while that of ASIC1, ASIC2, ASIC3, and ASIC4 decreased. The difference was statistically significant ($p < 0.05$). In conclusion, Sa12b can improve the biological activity of NP-MSCs in severely acidic environments of the intervertebral disc by reducing Ca^{2+} influx *via* AISC inhibition and, probably, the Notch signaling pathway. This study provides a new approach for the biological treatment of IVDD. Inhibition of AISCs by Sa12b may delay IVDD and improve low back pain.

Keywords: nucleus pulposus mesenchymal stem cells, acid-sensitive ion channels, Sa12b, acidsensitive ion channel inhibitors, intervertebral disc degeneration

INTRODUCTION

Low back pain (LBP) is one of the most common public health problems and is the leading cause of disability worldwide. It causes a severe social burden and mainly occurs among adults 25–49 years of age (Dieleman et al., 2020; O'Sullivan et al., 2020). Current treatments for LBP include mainly conservative management and surgery, which cannot cure the fundamental causes (Corp et al., 2021). According to previous reports, intervertebral disc (IVD) degeneration (IVDD) is the leading cause of LBP. In addition to standard conservative management and surgical treatment, experimental cell regeneration technology has also received extensive attention from researchers as a treatment strategy for lumbar IVDD (Kos et al., 2019).

The IVD is an avascular and non-renewable tissue composed of the central nucleus pulposus (NP), peripheral fibrous annulus, and upper and lower cartilage endplates. The NP plays a central role in IVD function and consists of nucleus pulposus cells (NPCs) and extracellular matrix (ECM) components. After the degeneration of the NP, nutrient metabolism is unbalanced, and the secretion of ECM components decreases, especially that of collagen II and aggrecan. This reduces the water content in the NP, increases the osmotic pressure, decreases the pH value, induces hypoxia, and accelerates internal disc disruption (IDD). In addition, the drop in disc height eventually leads to IVDD. Therefore, regulation of NPC function and restoration of the ECM metabolic balance is necessary to delay IVDD progression (Zhang et al., 2016). Recently, several studies have shown that nucleus pulposus mesenchymal stem cells (NP-MSCs) can be regenerated and differentiated into NP-like cells in IVDD. Biological treatments, including those based on cytokines, stem cells, and other biological materials, can promote the expression of proteoglycans in the degenerated NP, increase the water content in the IVD, and thus play a role in delaying IVDD (Tao et al., 2014; Tao et al., 2015; Bowles and Setton, 2017).

Relevant studies have shown that with IVDD, many changes occur in the internal environment of IDD, resulting in hypoxia, low pH, hypertonicity, and low nutrition (Colombier et al., 2014). A low pH can reduce the biological activity of NP-MSCs in IVDD. In addition, the change in pH *in vivo* is mainly controlled by acid-sensitive ion channels (ASICs) to regulate the biological activity of cells (Vullo and Kellenberger, 2020). ASICs are gated ion channels that belong to an essential member of the epithelial sodium channel/degenerin (DEG/ENaC) family of sodium-selective channels. They are important receptors for changes in the acidic environment *in vivo* and are widely distributed in various body tissues. ASICs are encoded by four genes and consist of seven subunits: ASIC1a, ASIC1b, ASIClb2, ASIC2a, ASIC2b, ASIC3, and ASIC4, which are expressed in both normal and degenerative NP and are positively correlated with the degree of degeneration (Cuesta et al., 2014). In addition, studies have found that changes in the acid environment of IDD regulate the biological activity of NP-MSCs through ASICs. Human degenerative NP-MSCs express ASIC1, ASIC2, ASIC3, and ASIC4, and this expression is inversely proportional to pH (Liu et al., 2017).

Carmen Hernández et al. purified and extracted the short peptide Sa12b (EDVDHVFLRF) from the venom of the solitary wasp Sphex argentatus and found that it belonged to a new type of non-specific ASIC inhibitor. Preincubation with Sa12b reversibly inhibited the amplitude of the ASIC current peak (IC50~81 nM) in rat DRG neurons in a concentration-dependent manner but had no consistent effect on the time course of desensitization or the persistent component of the current. Thus, the inhibitory effect of Sa12b (*IC50* = 81 nM) on ASIC current is equivalent to that caused by peptides of plant and animal origin, such as chlorogenic acid, gastrodin, phenol, APETx2, and mambalgines (Hernández et al., 2019). In addition, related studies have shown that when the pH value of the *in vivo* environment changes, the opening probability, ion permeability, and ion selectivity of ASICs can be used to adjust the amount of Ca^{2+} transported into cells, thereby adjusting the biological activity of cells (Zhou et al., 2016). However, it is unclear whether Sa12b mediates Ca^{2+} influx through ASICs, thereby affecting the biological activity of NP-MSCs and alleviating or inhibiting IVDD. Thus, this study aimed to investigate the effect of Sa12b on the biological activity of NP-MSCs through ASICs in the acidic environment of intervertebral disc degeneration (IVDD).

MATERIALS AND METHODS

Preparation of Sa12b Mother Liquor

The Sa12b mother liquor used in this study was custom-synthesized at Sangon Biotech (purity >90%, Shanghai, China). The peptide sequences are presented in **Table 1**. This

TABLE 1 | The peptide sequences of Sa12b.

name	Sequence
Sa12b	EDVDHVFLRF-

TABLE 2 | Sample information.

Case NO.	Age (years)	Gender	Pfirrmann grading
Case 1	32	female	V
Case 2	26	male	V
Case 3	23	male	IV
Case 4	26	female	IV

peptide powder was dissolved in phosphate buffered saline (PBS; Gibco) at a final concentration of 1% (w/v, 10 mg/ml) and sonicated on ice for 5 min. The peptide solution was filter-sterilized using syringe-driven filter units (0.22 μm HT Tuffryn membrane, Pall Corp., Ann Arbor, MI, United States) prior to use.

Isolation and Culture of Cells

Isolation of cells from patients with lumbar disc herniation was performed; **Table 2** provides detailed information about the age, sex, and disease status of the subjects. In accordance with the Declaration of Helsinki, all procedures for the study were approved by the local ethics committees of our institution and were performed with the informed consent of the patients. After washing the sample twice with PBS (Gibco), the annulus fibrosus and cartilaginous endplate were carefully removed, and the NP tissue was cut into approximately 1 mm^3 sections and digested in 5% CO_2 at 37°C with collagenase II solution (0.02 mg/ml). After 8 h, the cells were precipitated by centrifugation at 1,500 rpm for 5 min. The supernatant was removed, and the free cells and partially digested tissue were cultured as explants in a standard MSC expansion medium consisting of Dulbecco's modified Eagle's medium–low glucose (Hyclone, United States), 10% fetal bovine serum (FBS; Gibco, United States), and penicillin/streptomycin (United States, Gibco) in a humidified incubator at 37°C and 5% CO_2. After 24 h, suspended cells and debris were removed from the medium, and the medium was completely replaced every 3–4 days to promote adherent cell growth. When the cells reached approximately 90% confluence, the culture was passaged at a ratio of 1:3. The second-generation cells were harvested using trypsin-EDTA solution (United States, Gibco) and tested.

Identification of NP-MSC

After digesting NP-MSCs with 0.25% trypsin (Biosharp, United States), the cells were washed and resuspended in 100 μl PBS (Sigma, United States) containing 1% FBS. Each tube contained 5 μl of the following antibodies, according to the recommendations of the International Society for Cell Therapy: CD34-APC, CD73-FTTC, CD45-PE, CD90-FTTC, CD105-PE, and HLA-DR-APC (eBioscience, United States). In each case, isotype control (eBioscience, United States) was used. After incubation with the antibody for 30 min at room temperature, the cells were washed with PBS, and the supernatant was discarded. The cells were resuspended in 200 ul of PBS (Sigma, United States) and analyzed using a flow cytometer (BD, United States). Immunophenotyping analysis was performed to identify the percentage of positive cells and the fluorescence intensity.

Osteogenic Differentiation

The normal medium was replaced with an osteogenic differentiation medium (Cyagen, United States). The differentiation process lasted for 14 days, and the medium was changed every 2–3 days. After differentiation, the medium was removed, and the cells were washed with PBS and fixed with 4% paraformaldehyde solution (Sangon Biotech, China) at room temperature for 30 min. After washing twice with PBS, the cells were stained with alizarin red working solution (Cyagen, United States) for 15 min. Finally, the culture was washed three times with PBS and observed under an optical microscope.

Adipogenic Differentiation

NP-MSCs from the second passage were seeded in a six-well plate at a density of 5 × 104 cells/cm^2. When the cells reached 100% confluence, the normal medium was replaced with adipogenic differentiation medium A (Cyagen, United States). After 3 days, differentiation medium A was replaced with adipogenic differentiation medium B (Cyagen, United States) for 24 h. Then, medium B was replaced with medium A again. This cycle was repeated for three to four cycles for a total of 28 days. After differentiation, the medium was removed from the wells, and the cells were washed with PBS and fixed with 4% paraformaldehyde solution for 30 min. After washing twice with PBS, the cells were stained with Oil Red O working solution (Cyagen, United States) for 30 min at room temperature. The culture was then washed three times with PBS and observed under an optical microscope.

Chondrogenic Differentiation

NP-MSCs from the second passage were resuspended in chondrogenic differentiation basal medium (Cyagen, United States), centrifuged at 1,500 rpm for 5 min, and the supernatant was discarded. The cells were resuspended in a complete chondrogenic differentiation medium (Cyagen, United States) at a density of 5 × 105 cells/ml. Next, 500 μl of the cell suspension was placed in a 15 ml centrifuge tube containing 2.5 × 105 cells and centrifuged at 1,500 rpm for 5 min to form a pellet. The pellet was cultured at 37°C in 5% CO_2 in a complete chondrogenic differentiation medium without interference. After 3 days, the medium was changed, and the bottom of the test tube was flicked to ensure that the cartilage ball floated freely. After that, the differentiation medium was changed every 2 days, and the differentiation process lasted for 28 days. When the diameter of the cartilage ball increased to 3 mm, tissue sections were fixed in formalin and embedded in paraffin (Sangon Biotech, China). Finally, the sections were deparaffinized and hydrated with distilled water. The sections were then stained with Alcian Blue (Cyagen, United States) for 30 min. They were washed with running tap water for 2 min and then with distilled water several times. Finally, the sections were observed under an optical microscope.

Preparation of Media With Different pH Values

Media with different pH values were prepared by adding an appropriate amount of sterilized HCl (1 mol/L) and NaOH (1 mol/L) to the culture medium and monitoring the pH values using a pH microelectrode (Ramagnetic phs-25, China). After pH values (7.4, 6.2) were obtained, the medium was kept in 5% CO_2 at 37°C for 3 days to establish a pH balance (CO_2-dependent).

Cell Proliferation Assay

The second-generation NP-MSCs were inoculated in 96-well plates (200 μl medium per well) at a density of 3 × 104 cells/ml. These cells were cultured in a complete medium with a pH of 6.2 at 37°C with 5% CO_2. To observe the effect of Sa12b on proliferation, the experimental group also contained 20, 40, 60, and 80 μg Sa12b. Sub-samples were collected from three wells on days 1, 3, 5, 7, and 9, and 20 μl of CCK-8 (Japan, DOJINDO) was added to the cells, which were then incubated for 2 h. The isotype group did not contain any cells. The absorbance of each group was measured using a Spectrum MAX microplate reader.

Cell Cytotoxicity Assessment

NP-MSCs from the second passage were seeded in a 12-well plate at a density of 1 × 105 cells/ml. These cells were cultured for 2 days at different pH levels at 37°C and 5% CO_2. CAM and the nucleic acid dye propidium iodide (PI, Sigma, United States) were used to label live and dead NP-MSCs, respectively. Briefly, the cells were incubated with 2 μmol/L CAM and 5 μmol/L PI at room temperature for 30 min in the dark and then gently rinsed with PBS three times. A fluorescence microscope was used to collect the images.

Apoptosis Measurement of NP-MSCs

NP-MSCs from the second passage were seeded in a 12-well plate at a density of 1 × 105 cells/ml. These cells were cultured for 2 days at different pH levels at 37°C and 5% CO_2. To observe the effect of Sa12b on cell apoptosis, the experimental group also contained Sa12b at various concentrations (2, 4, 6, and 8 μg/μl), with four replicate wells in each group. The adherent cells were collected by trypsinization without EDTA (Biosharp, United States) and washed twice with PBS. In addition, Annexin V-FITC (5 μL) and PI (KeyKey BioTECH, China) were added to each group and incubated at room temperature in the dark for 15–20 min, and flow cytometry (United States, BD) was used within 1 h to detect the percentage of apoptotic cells.

Quantitative Real-Time PCR Analysis of Gene Expression

NP-MSCs from the second passage were seeded in T-25 culture flasks at a density of 5 × 105 cells/ml and cultured in 5% CO_2 at 37°C at different pH levels for 7, 14, and 28 days. To observe the effect of Sa12b on cell apoptosis, the experimental group also contained Sa12b at various concentrations (2, 4, 6, and 8 μg/μl). TRIzol (Ambion, United States) was used to extract total RNA according to the manufacturer's instructions, and the RNA samples were treated with DNase/RNase-free water. A Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, United States) was used to determine the quality and quantity of RNA. A reverse transcription reagent (Takara, Japan) was used to obtain cDNA from total RNA. A total of 1,000 ng of RNA was mixed with 2 μl of 5× PrimeScript RT®MasterMix and RNase-free ddH2O (the total system volume was 10 μl). The mixed solution was first incubated at 37°C for 15 min and then at 85°C for 5 s, and stored at −80°C for qPCR. All genes were analyzed by qPCR, and GAPDH was used as a control. SYBR Premix Ex Taq PCR kit (Takara, Japan) and LightCycler (Roche, Switzerland) were used for qPCR analysis. Premier software (version 5.0) was used to design the primers for all genes, as shown in **Table 3**.

Western Blot Analysis

NP-MSCs from the second passage were seeded in a 6-well plate at a density of 1 × 105 cells/ml. These cells were cultured in 5% CO_2 at 37°C at different pH levels for 14 days. To observe the effect of Sa12b on cell apoptosis, the experimental group also contained Sa12b at various concentrations (2, 4, 6, and 8 μg/μl). The cells were washed three times with ice-cold PBS, and total protein was extracted using RIPA buffer containing 1% PMSF. Protein concentration was measured using a BCA protein quantification kit (Takara, Japan). The protein was electrophoresed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Massachusetts, United States). After blocking with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h, the membrane in TBST was incubated with appropriate primary antibodies: anti-aggrecan antibody, anti-collagen II antibody (maintained at 4°C overnight), and anti-GAPDH antibody (1:1,000; Santa Cruz). The membrane was then incubated with horseradish peroxidase (HRP)-labeled secondary immunoglobulin G (1: 1,000, Santa Cruz) at room temperature for 1 h. After washing the membrane three times with TBST, the immunoreactivity was detected with an enhanced chemiluminescence (ECL, Millipore) substrate, and the optical density was measured using Quantum One software (BioRad Laboratories Inc., Munich, Germany). GAPDH was used as the loading control.

Calcium Imaging

NP-MSCs from the second passage were seeded at a density of 1 × 105 cells/ml in a special cell culture dish for confocal microscopy and cultured in an acidic environment (pH = 6.2) in 5% CO_2 at 37°C. The cells were then washed three times with D-Hanks' solution and incubated with 5 μM Fluo-3-AM (Dojindo Laboratories, Japan) for 30 min at 37°C, followed by three washes and an additional incubation in normal Hanks' solution for 15 min. Fluo-3-AM was excited at 488 nm using a laser scanning confocal microscope, and the emission was measured at 510 nm.

Detection of $[Ca^{2+}]$ i Using Flow Cytometry

Cell suspensions in Eppendorf tubes were washed three times with D-Hanks' solution and incubated with 5 μM Fluo-3-AM (Dojindo Laboratories, Japan) for 30 min at 37°C. Then, the cells were washed three times for 5 min and incubated in normal Hanks'

TABLE 3 | Primers used in qPCR.

Genes	Sense primer	Antisense primer
DAPDH	GAAGGTCGGAGTCAACGG	GGAAGATGGTGATGGGATT
ASIC1	TTCAAGGTGGTCTTCACACGCTATG	AGGTACTCGTCCTGCTGGATGTC
ASIC2	TCCTACTACTTCTCCTACCAGCATGTC	CGGAAGCCATTCAGGTTACAGAGG
ASIC3	GCCTGAGAACTTCACCACGATCTTC	GCACGTCCAGCATGATGTCCAG
ASIC4	ACCATCTGCCCACCAAATATCTACATC	CTCTTTCCCATAGCGTGTCAGGTTG
Aggrecan	ACGGCTTCTGGAGACAGGACTG	CTGGGATGCTGGTGCTGATGAC
SOX-9	AGGAGAGCGAGGAGGACAAGTTC	TGTTCTTGCTGGAGCCGTTGAC
Collagen II	GGAGCAGCAAGAGCAAGGAGAAG	TCATCTGGACGTTGGCAGTGTTG
Notch1	GCCTCAACATCCCCTACAAG	CACGAAGAACAGAAGCACAAA
Oct4	ACACTGCAGCAGATCAGCCAC	CCAGAGGAAAGGACACTGGTC
Nanog	GCTTTGAAGCATCCGACTGT	TTTGGGACTGGTGGAAGAAT
Jagged	CGAGGACTATGAGGGCAAGA	CTTCAGGTGTGTCGTTGGAA

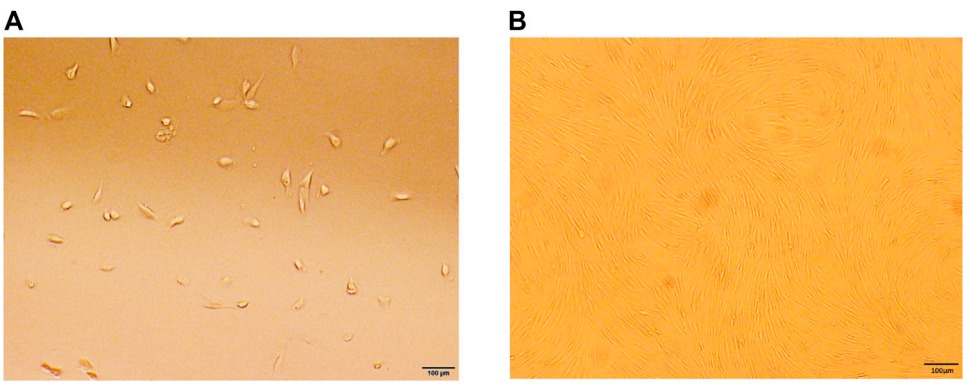

FIGURE 1 | Characteristics of cell culture *in vitro*. **(A)** Human degenerative NP-MSCs exhibited a short, rod-like shape in primary culture. **(B)** Human degenerative NP-MSCs showed uniform spindle filament spiral growth after subculture.

solution for 15 min. Fluo-3-AM was excited at 488 nm using a flow cytometer, and emission was measured at 510 nm.

Statistical Analysis

All data were statistically analyzed using SPSS 23.0 and are presented as the mean ± standard deviation. ANOVA of the factorial design was performed to analyze the main effect and the interaction between the groups and the time periods, and one-way ANOVA was performed for multiple-group comparisons. The Student-Newman-Keuls test (homogeneity of variance) or Tamhane's test (heterogeneity of variance) was performed to compare any two groups. Statistical significance was set at $p < 0.05$.

RESULTS

The Experimental Cells Were Identified as NP-MSCs Through Immunophenotyping and *In Vitro* Pluripotent Differentiation

The cells were extracted from human IVDs, isolated, and cultured *in vitro*. The primary cells showed a short spindle shape after 3–5 days (**Figure 1A**). After approximately 2 weeks of passage, the growth rate of the primary cells was significantly faster than that of the primary cells, with spiral growth, uniform morphology, and a characteristic spindle shape (**Figure 1B**). Flow cytometry showed that the cultured cells had high expression of CD73, CD90, and CD105 and low expression of CD34, CD45, and HLA-DR (**Figure 2A**). In addition, after the cells were incubated in differentiation induction culture, strongly stained mineralized nodules indicated osteogenic differentiation, oil red O staining of intracellular lipid vacuoles indicated adipogenic differentiation, and staining with sulfated proteoglycans indicated chondrogenic differentiation (**Figure 2B**). In summary, the cells met the evaluation criteria of the International Society for Cellular Therapy (ISCT) for NP-MSCs.

Sa12b Alleviated Proliferation Inhibition of NP-MSCs in a Severely Acidic Environment

As shown in **Figure 2A**, compared with the normal culture environment ($pH = 7.4$), the proliferation of human degenerative NP-MSCs cultured in an acidic environment ($pH = 6.2$) was significantly inhibited ($p < 0.001$); however,

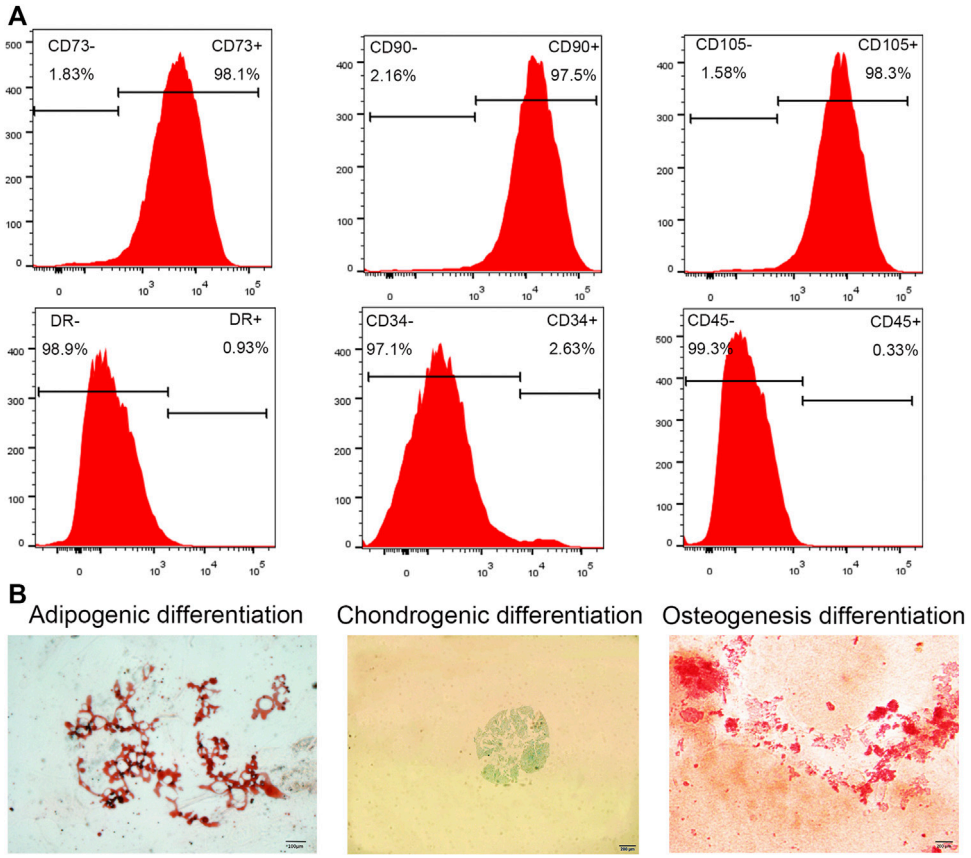

FIGURE 2 | Cell phenotype identification and cell differentiation. **(A)** Flow cytometry analysis showed high expression of the cell surface markers CD105, CD90, and CD73, while CD45, CD34, and HLA-DR expression was absent. **(B)** Three lineage differentiation of NP-MSCs. Oil red O staining of NP-MSC intracellular lipid vacuoles indicated adipogenic differentiation; Alcian blue staining of cartilage indicated chondrogenic differentiation; Alizarin red staining of calcium nodules indicated osteogenic differentiation.

after adding different concentrations of Sa12b (0, 2, 4, 6, 8 μg/μl) to the environment, the proliferation inhibition was significantly improved ($p < 0.05$), and this effect was strongest at 8 μg/μl. In addition, confocal microscopy was used to evaluate cytotoxicity in cells treated with different concentrations of Sa12b. The results indicated that Sa12b could alleviate the proliferation inhibition of NP-MSCs in a severely acidic environment without inducing cytotoxic effects (**Figure 2B**).

Sa12b Reduced the Apoptosis Rate of NP-MSCs in a Severe Acidic Environment

Human degenerative NP-MSCs were cultured in an acidic environment ($pH = 6.2$) for 2 days, and different concentrations of Sa12b (0, 2, 4, 6, and 8 μg/μl) were added. Flow cytometry was used to evaluate the percentage of apoptotic NP-MSCs after treatment under the above conditions. The results showed that compared with that in the control group, the apoptosis rate of NP-MSCs gradually decreased significantly after adding Sa12b (**Figures 3A,B**).

Sa12b Inhibited the Expression of ASICs in the Acidic Microenvironment Conditions and Promoted the Expression of ECM-Related Genes and Proteins

To evaluate the effect of Sa12b on the subunits of ASICs (ASIC1, ASIC2, ASIC3, and ASIC4) and ECM-related genes (aggrecan, collagen II, and SOX-9) in NP-MSCs in an acidic environment, we used different concentrations of Sa12b (0, 2, 4, 6, and 8 μg/μl). NP-MSCs were cultured for 7, 14, and 28 days. The expression of each gene was detected by qPCR, and GAPDH expression was used as a control. The results showed that, relative to the expression levels of each gene in cells that were not treated with Sa12b, the relative gene expression of the ASIC subunits gradually decreased with the increase in Sa12b concentration (**Figure 4**), while that of the ECM-related genes gradually increased ($p < 0.01$) (**Figure 5**). The results also showed that these differences were maintained at least until the 28th day. In addition, western blotting results showed that the expression of ECM-related proteins (aggrecan and collagen II) in

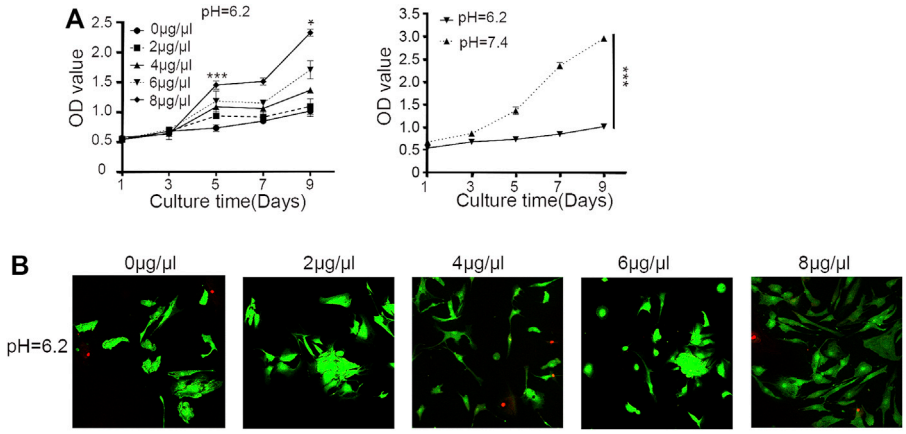

FIGURE 3 | Sa12b alleviated the proliferation inhibition of NP-MSCs in a severely acidic environment. **(A)** The proliferation of NP-MSCs in an acidic environment (*pH* = 6.2) was significantly lower than normal (*pH* = 7.4). When different concentrations of Sa12b (0, 2, 4, 6, and 8 μg/μl) were added, the proliferation inhibition of human degenerated NP-MSCs was significantly improved ($p < 0.05$). *$p < 0.05$ indicated statistical difference between the groups; ***$p < 0.001$ indicated significant difference between groups. **(B)** Live and dead cell imaging of NP-MSCs treated with different concentrations of Sa12b. The green fluorescent cells are the live cells labeled with CAM, and the red fluorescent cells are the dead cells labeled with PI.

NP-MSCs also increased with an increase in Sa12b concentration, which was consistent with the qPCR results.

Sa12b Increased the Expression of Stem cell-Related Genes Under Acidic Microenvironmental Conditions and Reduced the Influx of Ca^{2+} in NP-MSCs by Inhibiting ASICs

To explore the mechanism by which Sa12b improves the biological activity of NP-MSCs cultured in an acidic environment, we used cells without Sa12b treatment as a control and obtained the optimal Sa12b concentration (8 μg/μl) in the treatment group based on the results of proliferation and apoptosis experiments. As shown in **Figure 6**, the qPCR results showed that the expression of stem cell–related genes (Oct4, Nanog, Jagged, and Notch1) in the treatment group supplemented with Sa12b was significantly higher than that in the control group ($p < 0.05$) (**Figure 6A**). To measure Ca^{2+} concentration, we treated NP-MSCs cultured in an acidic environment with the following: no Sa12b, Sa12b at an optimal concentration, amiloride (an ASIC non-specific inhibitor), and Sa12b and amiloride together. The results showed that the Ca^{2+} concentration in the three treatment groups was lower than that in the control group. Interestingly, the Ca^{2+} concentration in the group treated with Sa12b and amiloride simultaneously was significantly less than that in the control group (**Figures 6B–D**).

DISCUSSION

During the process of IDD, the change in the microenvironment, especially the low pH, is one of the key factors that inhibit the differentiation of NP-MSCs into NP-like cells (Kanichai et al., 2008; Wuertz et al., 2008; Wuertz et al., 2009; Li et al., 2013; Colombier et al., 2014), mainly by activating ASICs (Liu et al., 2017). In our study, we found that the newly emerged wasp peptide Sa12b (EDVDHVFLRF-), a non-specific ASIC inhibitor, could effectively improve the biological activity of human degenerated NP-MSCs in harsh acidic environments (*pH* = 6.2). Compared with that in the control, the proliferation rate of NP-MSCs increased with the increase in Sa12b concentration, and the apoptosis rate decreased. Moreover, the relative expression of genes encoding ASIC subunits (ASIC1, ASIC2, ASIC3, and ASIC4) expressed in human degenerated NP-MSCs affected by Sa12b was reduced. On the contrary, the expression of genes related to the ECM (collagen II, aggrecan, and SOX-9) and the protein expression of collagen II and aggrecan increased. Therefore, Sa12b improved the biological activity of human degenerated NP-MSCs and is very likely to contribute to remodeling the ECM of the NP tissue and delaying IDD. Furthermore, Sa12b inhibits Ca^{2+} influx in human degenerated NP-MSCs by mediating ASICs to affect proliferation, differentiation, and apoptosis. In summary, our study indicated that the new ASIC non-specific inhibitor Sa12b could improve the biological activity of human degenerative NP-MSCs in a harsh acidic microenvironment and play a beneficial role in the process of IDD.

The chronic decrease in the number and function of NPCs in NP under conditions of IDD and loss of ECM components, especially collagen II and aggrecan, is one of the pathological features of IDD (Antoniou et al., 1996; Roberts et al., 2006; Nicholas and George, 2011; Colombier et al., 2014; Risbud and Shapiro, 2014). Studies have found that NP tissue contains MSC-like cells that can be isolated and proliferated *in vitro*, namely NP-

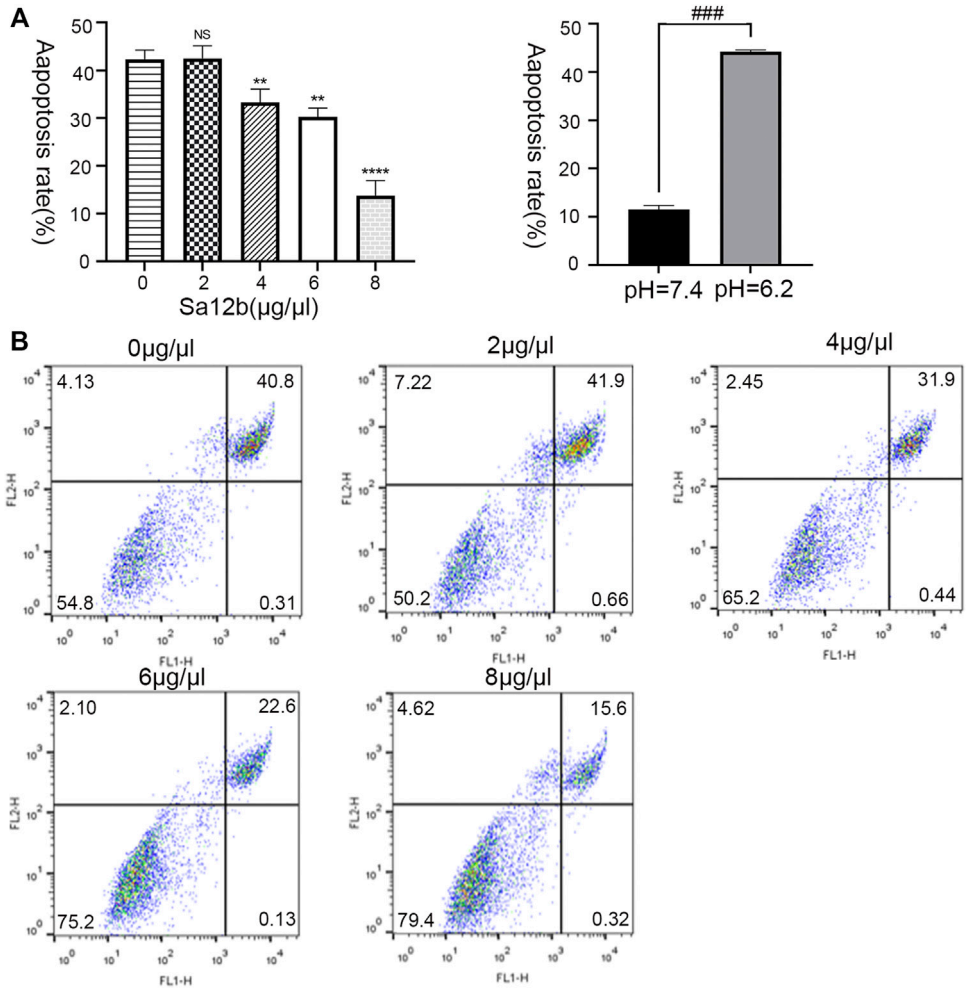

FIGURE 4 | Sa12b reduced the apoptosis rate of NP-MSCs in a severe acidic environment. **(A)** The apoptotic rate of NP-MSCs in an acidic environment (*pH* = 6.2) was significantly higher than normal (*pH* = 7.4). When different concentrations of Sa12b (0, 2, 4, 6, and 8 μg/μl) were added, the apoptotic rate of human degenerated NP-MSCs was significantly reduced. ** indicates $p < 0.01$; **** indicates $p < 0.0001$; NS indicates no statistical significance when the control group (*Sa12b* = 0 μg/μl) was compared with the other groups; ### indicates $p < 0.001$ when the control group (*pH* = 7.4) was compared with the treatment group (*pH* = 6.2). **(B)** Flow cytometry of NP-MSCs treated with different concentrations of Sa12b. The upper right corner indicates the proportion of late apoptosis.

MSCs, which have similar biological activity to bone marrow mesenchymal stem cells (BMSCs) and adipose-derived stem cells (ADSCs) (Blanco et al., 2010; Shen et al., 2015; Clouet et al., 2019), They can self-renew and differentiate into NPCs, promoting the regeneration of ECM after activation (Sakai et al., 2012). NP-MSCs were isolated from the NP in patients who underwent lumbar disc herniation surgery and then were extracted and purified. The homology observed *in vivo* and *in vitro* made this research highly relevant to clinical applications. In the cell identification stage, the cells presented the following characteristics: 1) adherent and long-term growth; 2) high expression of CD73, CD90, and CD105, and low expression of CD34, CD45, and HLA-DR; 3) osteogenic, adipogenic, and chondrogenic differentiation; and 4) expression of genes related to stem cells (Oct4, Nanog, Jagged, and Notch1), which met the international standards of MSCs and is consistent with the research of Shen et al. (Dominici et al., 2006; Shen et al., 2015).

An acidic environment is vital for the progression of IVDD. ASICs, which are closely related to the acidic environment, affect the biological activity of cells by regulating the flow of ions inside and outside the cell (Zhou et al., 2016). Relevant studies have shown that acidic conditions can significantly inhibit the proliferation of human degenerated NP-MSCs and increase cell apoptosis through ASICs. As the pH of the culture environment of human degenerated NP-MSCs decreases, the expression of ASIC1, ASIC2, ASIC3, and ASIC4 gradually increases (Liu et al., 2017). In this study, we found that when the non-specific ASIC inhibitor Sa12b was not added to the culture, the cell proliferation rate decreased as the pH value decreased, and apoptosis increased, which was most apparent when the pH was 6.2 and is consistent with the study by Liu et al. After increasing Sa12b concentration, the proliferation increased, and the apoptosis rate decreased significantly, reaching the

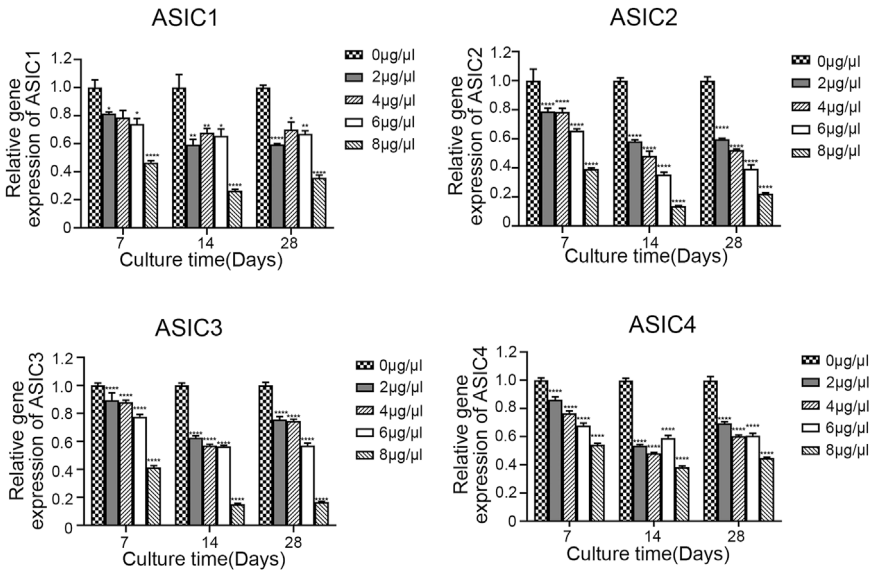

FIGURE 5 | The relative gene expression of ASIC subunits (ASIC1, ASIC2, ASIC3, and ASIC4) gradually decreased with increasing Sa12b concentration. *indicates $p < 0.05$; ** indicates $p < 0.01$; *** indicates $p < 0.001$ when the control group (*Sa12b* = 0 μg/μl) was compared with the other groups.

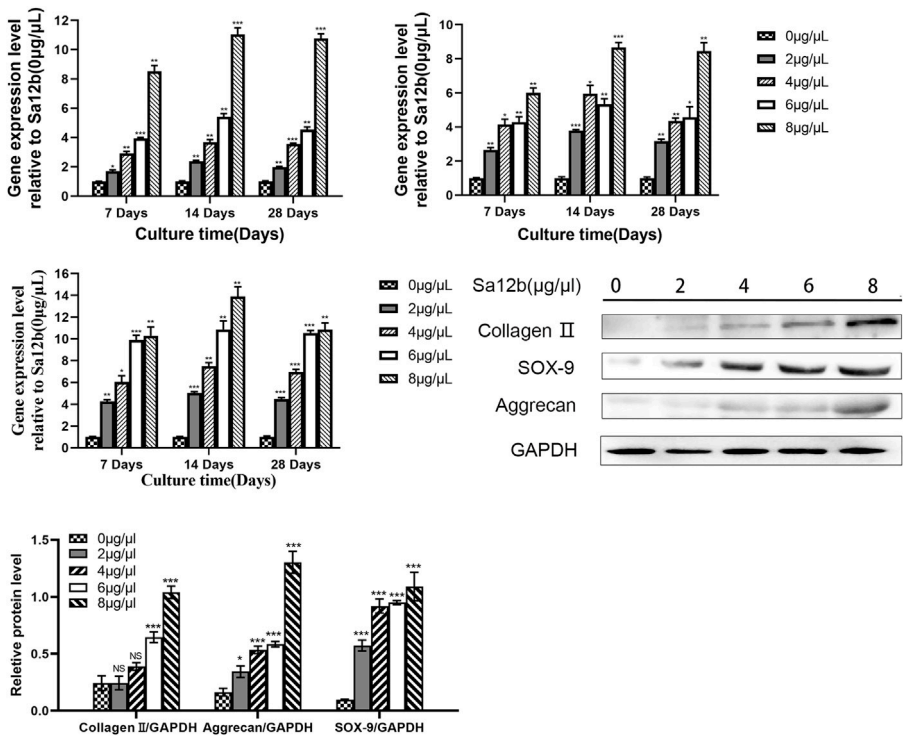

FIGURE 6 | Expression of ECM-related genes (Aggrecan, Collagen II, SOX-9) gradually increased with increasing Sa12b concentration. *indicates $p < 0.05$; **indicates $p < 0.01$; ***indicates $p < 0.001$; NS indicates no statistical significance when the control group (*Sa12b* = 0 μg/μl) was compared with the other groups.

optimum level when the Sa12b concentration was 8 μg/μL qPCR showed that when the pH of the complete medium was 6.2 and different doses of Sa12b were added, the relative expression levels of ASIC1, ASIC2, ASIC3, and ASIC4 in NP-MSCs were all reduced. This illustrated that Sa12b affected human degenerative NP-MSCs through ASICs. In

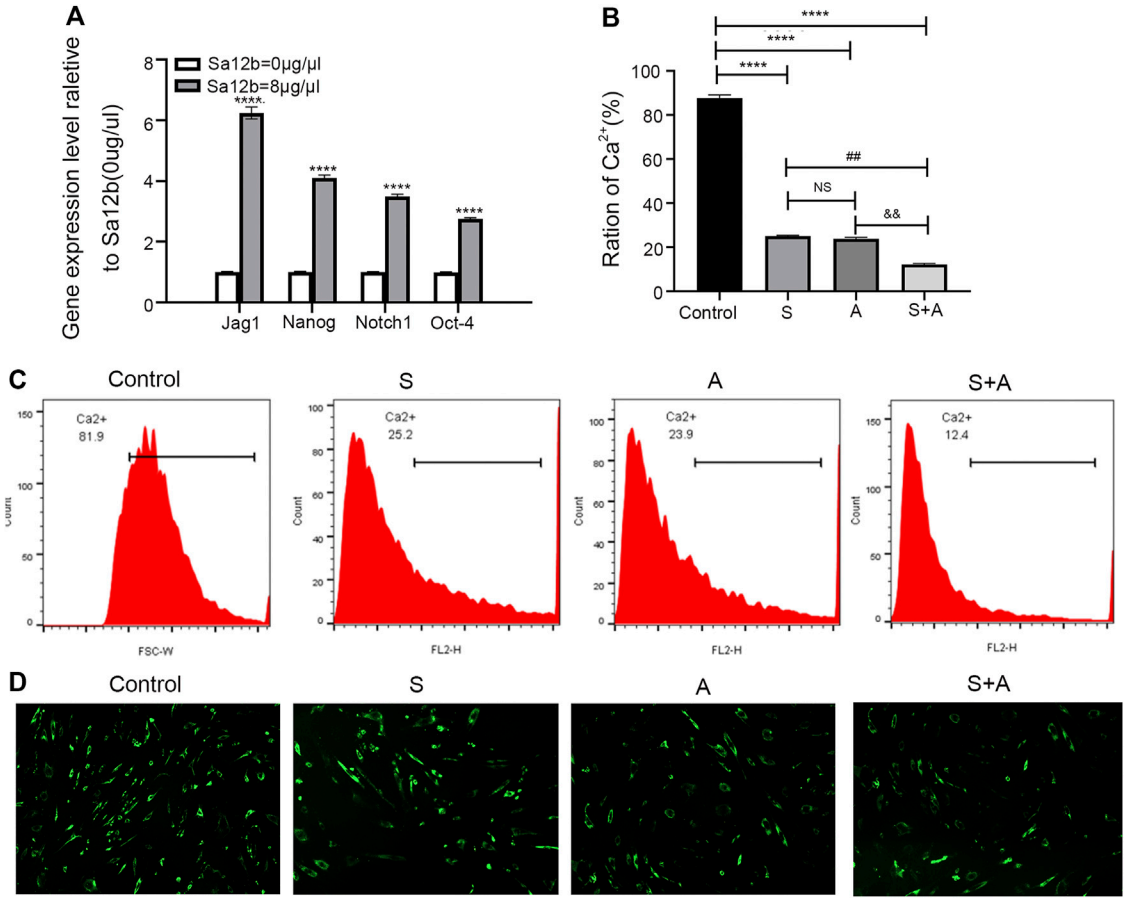

FIGURE 7 | Sa12b increased the expression of stem cell–related genes under acidic microenvironmental conditions and reduced the influx of calcium ions in NP-MSCs by inhibiting ASICs. **(A)** In an acidic environment, the optimal concentration of Sa12b was applied to NP-MSCs, and the expression of stem cell–related genes (Jag1, Nanog, Notch1, and Oct4) was significantly higher than that in the control group ($p < 0.001$). ****indicates $p < 0.0001$ when the control group (*Sa12b* = 0 μg/μl) was compared with the other groups; NS indicates no statistical significance when the Sa12b treatment compared with the group with amiloride. ##indicates $p < 0.01$ when the Sa12b treatment (*Sa12b* = 8 μg/μl) compared with the group with amiloride and Sa12b. &&indicates $p < 0.01$ when the Sa12b group compared with the amiloride and Sa12b treatment. **(B)** The Ca^{2+} concentration in the treatment groups (Sa12b, amiloride, and combined Sa12b/amiloride) was lower than that in the control group according to the results of flow cytometry. Furthermore, the Ca^{2+} concentration in the group administered Sa12b and amiloride simultaneously was significantly lower than that in the control group. **(C)** Ca^{2+} concentration was measured by flow cytometry. **(D)** Confocal laser scanning microscopy imaging analysis of Ca^{2+} concentration. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

addition, studies have shown that endogenous stem cells such as NP-MSCs can self-renew and differentiate into NPCs, which can promote the regeneration of ECM after activation (Sakai et al., 2012). ECM receptor interaction can also activate the AKT pathway to promote the differentiation of neuronal stem cells (He et al., 2021). In addition, liver-specific ECM (L-ECM) can promote the liver differentiation of BM-MSCs by activating specific types of integrins (ITG) and its downstream signal ITG pathways, and play a therapeutically beneficial effect on the liver regeneration of stem cells (Bi et al., 2017). It is consistent with our results, as the concentration of Sa12b increased, the relative expression levels of ECM-related genes and proteins in NP-MSCs, such as collagen II, aggrecan, and SOX-9, were all increased; thus, we speculated that Sa12b had a high probability of promoting the regeneration of ECM in IVDD, promote the differentiation of NP-MSCs through downstream AKT and ITG, and delay IVDD.

In addition, the Notch signaling pathway consists of four Notch receptors (Notch 1–4) and five Notch ligands (Jag1, Jag2, DLL-1, DLL-3, and DLL-4) (Siebel and Lendahl, 2017). The Notch signaling pathway, a key regulator of chondrogenesis, can regulate chondrocyte proliferation and differentiation and maintain the stromal metabolic balance of cartilage in articular chondrocytes (Liu et al., 2016). In addition, the study found that the binding of Notch1 and Jag1 activates the signal molecule HES1, and the activated HES1 inhibits the expression of downstream related genes, thereby inhibiting cell apoptosis and promoting cell cycle progression (Xue et al., 2014; Lee et al., 2016). This study found that the expression levels of Notch1 and Jag1 increased significantly when the Sa12b concentration was optimal (8 μg/μl). Moreover, the qPCR

results indicated that the expression levels of each ASICs subunit decreased. Therefore, we believe that Sa12b may activate the Notch signaling pathway by mediating ASICs, increasing the expression of downstream genes in the pathway, promoting proliferation, and inhibiting the apoptosis of NP-MSCs.

Oct4 and Nanog are homologous domain transcription factors that are the core transcription factors of human stem cells. Located upstream of genes responsible for totipotency regulation, Oct4 can maintain the pluripotency of stem cells (Saunders et al., 2017). Oct4 can be directly bound to the Nanog promoter to maintain the activation of Nanog and improve the proliferation ability of cells. Studies have found that upregulation of Oct4 and Nanog expression in dental pulp cells can promote cell proliferation, whereas downregulation can inhibit cell proliferation (Huang et al., 2014). In this study, the expression of Oct4 and Nanog was detected by qPCR, and the results showed that the expression was significantly increased at the optimal Sa12b concentration (8 μg/μl) compared with that in the control group. Therefore, Sa12b may promote the expression of Oct4 and then activate Nanog expression to enhance the pluripotency, proliferation, and differentiation of NP-MSCs.

ASIC1 and ASIC3 are crucial subunits in the cytomembrane and are key mediators of human NP-MSC aging during IVDD. They are affected by changes in the levels of extracellular lactate and induce Ca^{2+} influx. Ca^{2+}, a key second messenger in the signal transduction pathway, induces the activation of the NF-κB signaling pathway, leading to apoptosis (Ding et al., 2021; Zhao et al., 2021). *In vitro* animal experiments have also shown that ASIC1 mediates cartilage endplate apoptosis and matrix metabolism under acidic conditions (Yuan et al., 2016). In addition, the reduction in the pH of the articular fluid can lead to excessive apoptosis of chondrocytes. Wu et al. found that ASIC1a may promote $[Ca^{2+}]$ i and upregulate NLRP3 inflammasome expression, thus mediating cocaine death in AA rat chondrocytes (Wu et al., 2019).

Our results showed that the Ca^{2+} influx in the three treatment groups was lower than that in the control group. Interestingly, the Ca^{2+} influx in the group treated with Sa12b and amiloride simultaneously was significantly less than that in the control group. Studies have shown that in articular cartilage tissue and IVD, when the pH is within the physiological range, Ca^{2+} has a very high affinity for ASICs and can bind to the outside of the channel holes to close the channels; when pH decreases, H+ binds to acid-sensitive sites on the ASICs, reducing the binding affinity of Ca^{2+} to the ASICs, and Ca^{2+} leaves the channel pore, promoting the opening of the ASICs and entering the cell (Zhang et al., 2020). In addition, scientists speculated that the inhibitory effect of Sa12b on ASICs may occur because Sa12b has two positively charged residues (His5 and Arg9) that can bind to the binding site of the ASIC, especially the central vestibule of the channel, thereby blocking the channel (Hernández et al., 2019). Moreover, related studies have shown that ASIC1 and ASIC3 activate the senescence programming pathway of P53-P21/P27 and P16-RB1 signaling in an acid environment, and the expression of genes P53, P21, P27, P16 and RB1 increases, which promotes human degenerative disc NP- MSCs senescence and degeneration (Ding et al., 2021). The acid environment also regulates the level of reactive oxygen species between NP cells differentiated from human degenerated intervertebral disc NP-MSCs through ASIC1 and ASIC3, activates the NF-κB signaling pathway, and promotes intracellular activation of the NLRP3 inflammation group and IL-1β release, thereby promoting NP degeneration (Zhao et al., 2021). Therefore, we believe that Sa12b is very likely to reduce the Ca^{2+} influx by inhibiting AISCs, reduce the expression of genes related to the aging programming pathway of P53-P21/P27 and P16-RB1 signal transduction, and improve the biological activity of human degenerated intervertebral disc NP-MSCs. And inhibit or avoid activation of NF-κB signal to reduce the expression of inflammatory factors, thereby delaying IVDD.

Our study demonstrated that in the harsh acidic environment ($pH = 6.2$), the inhibition of AISCs reduced the influx of Ca^{2+}, thus improving the biological activities of NP-MSCs, such as proliferation, differentiation, and apoptosis. However, there were still some limitations in our study. First, we only studied the influence of Sa12b on the biological activity of NP-MSCs in a harsh acid environment, so further research on other IVD environments at different pH levels should be conducted. Second, although Sa12b reduces Ca^{2+} influx by inhibiting AISCs, thereby mediating the improvement of the biological activity of NP-MSCs, the specific signal pathways through which Sa12b affects NP-MSCs need further study. Third, we only carried out *in vitro* studies on the biological activity of NP-MSCs in the model of an acidic environment. We did not investigate the effects of various factors *in vivo*. Therefore, animal experiments should be conducted in future studies.

In summary, our study indicated that Sa12b reduces Ca^{2+} influx by inhibiting AISCs in the harsh acidic environment of IVD and may improve the biological activity of NP-MSCs through the Notch signaling pathway. This study provides a new perspective for the biological treatment of IVDD. Sa12b has enormous therapeutic potential for delaying IVDD and improving low back pain.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The First Affiliated Hospital of Anhui Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the

publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HT and JL was responsible for the conception, study design, and final decision of the manuscript. ZW was responsible for the study design, data collection and analysis, and manuscript writing and revision. LH, SuZ, and ShZ contributed to the experimental studies and data collection. HC, HZ, and YL were responsible for the data analysis.

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Cell-Free Fat Extract Prevents Tail Suspension–Induced Bone Loss by Inhibiting Osteocyte Apoptosis

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Introduction: As the space field has developed and our population ages, people engaged in space travel and those on prolonged bed rest are at increasing risk for bone loss and fractures. Disuse osteoporosis occurs frequently in these instances, for which the currently available anti-osteoporosis agents are far from satisfactory and have undesirable side effects. CEFFE is a cell-free fraction isolated from nanofat that is enriched with a variety of growth factors, and we aim to investigate its potential therapeutic effects on disuse osteoporosis.

Methods: A tail suspension–induced osteoporosis model was applied in this study. Three weeks after tail suspension, CEFFE was intraperitoneally injected, and PBS was used as a control. The trabecular and cortical bone microstructures of the tibia in each group were assessed by μCT after 4 weeks of administration. Osteocyte lacunar-canalicularity was observed by HE and silver staining. *In vitro*, MLO-Y4 cell apoptosis was induced by reactive oxygen species (ROSUP). TUNEL staining and flow cytometry were used to detect apoptosis. CCK-8 was used to detect cell proliferation, and Western blotting was used to detect MAPK signaling pathway changes.

Results: CEFFE increased the bone volume (BV/TV) and trabecular number (Tb.N) of the trabecular bone and increased the thickness of the cortical bone. HE and silver staining results showed that CEFFE reduced the number of empty lacunae and improved the lacuna-canalicular structure. CEFFE promoted osteocyte proliferative capacity in a dose-dependent manner. CEFFE protected MLO-Y4 from apoptosis by activating the serine/threonine-selective protein kinase (ERK) signaling pathways.

Abbreviations: BMD, bone mineral density; BS/TV, bone surface/total volume; BV/TV, bone volume; CBS, calf bovine serum; CCK-8, Cell Counting Kit-8; CEFFE, cell-free fat extract; Conn.Dens, connection density; ERK, the serine/threonine-selective protein kinase; FBS, fetal bovine serum; H&E, hematoxylin and eosin; MMP13, matrix metalloproteinase-13; LCN, lacunocanalicular networks; OP, osteoporosis; PFA, paraformaldehyde; PVDF, polyvinylidene difluoride; pERK ERK, phosphorylation; ROSUP, reactive oxygen species; Tb.N, trabecular number; qRT-PCR, quantitative real-time PCR; SDS-PAGE SDS-polyacrylamide gel electrophoresis; TS, tail suspension; μCT, microcomputed tomography; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; SMI, structure model index.

Conclusion: CEFFE attenuated immobilization-induced bone loss by decreasing osteocyte apoptosis. CEFFE increased the survival of osteocytes and inhibited osteocyte apoptosis by activating the ERK signaling pathway *in vitro*.

Keywords: cell-free fat extract, hind limb, osteoporosis, osteocyte, apoptosis, lacunar-canalicular system, ERK signaling pathway

INTRODUCTION

Osteoporosis (OP) is a metabolic bone disease in which bone mass is lost and the bone organic matrix and bone minerals are reduced (Lane et al., 2000), and it is mainly manifested as low bone mineral density and bone tissue structure destruction, which easily leads to fracture with the evolution of the disease (Conference, 2001). Long-term bed rest caused by trauma or spinal cord injury is a major cause of disuse osteoporosis (Bloomfield, 1997), and with the development of the space industry worldwide in recent years, astronaut's bone loss caused by a microgravity environment has gradually attracted attention (LeBlanc et al., 2000). Osteocytes are mechanosensitive cells and can sense mechanical changes in the environment (Klein-Nulend et al., 2012; Klein-Nulend et al., 2013); thus, in paralyzed people and astronauts, hypodynamic situations reduce the mechanical stimuli received by osteocytes and cause abnormalities in bone antiresorptive and bone metabolic activities.

In response to bone loss caused by long-term bed rest and weight loss, previous studies have found that bisphosphonates and traditional antiresorptive agents do not work well in disuse osteoporosis (Li et al., 2004). Therefore, an increasing number of studies have focused on osteocytes to rescue bone loss. Histone deacetylase 5 (HDAC5) in bone marrow mesenchymal stem cells play an important role in controlling bone remodeling. Recent studies used the tetrahedral nucleic acids framework (tFNAs) to transport miR-2861, which could inhibit HDAC5 expression, promoting osteogenic differentiation (Li et al., 2021a; Zhang et al., 2021). Sclerostin, an inhibitor of the Wnt/β-catenin signaling pathway that regulates bone growth, has emerged as an attractive therapeutic target for the treatment of osteoporosis (Suen and Qin, 2016). Dongye Zhang et al. used the sclerostin antibody to retain osteocytic micromorphology and function to rescue bone mass against prolonged mechanical unloading (Zhang et al., 2017; Zhang et al., 2020), while a phase 3 trial reported that patients administered with romosozumab (sclerostin monoclonal antibody) may experience some adverse events, such as nasopharyngitis, arthralgia, and hypercalcemia (Langdahl et al., 2017). Yi-Xian Qin et al. showed that low-intensity, high-frequency loading has the potential to mitigate regional bone loss induced by long-term bed rest (Qin et al., 2019). Likewise, J. Sibonga et al. found that advanced resistive exercise could attenuate bone mineral density defects caused by weightlessness but could not suppress elevated resorption biomarkers (Sibonga et al., 2019). Thus, seeking a better therapeutic strategy for disuse osteoporosis is still a continuously explored process.

Cell-free fat extract (CEFFE) was first described in our previous study (Yu et al., 2018; Xu et al., 2020). CEFFE is extracted from human adipose tissue, which seems to be the most convenient tissue for human separation because of its subcutaneous location, lower amount of trauma caused to the human body, and the need for minimally invasive techniques for the operator. Furthermore, CEFFE is a cell-free liquid that greatly reduces its immunogenicity and ensures its safety during treatment. Our previous studies have found it to have pro-angiogenic activity (Yu et al., 2018) and found that it improved skin flap survival (Cai et al., 2019), improved fat graft survival (Zheng et al., 2019), and promoted the healing of diabetic wounds (Wang et al., 2020; Yin et al., 2020). CEFFE contains cytokines and growth factors such as IGF1, bFGF, and other growth factors, which can promote bone growth (Pedersen and Febbraio, 2012). Therefore, it is worth exploring its therapeutic effect in osteoporosis.

The objective of this study was to evaluate the therapeutic effects of CEFFE in mitigating disuse bone loss in a tail suspension mouse model. Moreover, to investigate the underlying mechanisms by which CEFFE rescues bone mass, apoptosis-related proteins and matrix-degrading proteins were examined, and the ability of CEFFE to inhibit osteocyte apoptosis was evaluated in MLO-Y4 cells *in vitro*.

MATERIALS AND METHODS

CEFFE Preparation

CEFFE was provided by Shanghai Stem Cell Technology Co., Ltd. (Shanghai, China). The extraction of CEFFE was performed as described previously (Qin et al., 2019). In brief, the fresh fat obtained from healthy volunteers was mechanically emulsified after centrifugation and the third aqueous layer is retained after re-centrifugation, filtered using a 0.22-μm filter, and stored at −80 °C. The CEFFE protein concentration was 5,000 μg/ml detected using a bicinchoninic acid assay kit (Beyotime Biological Technology Institution, Shanghai, China).

Animals

The animal operation procedures were approved by the Committee of Ethics on Animal Experiments at the Shanghai Jiao Tong University School of Medicine. Eight-week-old C57BL/6 male mice (Shanghai SIPPR BK Laboratory Animals Ltd., Shanghai, China) were housed individually in a temperature-controlled animal facility with a 12-h light/dark cycle and free access to chow diet and water.

Tail Suspension Mice Model

Each mouse's tail was taped to a rope, and the mice were suspended through a pulley system on the top of a customized

cage. The mice could walk in the cage on their forelimbs, which remained in contact with the cage floor, while their hind limbs remained suspended with their body at a 30° head-down angle to mimic microgravity (Plotkin et al., 2005). Food and water were provided on the cage floor.

Eighteen mice were randomly divided into three groups (n = 6/group): a control group with normal gravity (Normal), a tail-suspended group that was injected intraperitoneal (i.p.) with 250 μl of PBS as vehicle (TS + vehicle), and a tail-suspended group that was injected i.p. with 250 μl [the administration dose of CEFFE was referred to our previous published article (Cai et al., 2019)] of CEFFE (TS + CEFFE) twice a week for 4 weeks after suspension for 3 weeks, and the mice were still in tail suspension during the administration period. The extraction of CEFFE was performed as described previously (Yu et al., 2018). The mice were sacrificed by cervical dislocation after anesthetization with pentobarbital. Left and right tibias were isolated.

Microcomputed Tomography Analysis

Right tibias were scanned by a high-resolution μCT scanner (μCT 80; Scanco, Zurich, Switzerland) to obtain the trabecular and cortical bone microstructure. Tshe scanning parameters were set as follows: voltage, 70 kV; electric current, 114 μA; and resolution, 10 μm per voxel. For trabecular measurements, a region of interest was defined at 1.9 mm from the proximal tibial condyles, immediately distal to the growth plate, and extended to 100 slices. For cortical bone analyses, a region of interest was defined at mid-diaphysis, starting 4.5 mm from the proximal tibial condyles and extended to 100 slices. The microarchitecture parameters included bone volume fraction (BV/TV, %), trabecular number (Tb.N, 1/mm), trabecular separation (Tb.Sp, mm), connection density (Conn.Dens, 1/mm^3), and cortical bone thickness (Ct.Th).

Histomorphological Analysis of the Lacunar-Canalicular System

The left hind limbs were fixed with 4% paraformaldehyde (PFA) for 24 h, followed by running water for 4–8 h, transferred to 10% EDTA (pH 7.4), and placed in a 4 oC refrigerator for decalcification for 28 days. Bone samples were embedded in paraffin and sectioned (5 μm thick). Tissue sections were stained with hematoxylin and eosin (H&E). Images were obtained at 20x and 40x magnification for analysis, and the proportion of empty bone lacunae in each group was counted.

Silver staining was performed as previously reported (Feng et al., 2020). In brief, the sections were deparaffinized and incubated for 55 min in a gelatin solution containing two parts 50% silver nitrate and one part 1% formic acid. Stained slides were then washed in 5% sodium thiosulfate for 10 min and subsequently dehydrated, clarified, and fixed. Consistent cortical regions were selected for assessment in the medial and lateral regions of the femur midshaft in each specimen. Images were obtained at 100x magnification for analysis. In the cortical bone regions, canalicular length was quantified using ImageJ (NIH, Bethesda, and Maryland) (Java 1.8.9_66).

Immunohistochemistry Stain

After deparaffinization and hydration with distilled water, the antigens were retrieved with 0.25% trypsin, and the peroxidase was inactivated (3% H_2O_2). Sections were incubated overnight at 4°C with primary antibodies against cleaved caspase-3 (1:1,000, CST) and matrix metalloproteinase-13 (MMP13) (1:1,000, CST), followed by horseradish peroxidase–conjugated secondary antibody. Peroxidase was reacted with 3,3′-diaminobenzidine. Consistent cortical bone regions were selected in the medial and lateral regions of the femur midshaft in each specimen. Total osteocytes and positively stained cleaved caspase-3 and MMP-13 osteocytes were counted.

Cell Culture

MLO-Y4 cells were generously provided by Dr. Lynda F. Bonewald. MLO-Y4 cells were cultured in *a*-MEM with 5% fetal bovine serum (FBS), 5% calf bovine serum (CBS), and 50 μg/ml gentamicin sulfate under a humidified atmosphere (37 °C, 5% CO_2). Cells were seeded in culture dishes preplated with rat tail type I collagen (Corning Inc., Corning, NY, United States), the medium was replaced every 2 days, and cell passaging was performed when the monolayer of adherent cells reached 80–90% confluence. An osteocyte apoptosis model was established with Rosup (50 μg/ml), and cells were treated with CEFFE (250 μg/ml).

Cell Proliferation Assays

MLO-Y4 cells were seeded in a 96-well plate at 8×10^3 cells per well and maintained in a complete medium. After 12 h, the cells were incubated with different concentrations of CEFFE (0, 25, 50, 100, 250, and 500 ng/ml) for 24 h. A cell Counting Kit-8 (CCK-8; Weiao Biotechnology, Shanghai, China) was used to evaluate cell proliferation. The absorbance spectrum at 450 nm was recorded using a microplate reader, and the absorption spectrum at 620 nm was recorded as the reference wavelength (SpectraMAX i3x; Molecular Devices, Sunnyvale, CA, United States). The data are presented as the ratio of the O.D. value relative to the control group without CEFFE.

Flow Cytometry

MLO-Y4 cells were cocultured with different concentrations of CEFFE (50, 250, and 500 μg/ml), and cell cycle analysis was performed after 24 h. Cultured cells were collected and fixed with 70% ethanol overnight, followed by incubation with RNase A (Beyotime Biological Technology Institution, Shanghai, China) and propidium iodide (Beckman-Coulter, Brea, CA, United States).

Apoptosis of MLO-Y4 cells induced by Rosup (a compound mixture with 4-butylhydroperoxide included) was determined by flow cytometry using the Annexin-V/PI Apoptosis Detection Kit (Becton Dickinson and Co., Franklin Lakes, NJ, United States). In brief, MLO-Y4 cells were stimulated for apoptosis with Rosup, and the experimental group was incubated with CEFFE (250 μg/ml) for 8 h. In subsequent experiments to examine the mechanism of CEFFE anti-apoptosis, ERK, p38, and JNK inhibitors (10 μM), that is, GDC-0994, SB203580, and PD98059, were added to the cells incubated with Rosup and

TABLE 1 | Primers used in real-time PCR.

Gene	Forward primer	Reverse primer
Gapdh	ATGGTGAAGGTCGGTGTGAA	TGAGTGGAGTCATACTGGAACA
MMP13	TTTCTTTATGGTCCAGGCGATGA	AGGCGCCAGAAGAATCTGTC
Sost	AGGCGCCAGAAGAATCTGTC	AGGCGCCAGAAGAATCTGTC
Rankl	CAGCATCGCTCTGTTCCTGTA	CTGCGTTTTCATGGAGTCTCA

CEFFE for 8 h. Then the cells were harvested, washed twice with cold PBS, and labeled with FITC Annexin V and PI in a binding buffer. The cells were then submitted to flow cytometry using a BD LSR Fortessa system (Becton Dickinson and Co.) to detect the fluorescence intensity of the cells. The experiment was repeated three times, and the apoptosis rate (%) of each group was calculated.

RNA Extraction and qRT-PCR

Total RNA from cells was extracted using the TRIzol reagent (Thermo Fisher Scientific, 15596026), according to the manufacturer's instructions. Reverse transcription was performed using a Biomake Supermix Kit (Bimake, Houston TX, United States). Diluted complementary cDNA was analyzed by qPCR using the SYBR Green reagent (Bimake). Quantitative

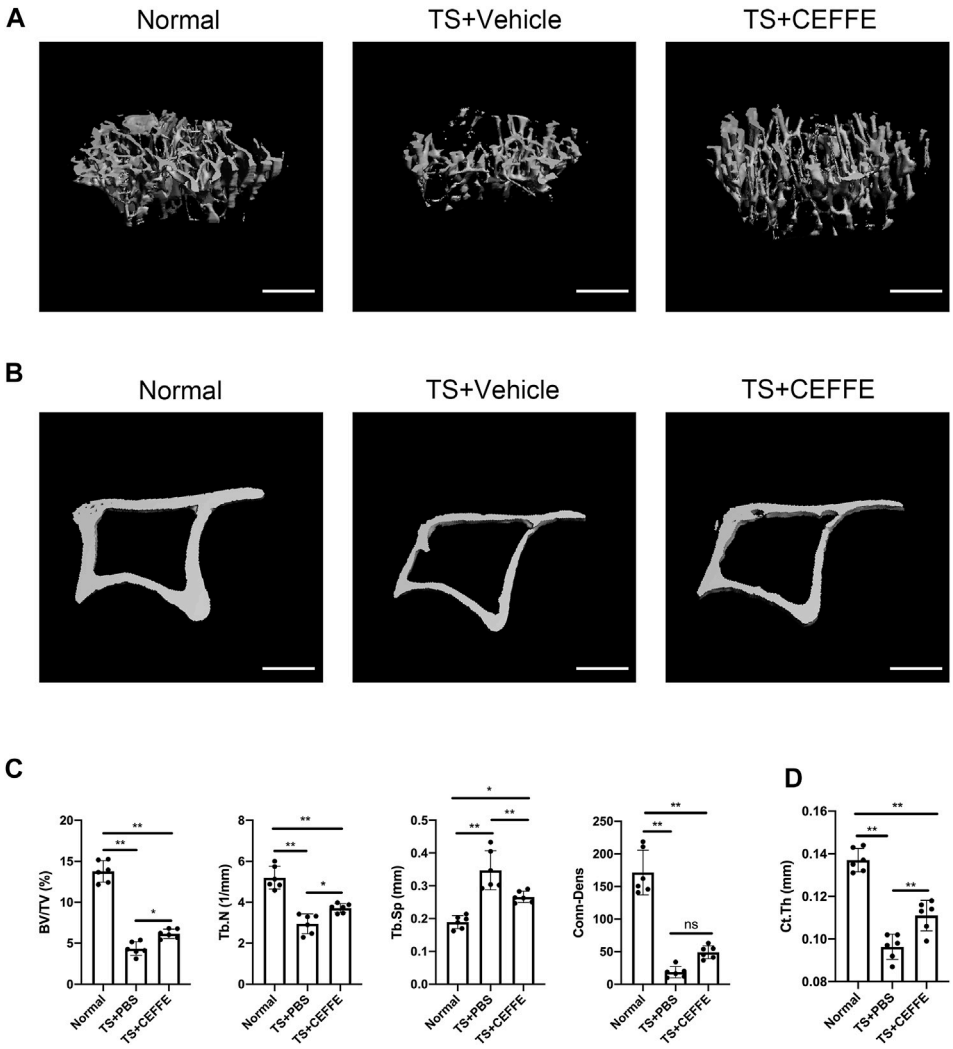

FIGURE 1 | CEFFE improved bone mass in osteoporotic mice induced by the tail suspension model. Three-dimensional (3D) reconstruction from microcomputed tomography (μC+T) scans of proximal tibia from normal mice, mice submitted to tail suspension + vehicle, and mice submitted to tail-suspension + CEFFE **(A)**. Representative μCT images of the cortical bone **(B)**. Quantitative analyses of the trabecular bone of the proximal tibia **(C)**, and the cortical bone thickness in all groups **(D)**. Scale bar, 500 μm $^{*}p < 0.05$, and $^{**}p < 0.01$.

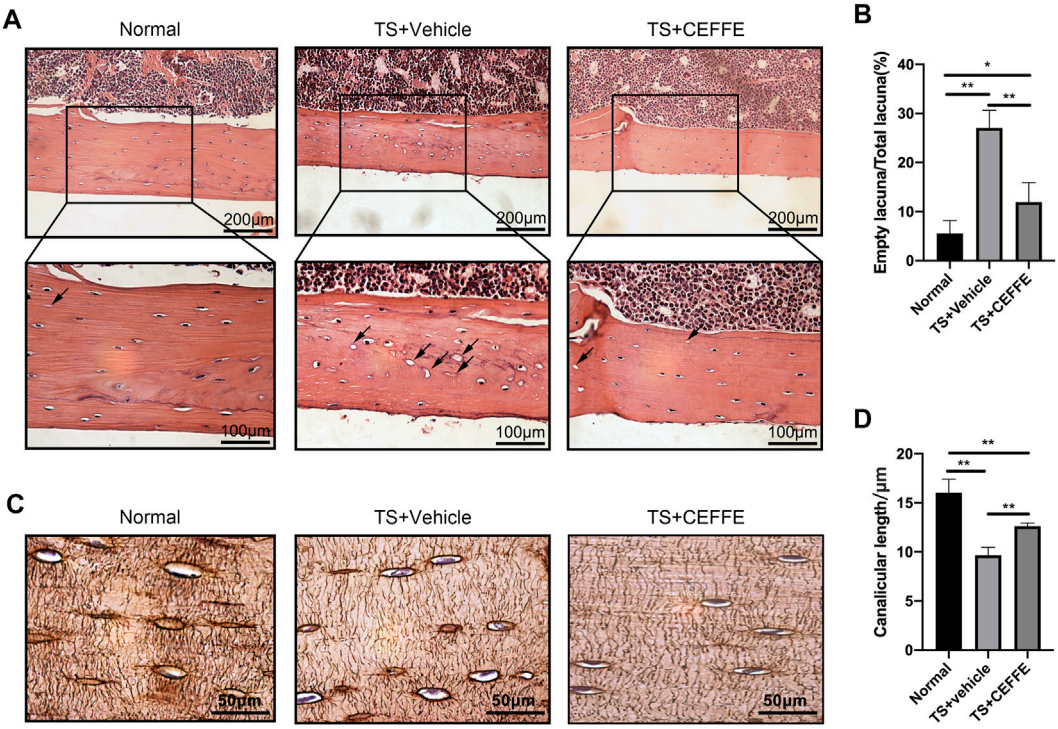

FIGURE 2 | CEFFE improved the lacunocanalicular microstructure in tail-suspended mice. Representative pictures of H&E staining in the femurs **(A)** and quantification of empty bone lacunae **(B)**. Representative pictures of silver staining in the femurs **(C)** and quantification of empty bone lacunae **(D)**. $^{*}p < 0.05$, $^{**}p < 0.01$.

real-time PCR (qRT-PCR) primers used in the study were designed using PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in **Table 1**.

TUNEL Assays

TUNEL assays were performed using a One-Step TUNEL Apoptosis Assay Kit (Beyotime Biological Technology Institution, Shanghai, China) to detect apoptotic cells. In brief, cells were fixed with 4% PFA for 30 min. PBS containing 0.3% Triton X-100 was added and incubated for 5 min at room temperature. Fifty microliters of TUNEL detection solution was then added to the samples and incubated for 60 min at 37 °C in the dark. The slides were mounted with anti-fluorescence quenching mounting solution and observed under a confocal fluorescence microscope. The excitation wavelength range was 450–500 nm, and the emission wavelength range was 515–565 nm (green fluorescence).

Western Blotting

MLO-Y4 cells were seeded in collagen-coated 12-well plates at 2.0×10^5 cells per well, maintained in *a*-MEM containing 0.5% FBS and 0.5% CBS for 3 h, and then transferred into a medium containing 5% FBS, 5% CBS, and CEFFE (250 μg/ml). Total protein was collected from each group of MLO-Y4 cells at different time points (0, 5, 10, 15, 30, and 60 min) after changing the medium. Proteins were cleaved with the SDS lysis buffer (Beyotime Biological Technology Institution), extracted, subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE, 15%), and then transferred to polyvinylidene difluoride (PVDF) membranes. Incubation with the primary antibodies anti-pERK, anti-Erk, anti-p38, anti-p-p38, and β-actin was performed overnight at 4 oC. Subsequently, the membranes were incubated with anti-rabbit IgG (CST, Danvers, MA, United States) for 1 h at room temperature. Finally, protein bands were visualized with an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, United States).

Statistical Analysis

The results are expressed as mean ± SD. All data were analyzed with GraphPad Prism 9 (GraphPad Software, United States), and differences were analyzed by one-way ANOVA, followed by Tukey's post hoc test (group >2). All tests were performed with significance levels of $p < 0.05$ and $p < 0.001$.

RESULTS

CEFFE Improved Bone Mass in Osteoporotic Mice Induced by the Tail Suspension Model

As shown in **Figures 1A,C**, tail suspension caused significant trabecular bone loss compared to wild-type mice. The trabecular bone impairment was ameliorated in the TS + CEFFE group, with increased BV/TV (%), Tb.N (1/mm), and Conn.Dens levels

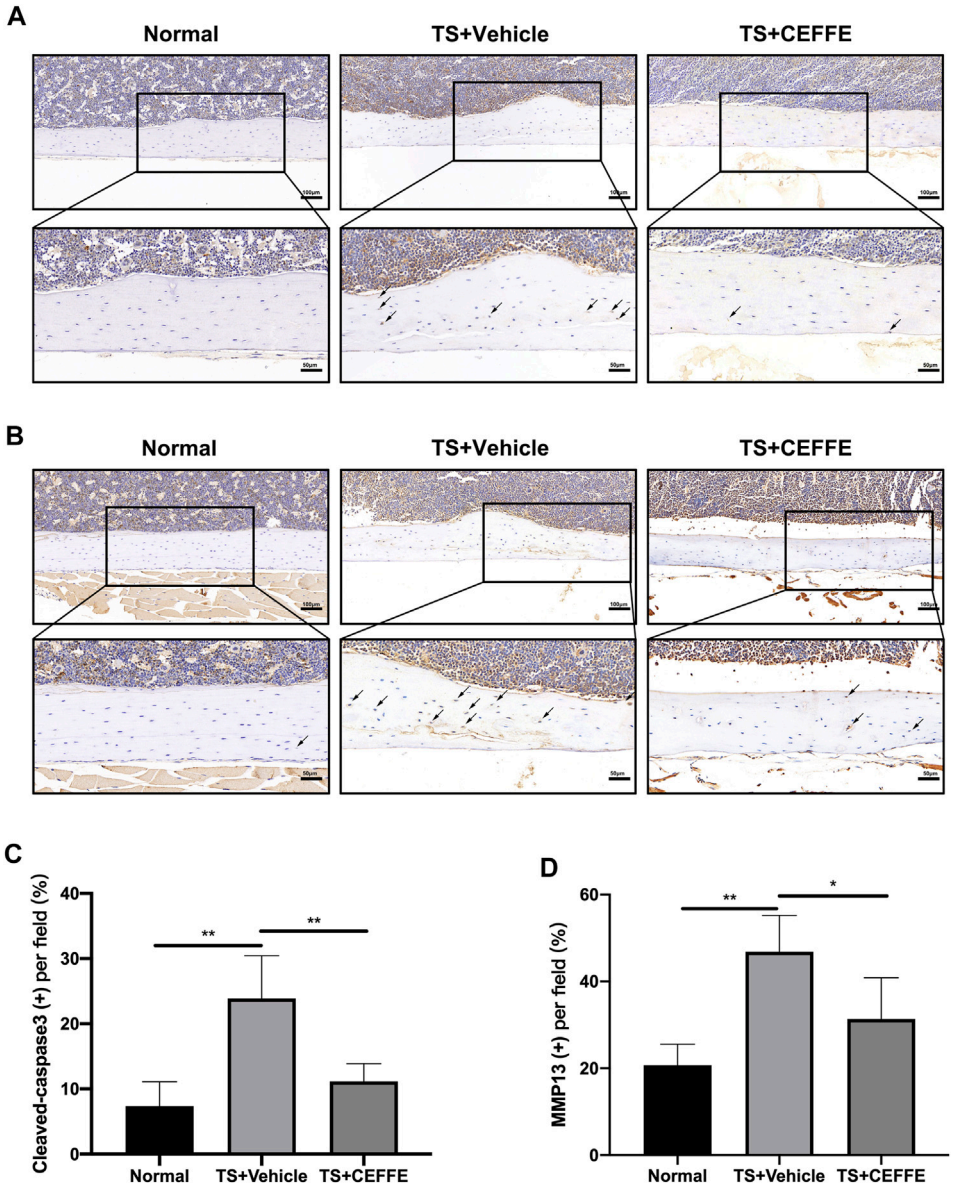

FIGURE 3 | CEFFE attenuated osteocyte apoptosis and extracellular matrix degradation. Immunohistochemical staining and quantitation of femurs shows the expression of cleaved caspase-3 **(A,C)** and MMP-13 **(B,D)**. *$p < 0.05$, **$p < 0.01$.

compared with the TS + vehicle group, but Conn.Dens was not different between the TS + CEFFE and TS + vehicle group. Meanwhile, the Tb.Sp (mm) was apparently decreased (**Figure 1B**). CEFFE also increases the cortical bone thickness of the tibia after tail suspension (**Figures 1B,D**).

CEFFE Improved the Lacunocanalicular Microstructure in Tail-Suspended Mice

H&E staining showed that there were more empty bone lacunas in the cortical bones of tail-suspended mice, which was significantly reduced in the TS + CEFFE group (**Figures 2A,B**). Tail suspension injured the lengths of the lacunar canaliculi in mice according to silver staining of osteocytes, while the TS + CEFFE group obviously improved the shortening of the lacunocanalicular length caused by tail suspension (**Figures 2C,D**).

CEFFE Attenuated Osteocyte Apoptosis and Extracellular Matrix Degradation

Immunohistochemical staining showed that the TS + CEFFE group had lower levels of cleaved caspase-3 expression in osteocytes than the TS + vehicle group (**Figures 3A,B**). This is

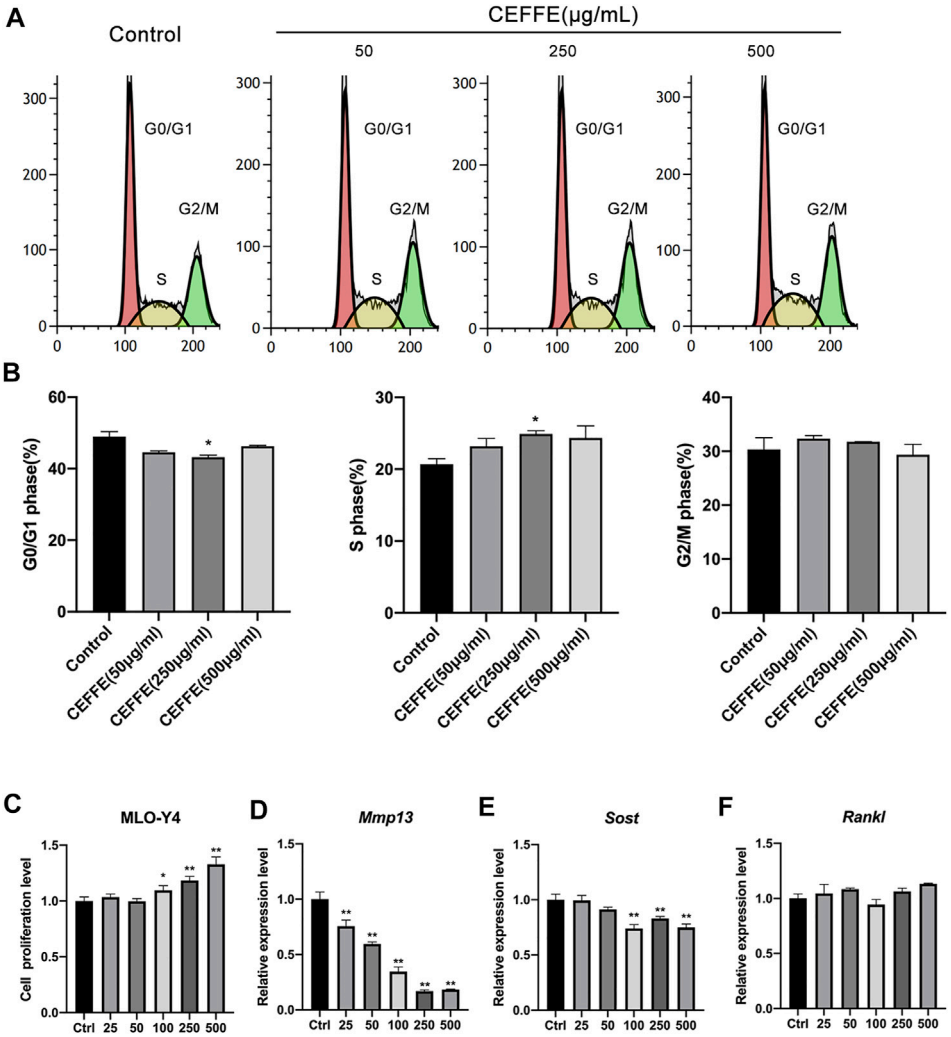

FIGURE 4 | CEFFE promotes the proliferation of osteocytes *in vitro*. Incubation with various concentrations of CEFFE promoted MLO-Y4 proliferation in a dose-dependent manner using flow cytometry **(A,B)** and CCK-8 **(C)**. CEFFE (250 μg/ml) promoted the viability and proliferation of MLO-Y4 cells. Expression of osteocyte-specific and remodeling-related genes, Mmp13 **(D)**, Sost **(E)**, and Rankl **(F)** in MLO-Y4 cells treated with different concentrations of CEFFE for 24 h. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

consistent with previous results illustrating that CEFFE resisted osteocyte apoptosis caused by tail suspension. Another surrogate marker of extracellular matrix degradation, MMP13, was also expressed at higher levels in TS + vehicle group mice than in normal mice but not in CEFFE-treated mice (**Figures 3C,D**). The lower expression of MMP13 may illustrate why the mice in the TS + CEFFE group had a better lacunocanalicular microstructure.

CEFFE Promotes the Proliferation and Reduces the Expression of MMP13 in Osteocytes *In Vitro*

According to the flow cytometry results, CEFFE (250 μg/ml) increased the number of cells in the S phase (**Figures 4A,B**). Likewise, CEFFE promoted MLO-Y4 proliferation in a dose-dependent manner at concentrations greater than 100 μg/ml (**Figure 4C**). After incubation with different concentrations of CEFFE for 24 h, MMP13 expressed by MLO-Y4 was significantly decreased in a dose-dependent manner (**Figure 4D**), and the expression level of SOST was reduced when the concentration of CEFFE was greater than 100 μg/ml (**Figure 4E**); however, CEFFE culture did not affect RANKL expression (**Figure 4F**).

CEFFE Rescued Osteocyte Apoptosis Induced by Reactive Oxygen Species

After treatment with ROS for 8 h, MLO-Y4 showed a marked increase in apoptotic cells according to TUNEL staining and flow cytometry (**Figure 5A**). When incubated with CEFFE during Rosup administration, the number of apoptotic cells was decreased dramatically, and quantitative differences were detected (**Figure 5B**).

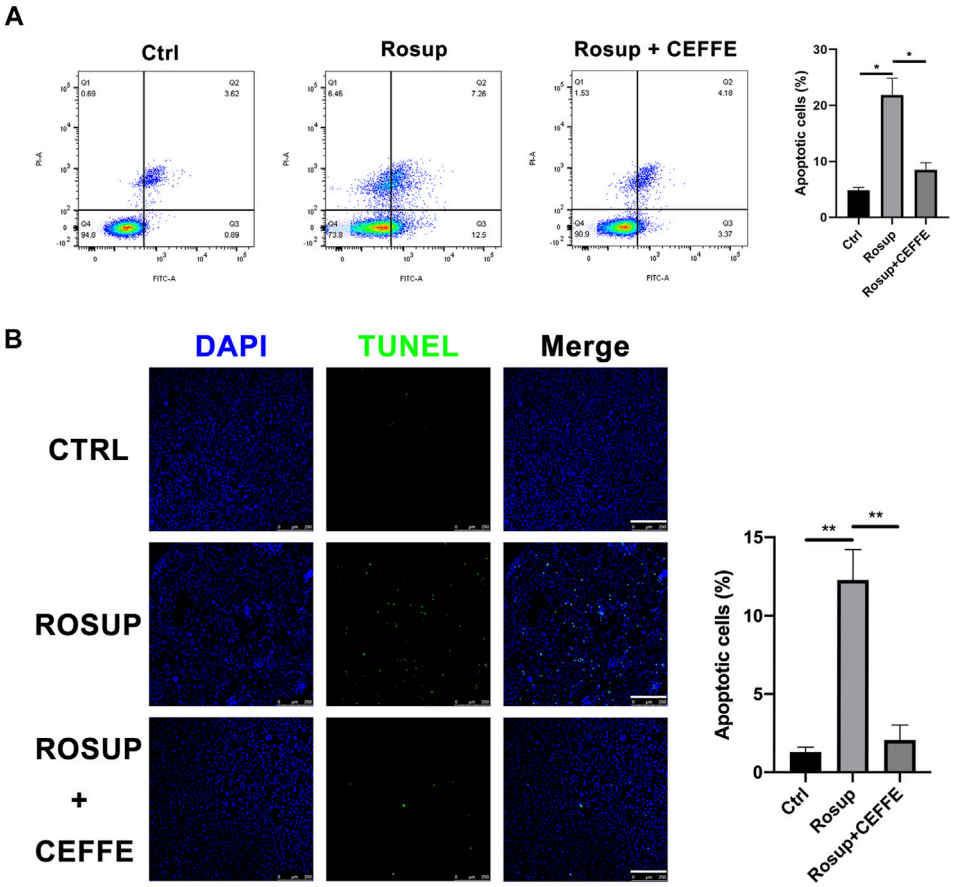

FIGURE 5 | CEFFE rescued osteocyte apoptosis induced by reactive oxygen species. Flow cytometry findings show that CEFFE (250 μg/ml) significantly decreased apoptosis ratios in MLO-Y4 cells induced by Rosup for 8 h **(A)**. Fluorescent green TUNEL staining **(B)**, corresponding blue nuclear counterstaining and merged channels of representative live sections (magnification ×100); ratio (%) of TUNEL-positive cells (N = 3). Scale bars, 200 μm. Data are shown as means ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$ vs control.

CEFFE Inhibited Osteocyte Apoptosis Through the ERK and p38 Signaling Pathways

To further explore the antiapoptotic mechanism of CEFFE, Western blotting was applied to assess activation of the MAPK signaling pathway in MLO-Y4 cells. CEFFE significantly induced rapid increases in the phosphorylation of ERK and p38 at 250 μg/ml, which lasted for more than 1 h (**Figures 6A,B**). Flow cytometry results showed that CEFFE-induced phosphorylation of ERKs was abrogated by treatment of the cells with PD98059 (ERK inhibitors) (**Figure 6C**).

DISCUSSION

In the present study, we demonstrated that CEFFE can rescue tail suspension–induced bone loss and recover the lacunocanalicular microstructure. CEFFE was capable of suppressing the pro-apoptosis of MLO-Y4 cells exposed to reactive oxygen species *in vitro*, which was accompanied by an increase in ERK phosphorylation (pERK). Due to the wild source and the effect of anti-bone loss, CEFFE may have potential in treating osteoporosis.

Since CEFFE is extracted from human adipose tissue, many past studies have shown an inverse correlation between adipose tissue content and bone mass. *In vivo*, adipose tissue can affect the growth and development of bone through endocrine pathways. For example, leptin secreted by adipose tissue promotes adipogenesis and reduces osteogenesis in high-fat diet–induced bone-fat imbalance (Yue et al., 2016). This evidence seems to indicate that adipose tissue has a negative effect on bone formation. However, the relationship between bone marrow formation and adipose tissue accumulation in the bone marrow is not always mutually exclusive. Some recent studies have found that adipose tissue plays an indispensable role in maintaining bone mass. For instance, C3H/HeJ mice have both high proximal tibial rBMAT and bone mass (Scheller et al., 2015), although it is difficult for us to clarify the causal relationship between them. Likewise, some newly identified adipocytokines, such as omentin-1, play an essential role in the maintenance of normal bone mass and are able to alleviate magnesium silicate–induced inflammation and osteoporotic bone loss (Rao

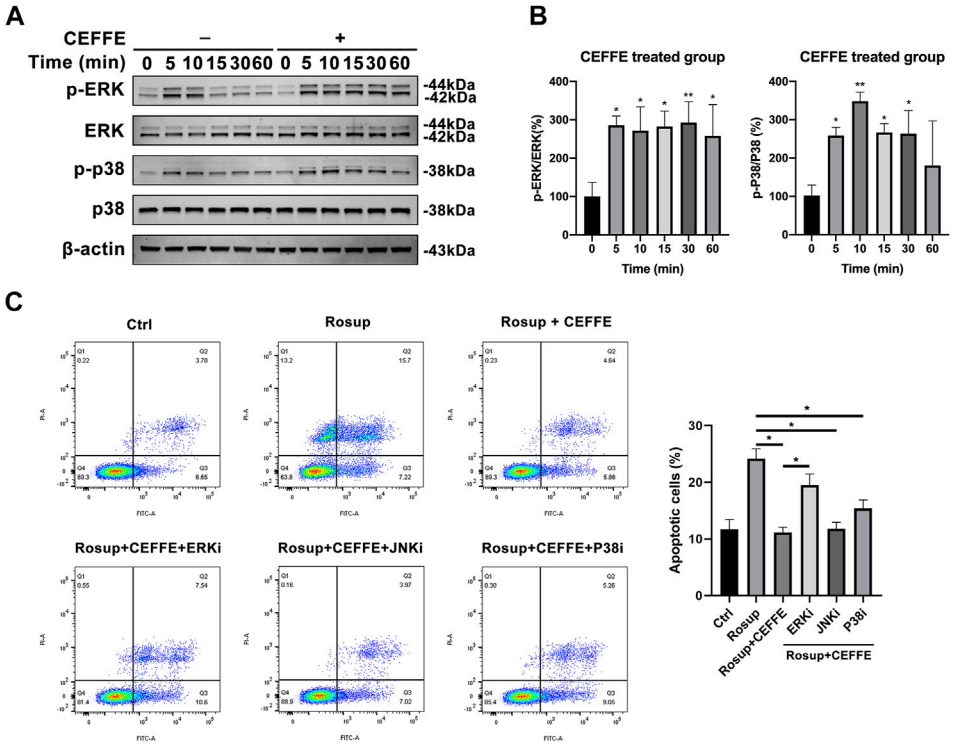

FIGURE 6 | CEFFE inhibited osteocyte apoptosis through ERK and p38 signaling pathway. Cells were incubated with CEFFE (250 μg/ml) for the indicated periods, and proteins were collected within 1 h to assess activation of MAPK signaling pathways, including ERK and p38 **(A,B)**. Incubation with small-molecule inhibitors of MAPK signaling pathways significantly counteracted anti-apoptosis induced by CEFFE **(C)**. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

et al., 2018). Thus, some components in adipose tissue may have a promoting effect on bone mass. Our previous study found that CEFFE is abundant in cytokines, including IGF-1, BDNF, GDNF, TGF-β, HGF, bFGF, VEGF, PDGF, EGF, NT-3, and G-CSF (Yu et al., 2018). These cytokines may affect bone mass; for example, overexpression of IGF-1 upregulated the expression of nuclear β-catenin *via* the AKT pathway, which enhanced cell survival (Lin et al., 2020). Likewise, prior studies validated that VEGF is overexpressed in response to mechanical stimulation and promotes osteocyte survival through a caveolin-1–dependent mechanism (de Castro et al., 2015).

Osteocytes serve as mechanosensitive cells (Li et al., 2021b), and weight loss induces cell apoptosis due to the withdrawal of mechanical stimuli (Basso and Heersche, 2006). These cells have been recognized as multifunctional cells that can regulate osteoclasts through RANKL expression and osteoblasts through sclerostin expression (Robling and Bonewald, 2020); therefore, the death/apoptosis of osteocytes can serve as a signal of resorption and remodeling of bone. According to the immunohistochemistry findings, CEFFE protected osteocytes from apoptosis by reducing the expression of cleaved caspase-3 and MMP13 in cortical bone regions, which indicated that more osteocytes survived and a less number of proteoglycan-rich matrix was degraded (Van Tol et al., 2020). More empty bone lacunae and damaged lacunocanalicular networks (LCN) in unloaded hind limbs of tail-suspended mice were observed, whereas mice treated with CEFFE had more osteocyte survival, and better bone microarchitecture. According to the fluid flow hypothesis, it is difficult for external loading to force bones to deform. Due to the high stiffness of bones, the stress causes the fluid in the LCN to oscillate, and the oscillating fluid flow generates sufficiently strong resistance on the osteocytes to trigger a mechanical reaction (Van Tol et al., 2020). Since mice treated with CEFFE have a more intact LCN structure, osteocytes also have better mechanical sensitivity in this group.

A previous study found that CEFFE significantly upregulates the protein expression of the intracellular antioxidant enzyme glutathione peroxidase-1 and significantly blocks the accumulation of ROS in dermal fibroblasts in UVB-induced cell death (Deng et al., 2019). Yukiko Kitase et al. identified a new function for the muscle-derived metabolite L-BAIBA on osteocyte viability, which protects osteocytes from ROS-induced apoptosis through MRGPRD and through maintaining mitochondrial integrity (Kitase et al., 2018). Therefore, ROS appear to be an intermediate mediator against the protective effects of CEFFE on osteocytes. In a recent study, Rekha Kar et al. found that glucocorticoids activated the MAPK/ERK signaling pathway and increased autophagy and osteocyte survival under oxidative stress (Kar et al., 2019). L. I. Plotkin et al. demonstrated that mechanical stimuli preserve osteocyte viability via activation of the ERK signaling pathway (Plotkin et al., 2005). As a consequence of this finding, we observed that osteocytes treated with CEFFE significantly activated the ERK and p38 signaling pathways. Indeed, rapid phosphorylation of ERK by CEFFE was indispensable for the effects of CEFFE, as their antiapoptotic effects on osteocytes could

be almost prevented by a specific inhibitor of ERK activation, GDC-0994. However, CEFFE contains an abundance of growth factors, including IGF-1, TGF-β, VEGF, HGF, bFGF, and many mitogens. Then, we also showed that cell proliferation was significantly increased after 24 h of CEFFE treatment in a dose-dependent manner. Sost is mainly expressed in osteocytes, exhibiting significant inhibition of osteoblast activity and bone formation *in vivo* (Robling and Bonewald, 2020). Hence, the reduction of sost secreted by osteocytes could regulate the survival and differentiation of osteoblasts. We found that incubation with CEFFE could significantly reduce the expression levels of MMP13 and Sost in MLO-Y4 cells, indicating that CEFFE could indeed reduce the degradation of pericellular matrix by osteocytes and reduce the inhibition of the Wnt/β-catenin pathway by Sost to promote anabolism.

Due to the variety of growth factors, a previous study demonstrated that CEFFE was capable of attenuating ischemic injury and stimulating angiogenesis in ischemic tissues (Qin et al., 2019). Therefore, it is likely that the protective effect of CEFFE on osteocytes reported here is also mediated by attenuating limb ischemia from tail suspension or disuse. Based on these observations, we speculate that CEFFE may exert indirect effects on osteocytes by improving the pericellular microenvironment.

There are still many limitations in our study. In this study, although CEFFE treatment rescued bone loss caused by tail suspension, CEFFE treatment did not completely recover the bone mass because of a short-term CEFFE administration. As the treatment time is extended, the increase in bone mass will be more pronounced. Since CEFFE is a mixture rich in various cytokines extracted from adipose tissue, the specific components in CEFFE that have an effect on osteocyte survival are not yet understood, and the specific mechanism remains to be further studied. There are many causes of osteoporosis, in which osteoclasts are hyperactivated in patients with postmenopausal osteoporosis, and whether CEFFE can treat osteoporosis by affecting osteoclast function deserves further study. People may experience bone loss after discontinuation of many osteoporosis drugs, and whether there is a similar discontinuation response after CEFFE treatment requires further study.

CONCLUSION

In this study, we showed that CEFFE protected against disuse-induced osteoporosis and that CEFFE increases the survival of osteocytes by activating the ERK pathway. Therefore, CEFFE may be used as a potential drug for the treatment of osteoporosis due to its rich source availability, ease of preparation, and absence of immunogenicity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Committee of Ethics on Animal Experiments at the Shanghai Jiao Tong University School of Medicine.

AUTHOR CONTRIBUTIONS

MX, JD, and JC contributed to conceptualization and design, collection of data, and manuscript writing. SZ and SZ helped with data analysis and interpretation. MD and WZ assisted with CEFFE preparation. HL and ZY involved in conceptualization and approval of the final manuscript version.

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SYSTEMATIC REVIEW
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A Decade of Progress in Gene Targeted Therapeutic Strategies in Duchenne Muscular Dystrophy: A Systematic Review

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As one of the most severe forms of muscle dystrophy, Duchenne muscular dystrophy (DMD) results in progressive muscle wasting, ultimately resulting in premature death due to cardiomyopathy. In the many years of research, the solution to DMD remains palliative. Although numerous studies including clinical trials have provided promising results, approved drugs, even, the therapeutic window is still minimal with many shortcomings to be addressed. Logically, to combat DMD that arose from a single genetic mutation with gene therapy made sense. However, gene-based strategies as a treatment option are no stranger to drawbacks and limitations such as the size of the dystrophin gene and possibilities of vectors to elicit immune responses. In this systematic review, we aim to provide a comprehensive compilation on gene-based therapeutic strategies and critically evaluate the approaches relative to its efficacy and feasibility while addressing their current limitations. With the keywords "DMD AND Gene OR Genetic AND Therapy OR Treatment," we reviewed papers published in Science Direct, PubMed, and ProQuest over the past decade (2012–2021).

Keywords: Duchenne muscular dystrophy, DMD, gene therapy, CRISPR/Cas9, exon skipping

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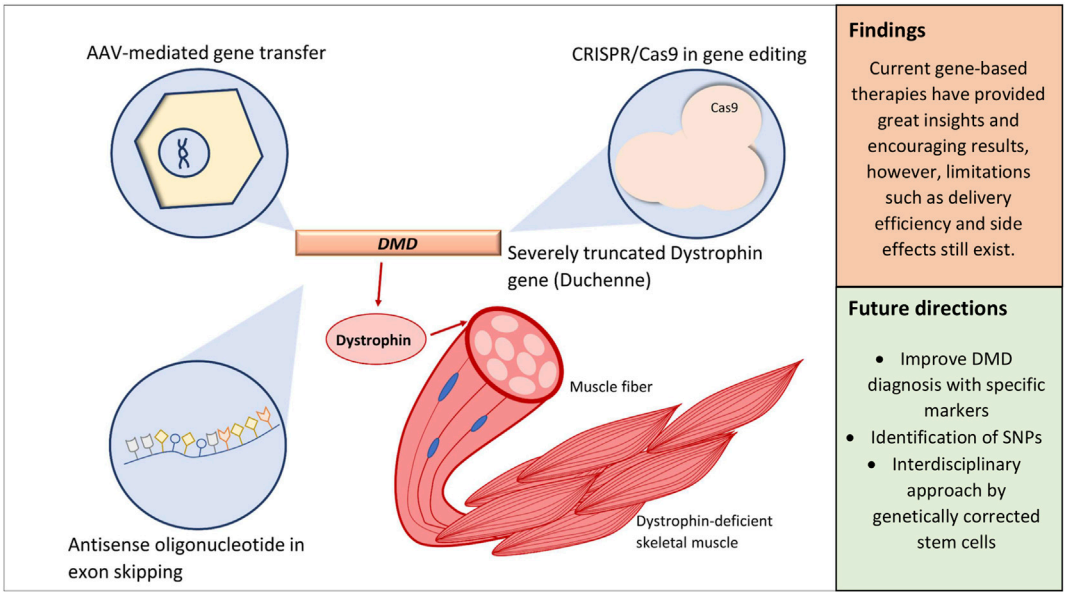
INTRODUCTION

The DMD gene in its entirety covers 2.4 Mb in size, making it the largest known human gene with 79 exons (Gao and McNally, 2015; Kumar et al., 2020). At such size, this gene provides the instruction to give rise to a 427 kD protein, dystrophin, that is integral to sarcolemmal integrity (Hoffman, 2020). Such size also meant that spontaneous mutations occur at a high rate, which is evidenced by *de novo* mutations reported in one-third of cases (Juan-Mateu et al., 2015). In general, large mutations involving deletion or duplication of one of more exons account for an approximate of 60–70%, while the remaining 25–35% are small mutations comprised of missense, nonsense, and frame-shift mutations (Kong et al., 2019). The mutational spectrum of DMD exhaustively varies; however, it was discovered that mutational hotspots exist along the gene, notably duplication clusters in exons 2–10 and deletion clusters in exons 43–55 (Aartsma-Rus et al., 2016; Niks and Aartsma-Rus, 2017; Min et al., 2020). In rare cases, deep intronic copy number variations (CNVs) and mid-intronic mutations that produce cryptic exons or pseudoexons may potentially be deleterious in nature disrupting the normal reading frame of the DMD gene (Khelifi et al., 2011; Trabelsi et al., 2014; Greer et al., 2015; Keegan et al., 2019).

While it is known that DMD is an X-linked recessive disorder that predominantly affects males whereas females largely remain as asymptomatic carriers, the phenotypes of DMD are at times



GRAPHICAL ABSTRACT |

expressed in female carriers manifesting (2.5–10%) muscle weakness and cramps (Lee et al., 2015; Zhong et al., 2019). This can be associated with a skewed inactivation of the X chromosome carrying the normal gene, or in the case of chromosomal aberrations in carriers with Turner syndrome (Giliberto et al., 2014; Lee et al., 2015). Interestingly, the aforementioned association was challenged by a study conducted by Brioschi et al. (2012), in which it was reported that there was a lack of relationship between X-chromosome inactivation and dystrophinopathic phenotype observed in female carriers. The findings, therefore, suggest that the dystrophin level may be the key behind the expression of the DMD phenotype.

This brings us into emphasizing the role of dystrophin and its expression, which, irrespective of gender, has an impact on the phenotype and progression of the disease. The loss of functional dystrophin expression has a direct effect on the dystrophin-associated glycoprotein complex (DGC) resulting in membrane instability and increased susceptibility to injury that will eventually be replaced by fibroadipose tissues (Falzarano et al., 2015; Niks and Aartsma-Rus, 2017). That said, therapeutic strategies focusing on correcting and restoring dystrophin expression have been in development rigorously.

When Rosenberg et al. (1990) first demonstrated a retroviral-mediated gene transduction in modifying tumor-infiltrating lymphocytes (TILs), it was both a breakthrough and a landmark. The study had proven two key events: 1) It is possible to genetically modify human cells, and 2) the approach was safe and feasible that no adverse effects were observed upon the introduction of the modified cell. Cumulatively as of 2017, there are almost 2,600 clinical trials employing gene-based strategies, which are either approved, completed, or still ongoing (Ginn et al., 2018). While success stories along the years were not without setbacks, limitations, and public prejudice, numerous studies emerging in the third decade have achieved in-depth understanding, demonstrating feasibility of gene therapy and at the same time pinpointing current limitations to be revisited and improved.

The trend in current DMD research can be distinguished based on distinct niches: gene therapy, which is of our interest, pharmacological therapy (Guglieri et al., 2017; Bhattacharya et al., 2018), cell therapy (Dai et al., 2018; Siemionow et al., 2018; Jelinkova et al., 2019), improved disease management (Sheehan et al., 2018; Adorisio et al., 2020), and therapy on the downstream pathology of DMD (Guiraud and Davies, 2017). Efforts to combat DMD through gene-based intervention are of exceptional interest as the disease itself is due to single-gene mutations. The idea of correcting specific mutations also draws in opportunities for a personalized approach; for example, the correction of exon 51 via exon skipping is applicable to about 14% of patients (Lim et al., 2017; Dzierlega and Yokota, 2020). These strategies include, but are not limited to, employing the CRISPR/Cas9 system, adeno-associated virus (AAV) vectors, and antisense oligonucleotides (AOs) of various constructs (Łoboda and Dulak, 2020).

In justifying the focus toward gene therapy, this approach allows defective genes to be corrected at an earlier stage (Wolf et al., 2019; Palanski et al., 2020). In contrast, the emerging stem cell-based therapy in DMD, which also predominantly exists in laboratories, is impeded by poor systemic delivery efficacy and reliance of immunosuppressants over graft rejection (Biressi et al., 2020). While sharing almost the same set of challenges, the plasticity and flexibility of gene-based therapies over the maturation of this field have proven its ability to alternatively overcome some of these challenges, providing promise in treating diseases that were once incurable (Dunbar et al., 2018). Although the trajectory of DMD research with regard to gene therapy has

dramatically progressed in recent years, realistic therapeutic efficacy is still minimal considering that challenges and limitations still exist. Therefore, our objective in this systematic review is to provide critical assessment of current gene-based therapeutic strategies while addressing their limitations. Through systematic reviews, relevant data could be reliably curated and analyzed using scientific strategies such as risk of bias analysis and guidelines set. Ultimately, this avoids the reporting of biased results and retains the presentation of high-quality results (Ahn and Kang, 2018; Henry et al., 2018).

MATERIALS AND METHODS

Search Strategy

In this systematic review, we assemble and report in accordance with the guidelines set in Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (**Supplementary Table S1**). For the retrieval of articles, a comprehensive systematic screening through three online databases, Science Direct, PubMed, and ProQuest, was performed in accordance with PRISMA guidelines (Page et al., 2021). Original research articles relevant to gene-based therapeutic strategies on DMD were searched employing the following keywords: DMD AND Gene OR Genetic AND Therapy OR Treatment.

Inclusion and Exclusion Criteria

Full-text articles in English with publication period set from 2012 to 2021 were included. Articles obtained were exclusively original research articles including *in vitro*, *in vivo*, and clinical trials. While adhering to the exclusion criteria (**Figure 1**), the reference lists in systematic reviews were screened to identify potential studies prior to exclusion. Articles relevant to the gene-based therapeutic strategies irrespective of the type of intervention were selected to be evaluated thoroughly and independently by three reviewers.

Data Extraction, Quality Evaluation, and Risk-Bias Assessment

Similarly, data were extracted from each eligible article by three reviewers working independently. The selected articles were screened to meet specific selection criteria. Articles with outcomes that were not relevant or with studies that were out of our scope of interest were excluded. The extracted data are tabulated concisely in the following order: 1) References, 2) Aim, 3) Tested Compound/Treatment Strategy, 4) Method of Intervention, 5) Additional Information, 6) Findings, and 7) Conclusion and Impact on DMD Therapy. The selected studies were validated with a modified version of Office of Health Assessment and Translation (OHAT). The study quality relative to risk of bias was classified as definitely low risk, probably low risk, probably high risk, definitely high risk, and not applicable concerning the following fields: reporting bias, performance bias, detection bias, and selection bias.

RESULTS AND DISCUSSION

Search Result and Study Characteristics

The initial search has identified 36,590 articles, to which only 960 remain after removal based on the criteria mentioned; duplicates were also removed in this stage. After initial screening for eligibility, 79 (8.2%) articles were retained for further evaluation of the title and abstract on the basis of relevance. Of these 79, 42 (53.2%) met all the eligibility criteria set and hence were included (**Figure 1** and **Supplementary Table S2**). Pertaining to our selection strategy based on relevance, only studies centered on gene therapy on DMD were selected, while articles with only brief mentions of the aforementioned focus, genetic interventions on other types of muscular dystrophies, and studies on cellular signaling events in DMD were deemed as articles with outcomes not relevant.

In summary, there are 23 studies on exon skipping, 8 on gene editing, 5 on gene transfer as means of intervention, 5 studies that used microdystrophin gene therapy, and 1 on RNA interference (**Supplementary Table S3**). It is, however, necessary to underline that the frequency of studies does not indicate superiority over any of their counterparts. Most of the studies were performed *in vivo* (n = 22; 52.4%), while 13 (31%) were performed in combination of *in vitro* and *in vivo*. In one of the studies, *in silico* analysis was performed in order to predict individual exon skipping efficiencies prior to selecting the AOs for *in vitro* and *in vivo* studies (Echigoya et al., 2017).

Quality Evaluation

Upon evaluation, all of the studies show low risk of bias. However, it is noteworthy that although the study by Hayashita-Kinoh et al. (2015) did not perform randomization of microdystrophin gene administration on the canine models, it was ruled to be of low bias as the parameters and the result reported were all quantitative. Out of 42 studies, 9 were evaluated to carry the possibility of a low-risk bias (+), which were all due to indirect and unclear mention of statistical tests performed, therefore also slightly affecting the confidence of the outcome assessment of 4 studies (**Supplementary Table S4**).

Exon Skipping

DMD pathology is directly associated with loss of dystrophin expression due to mutations of the normal reading frame. Restoration of the disrupted reading frame can be achieved through exon skipping that utilizes AOs, short single-stranded deoxynucleotides that induce endonuclease-mediated knockdown in the DMD transcript by targeting dystrophin pre-mRNA and inhibit mRNA translation. Consequently, this mechanism converts the severe out-of-frame mutation into an in-frame mutation (Crooke, 2017; Rinaldi and Wood, 2018). In this approach, functional dystrophin, though still internally truncated, can be produced, essentially converting the DMD phenotype to the milder Becker muscular dystrophy (Findlay et al., 2015). Given such specificity, exon skipping can be personalized to benefit larger groups of DMD patients. Eteplirsen, which is an AO that was granted accelerated

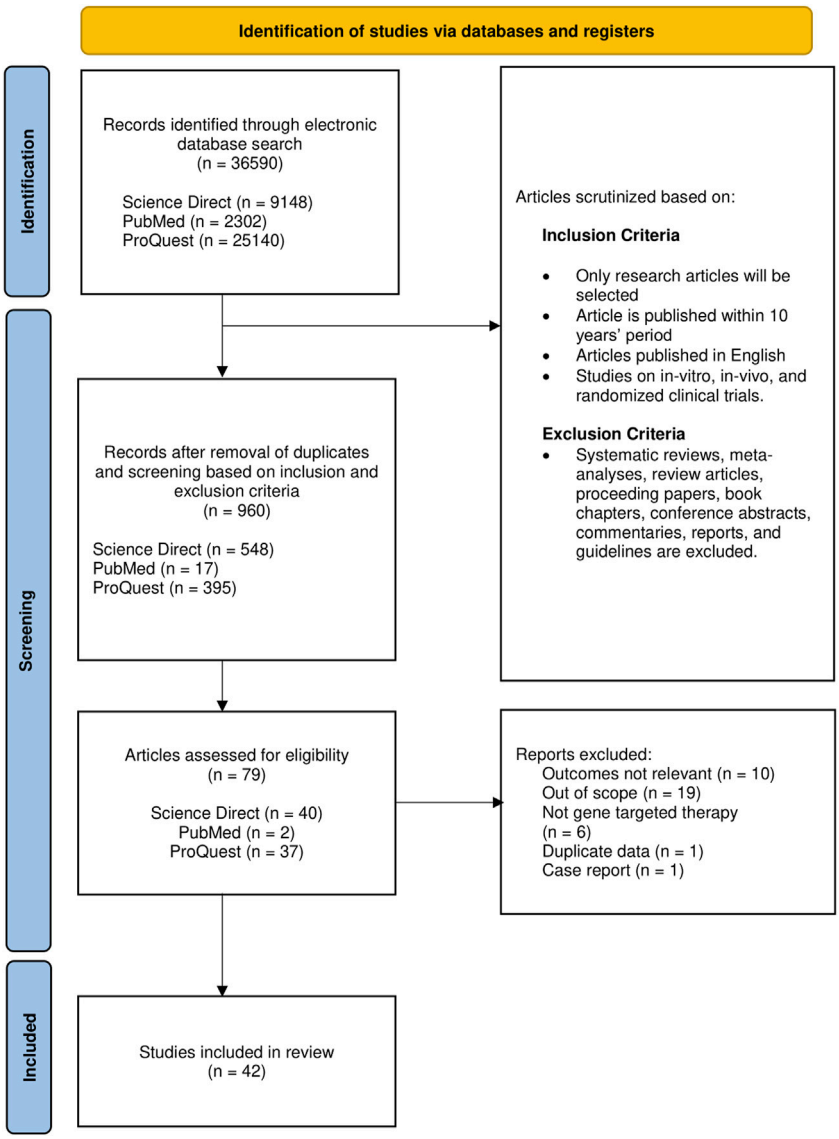


FIGURE 1 | Flow chart representing article selection process based on PRISMA.

approval by the FDA in 2016, induces exon 51 skipping that could benefit around 14% of DMD patients (Syed, 2016; Łoboda and Dulak, 2020). However, the efficacy of Eteplirsen is rather controversial as claims of Eteplirsen demonstrating significant dystrophin restoration in phase III of the PROMOVI trial (NCT02255552) were only evidenced by 12 evaluable patients (Syed, 2016; Akpulat et al., 2018). The relatively short half-life of AOs and its rapid clearance from the circulation were two challenges that were continuously addressed; the solution would be to increase AO dosage, but this, too, is faltered by excessive cost. To remedy this issue, structural modification such that was done by Akpulat et al. (2018) in shortening the commercial 30-mer Eteplirsen into 25-mer AOs could allow cost-efficient administration of higher doses.

One clear advantage of AOs in exon skipping provides the basis for modeling, significantly enhancing its ability to be tailored with, thus improving delivery efficiency and exon skipping efficacy. In this review, 15 out of 23 studies have employed various constructs of different backbones comprising of either phosphorodiamidate morpholino oligomers (PMOs) (Akpulat et al., 2018; Lee et al., 2018), 2′-O-methyl phosphorothioate (2OMePS) AO (Van Putten et al., 2019), 2′-deoxy-2′-fluoro phosphorothioate (2FPS) AO (Jirka et al., 2015), or 2′-O-methoxyethyl oligonucleotide (MOE) (Yang et al., 2013). The results were encouraging, supporting the use of these constructs as possible alternatives. However, it is noteworthy to mention that 2FPS necessitates improvement in the future due to its contradicting results—2FPS had outperformed 2OMePS in

the *in vitro* evaluation but was not tolerated in the mdx mouse model (Jirka et al., 2015).

In addition, delivery efficiency was addressed in several studies that introduces the conjugation of these oligomers with peptides, and with the use of delivery agents such as saponins and aminoglycosides (Wang et al., 2018a; Wang et al., 2018b; Wang et al., 2019). While peptide-conjugated PMOs had enabled significant dystrophin overexpression, this approach still requires optimization due to its renal toxicity that prevents escalations in dose-dependent therapies (Roberts et al., 2020). Concordantly, in a study investigating the efficacy of the peptide-conjugated PMO, Pip6a-PMO, death of 5 mdx mice was reported immediately after repeated injections (Blain et al., 2018).

As an amphiphilic naturally occurring compound, saponin has attracted the potential as carriers in drug delivery where studies have reported improved bioavailability (Liao et al., 2021). In exon skipping, the interesting use of saponins as vehicles was first demonstrated and had provided exciting results, digitonin being the most effective. Wang et al. (2018a) reports a 26-fold increase in digitonin-mediated delivery *in vitro* with no obvious cytotoxicity.

Gene Transfer (AAV and Microdystrophin)

Systemic delivery of functional gene aimed to restore dystrophin expression, most utilizing AAV as a means of mediated delivery, is another interesting chapter broadly studied since it promises high transduction efficiency and stable long-term expression (Kimura et al., 2019; Łoboda and Dulak, 2020). Ideally, recombinant AAVs are best suited for DMD therapy as they exhibit strong tropism toward skeletal muscles (Wasala et al., 2019; Muraine et al., 2020).

Realistically, AAV-mediated gene transfer is impeded by the large size of the dystrophin gene, which is almost three times the capacity of a single AAV virion's payload (Colella et al., 2018). Realizing such limitations, dual AAVs were adopted along with the notion, from studies in animal models, that functional, though internally deleted dystrophin genes 6–8 kb in size are sufficient for body-wide dystrophin expression (Zhang and Duan, 2012; Duan, 2018). The latter option is seen to be more prominently studied. Specifically, Mendell et al. (2020) demonstrated the intravenous infusion of rAAVrh74. MHCK7. micro-dystrophin in ambulatory boys aged 4 to 7 up to which the results have shown robust transgene expression. In the 3-year follow-up, it was noted that the vector was well tolerated with minimal adverse effects. In addition, Hayashita-Kinoh et al. (2015), in an *in vivo* study of intra-amniotic administration of rAAV-CMV-microdystrophin in canine model, yielded long-term transgene expression and improvements to cardiac function.

In reference to heart failure as the main cause of death in DMD, the potential of this approach in ameliorating heart disease is explored by Bostick et al. (2012). An AAV9-mediated microdystrophin therapy was performed in end-stage models of cardiomyopathy in mdx mice. The study, although did not show significant improvements despite robust expression of micro-dystrophin in treated mice, revealed limitations that served as important findings. In terminally aged mdx mice, the benefit of such an approach is incomparable to what is administered in models of younger age. Indirectly, it answers the question of whether patients in advanced stages of the disease may still benefit from micro-dystrophin therapy, to which the answer is two-pronged: 1) Obviously it would be difficult to induce dystrophin expression in late stages of the disease where fibroadipose tissues would have already overwhelmed cardiomyocytes; however, 2) if the goal is to only alleviate heart disease, such an approach provides promise as a supplement to other interventions. The latter was proposed by Kolwicz et al. (2019) in a novel study employing cardiac troponin T-driven ribonucleotide reductase (RNR) gene transfer. As RNR solely is incapable of inducing statistically significant results, the authors suggested its role as supplemental to microdystrophin treatment.

Besides introducing micro-dystrophin, 2 studies have explored similar AAV-mediated delivery of the beta-1,4-N-acetyl-galactosaminyltransferase 2 (B4GALNT2) gene (formerly known as GALGT2) (Chicoine et al., 2014b; Xu et al., 2019). Xu et al. (2019) reported significantly improved cardiac output following the overexpression of GALGT2; however, Chicoine et al. (2014b) found no significant changes to the DMD phenotypes despite robust expression. It was revealed that there remains a possibility of preexisting antibodies triggered during the study. Pertaining to this obstacle, investigation on whether the removal of AAV binding antibodies would sustain and improve gene expression was necessary. In an attempt by Chicoine et al. (2014a), the removal of suspected AAV binding antibodies had improved gene expression by 4-fold.

Gene Editing Utilizing CRISPR/Cas9

The application of gene editing, specifically CRISPR/Cas9 system, can accurately correct gene mutations by using either a homology-directed repair (HDR) or nonhomologous end joining (NHEJ) approach (Mollanoori et al., 2021). This approach originates from the bacterial immune system against viruses, in which its discovery was adopted as a tool for gene editing. CRISPR/Cas9 is comprised of a CRISPR-associated endonuclease (Cas) and a guide RNA (gRNA) where both would form a Cas9-gRNA complex known as a ribonucleoprotein directed toward the target DNA. Upon recognition, RNA-guided endonuclease Cas9 produces site-specific double-strand breaks. During the formation of this RNA–DNA heteroduplex, nucleic acid recognition takes place (Palermo et al., 2018). The conformational dynamics within this heteroduplex is influenced by the bi-lobed architecture of the Cas9 protein that consists of recognition lobes (REC1-3) that mediate binding and a nuclease lobe with domains RuvC and HNH responsible in controlling cleavage activity (Nishimasu et al., 2014; Jiang et al., 2015; Sternberg et al., 2015).

With the capacity to permanently edit specific genes, numerous studies utilizing CRISPR/Cas9 to correct DMD mutation have been performed in recent years. Gene editing with regards to DMD is at large focused at generating an in-frame mutation similar to that of BMD (Chemello et al., 2020). Lattanzi et al. (2017) demonstrated restoration of wild-type dystrophin following the removal of a duplicated exon 2 by a single administration. Remarkably, no off-target effects were detected, although it was only expressed in 5% of total cell population. While delivery efficiency was not addressed, it

remains one of the many challenges of the CRISPR/Cas9 approach (Uddin et al., 2020). To drive better delivery, AAV is considered as a vehicle such that is performed by Koo et al. (2018) taking into advantage the tropism exhibited by AAV9. However, relying solely on AAV delivery poses more challenges such as preexisting immunity and possibilities of off-target effects from sustained expression (Lee et al., 2017). In spite of that, AAV vectors are still considered the key delivery vehicle due to their high efficiency (Xu et al., 2019).

As proposed by Lee et al. (2017), gold-conjugated delivery in lieu of viral delivery vehicles prompted HDR repair restoring wild-type dystrophin; however, they are still observing its cytotoxicity. Significant elimination of potential toxicity of mediated delivery was seen in the use of extracellular nanovesicles where little to no off-target cleavage was observed while inducing permanent exon 45 deletion (Gee et al., 2020). The approach is nontoxic, although it was noted that the expression of its single guided RNA was driven by HIV-1 Tat, posing the risk in the event of nonspecific incorporation. It was also reasoned that the limited capacity of AAV may hinder the delivery of the various components of CRISPR/Cas9, but ultimately proven that payload limitation could be resolved using significantly smaller protein such as SaCas9 (Duchêne et al., 2018).

One of the main concerns on gene editing via CRISPR/Cas9 lies in its off-target effects with frequencies of 50% or more (Zhang et al., 2015; Uddin et al., 2020). To remedy this, modifications were suggested such as desensitization of the REC3 as witnessed in the variants of Cas9 (Valkuskas et al., 2018). As an extension to the conformational dynamics mentioned earlier, it was revealed that REC participates in subsequent conformational activation of the highly flexible HNH domain (Sternberg et al., 2015). To prevent the possibilities of off-target genome editing, alterations to REC3 prevent downstream conformation in the HNH domain when bound to off-targets (Chen et al., 2017; Han et al., 2020). This was also demonstrated in a study by Koo et al. (2018) utilizing highly specific CjCas9.

From Drug Prescription to Gene-Based Therapies

Conventionally, the gold standard of DMD drug therapy is the administration of glucocorticoid steroids, either prednisone or deflazacort, that are routinely prescribed to prevent inflammation-associated tissue damage and, to an extent, effectively delay disease progression (Kharraz et al., 2014; Zhang and Kong, 2021). Glucocorticoids, as demonstrated by Morrison-Nozik et al. (2015), were able to enhance muscle strength and provide ergogenic effects. Furthermore, it was established that the downstream cascade after glucocorticoid receptor-mediated activation of Kruppel-like factor 15 (KLF15) mitigates dystrophic severity of the disease (Morrison-Nozik et al., 2015; Ahlskog et al., 2019). Although the findings illuminate the relationship between glucocorticoids and KLF15, and possibly the potential to improve DMD pathology in the event of overexpression, it was also noted that the downstream targets of this mechanism are insufficient (Chen et al., 2000; Morrison-Nozik et al., 2015).

Prolonged glucocorticoid treatment induces adverse effects including delayed puberty, adrenal insufficiency, cataracts, osteoporosis, and obesity (Zhang and Kong, 2021). To date, the most widely used regimen for DMD are corticosteroids routinely prescribed along with proper patient management in delaying the disease progression (Colella et al., 2018). While groundbreaking research and clinical trials in recent years have made interesting discoveries, it is still evident that there remains no cure for DMD yet. Now, with new platforms that provide intense research in gene-based strategies, the capacity of such approach in curing DMD demonstrates great potential. In this systematic review, we aimed to provide an overview of the most recent strategies in research, and highlighting the success, potential, and obstacles in each approach.

From our initial search until the inclusion of eligible articles, it is noticed that exon skipping remains fairly popular in research. This is evidenced by the indefinite avenues for engineering PMO constructs by attaching different backbones and conjugating the oligomers with various molecules that could enhance delivery and exon skipping efficiency. As discussed earlier pertaining to overcoming obstacles in AAV-mediated interventions, the incorporation of bioactive molecules in PMOs as carriers may significantly enhance exon skipping. In addition, antisense RNA sequences such that of U7snRNA were found to be exceptionally feasible considering their minimal efficacious dose establishing near full-length dystrophin restoration in both skeletal and cardiac muscles (Simmons et al., 2021).

Despite that, persisting challenges in exon skipping include chemical-dependent toxicity in the case of conjugated PMOs and short-lived effect *in vivo*. Several studies have already addressed the former obstacle by minimizing toxicity, while the latter, though interestingly preserved when delivered with a viral vector, posed another challenge with regard to AAV-mediated efficiency. As thoroughly discussed in the results, there are possibilities that viral vector-mediated approaches could be rendered ineffective due to preexisting humoral immunity (Giles et al., 2020). It is complicated since the full extent of such an immune response may not be fully reflected in *in vivo* studies employing DMD models as noted by Chicoine et al. (2014b). Surprisingly, regardless of such risk, there are still ongoing studies because AAV-mediated exon skipping provides the highest efficacy in dystrophin recovery. In this critical evaluation of gene-based therapies, it is eminent that with every proposed solution comes two obstacles. It is also necessary to underline that the objective of most therapies is to effectively dial down the severity of DMD to that of BMD's, and this too is faced with challenges. In our view, there are still avenues to which future research could expand on in CRISPR/Cas9. Several risks and challenges were already addressed with proposed modifications as previously mentioned. Owing to its relatively cheap cost and high specificity, it is opined that CRISPR/Cas9, though relatively new, may provide better opportunities in DMD treatment.

In summary, we provide a panoramic overview of the gene-based approaches in DMD treatment in the last decade to highlight individual progress and notable results while remaining critical in evaluating each approach. More importantly, we have highlighted the current limitations

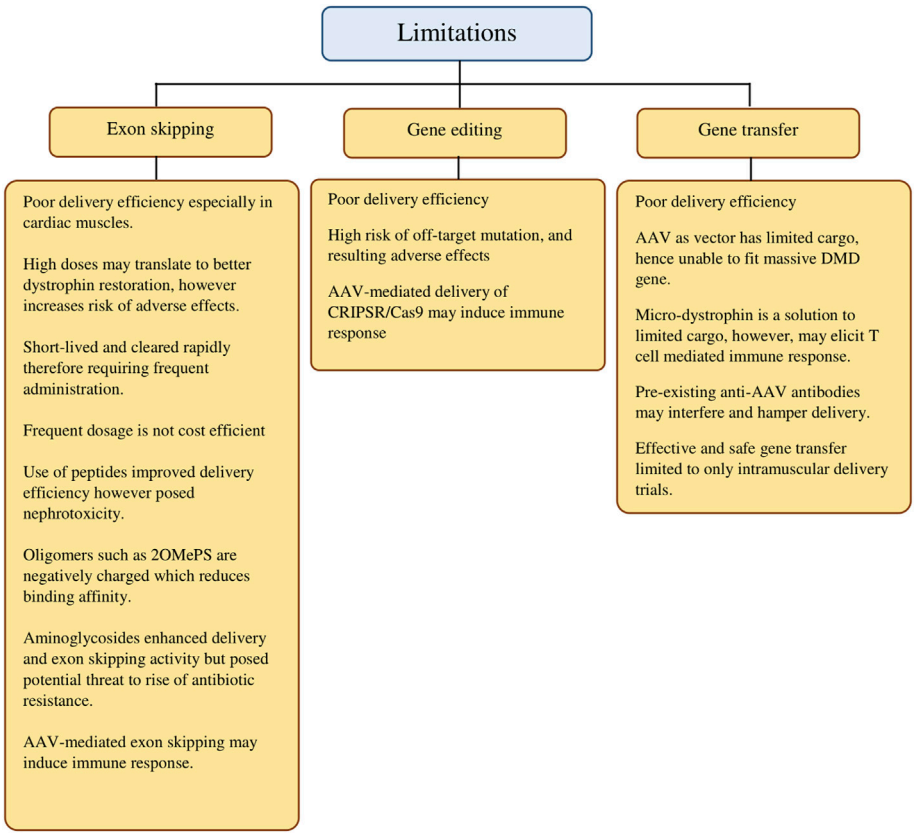


FIGURE 2 | Current limitations of gene-targeted therapeutic strategies in DMD treatment.

(**Figure 2**) and noted on alternatives evidenced by findings from the selected studies that were elaborated earlier. In addition, we intend to provide a concise point of reference that studies in the coming years may revisit the current shortcomings with more effective solutions.

Limitations of the Study

In this review, we recognize our limitations. We acknowledge the high number of articles that are deemed relevant. This is also due to our relatively large focus—gene-based interventions and therapies. That said, we have attempted to minimize the said limitation by having three authors to independently screen, select, and extract data, and subsequently also performing a risk-bias assessment on the selected articles. Having to include only original research articles of such focus, we acknowledge that at the time of writing, more recent studies are performed or are in the process of publication, which meant that key findings might have been missed out.

FUTURE RESEARCH DIRECTIONS AND PERSPECTIVES

Irrespective of the approach, the continuous advancement of technology and better grasp of novel techniques may provide answers to questions today with regard to improving overall efficacy and efficiency while not discounting the need to prioritize safety by scrutinizing possible adverse effects in long-term exposure of any of these gene-based approaches. With respect to CRISPR/Cas9, there have been already promising results in strengthening further research efforts in this direction. In line with that, we hope that the controversies surrounding the use of CRISPR/Cas9, especially the public's view, will be cleared off by outcomes of future clinical trials. Nevertheless, further work on improving delivery efficiency, feasibility, and safety of other approaches may also bring promise to other gene-based approaches. To this, we are looking forward to efforts in elucidating immunological responses toward various vector or vehicle-mediated delivery in microdystrophin gene delivery and AOs, performed both *in silico* and *in vivo* in larger samples of mice and nonhuman primates.

In addressing DMD as a whole, it is of paramount importance to provide better diagnosis of DMD and the need of discovering specific markers for accurate assessment. Proper assessment, in this sense, also meant the inclusion of data in real time, however requiring updated and routine data collection from various healthcare sources globally to provide better understanding in patient management and clinical progression of the disease. Stressing on the mutational spectrum of DMD, we hope that rigorous in-depth analysis can account for a personalized

medicine approach for inter-individual variability through means of identifying single nucleotide polymorphisms (SNPs). At the same time, we look forward to interdisciplinary research focused on the transplantation of patient-derived stem cells corrected either by exon skipping or the CRISPR/Cas9 system.

In conclusion, we hope that future research and clinical trials may provide a definite cure, if not better approaches, that will ultimately improve the quality of life for DMD patients.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LC conceived the concept and layout, and wrote the first draft of the manuscript. LC, MY and NS were responsible for preliminary data curation and extraction of screened data independently. LC performed risk-bias analysis, and the results were validated by MY and NS. Revisions and corrections were advised by MY and NS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.833833/full#supplementary-material

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REVIEW
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Adipokine Signaling Pathways in Osteoarthritis

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Osteoarthritis (OA) is a debilitating joint disease that affects millions of individuals. The pathogenesis of OA has not been fully elucidated. Obesity is a well-recognized risk factor for OA. Multiple studies have demonstrated adipokines play a key role in obesity-induced OA. Increasing evidence show that various adipokines may significantly affect the development or clinical course of OA by regulating the pro/anti-inflammatory and anabolic/catabolic balance, matrix remodeling, chondrocyte apoptosis and autophagy, and subchondral bone sclerosis. Several signaling pathways are involved but still have not been systematically investigated. In this article, we review the cellular and molecular mechanisms of adipokines in OA, and highlight the possible signaling pathways. The review suggested adipokines play important roles in obesity-induced OA, and exert downstream function *via* the activation of various signaling pathways. In addition, some pharmaceuticals targeting these pathways have been applied into ongoing clinical trials and showed encouraging results. However, these signaling pathways are complex and converge into a common network with each other. In the future work, more research is warranted to further investigate how this network works. Moreover, more high quality randomised controlled trials are needed in order to investigate the therapeutic effects of pharmaceuticals against these pathways for the treatment of OA. This review may help researchers to better understand the pathogenesis of OA, so as to provide new insight for future clinical practices and translational research.

Keywords: adipokine, osteoarthritis, signaling pathway, cartilage, degeneration, obesity

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1 INTRODUCTION

Osteoarthritis (OA) is the most prevalent arthritis worldwide and a leading cause of pain and physical disability in nearly 10% of males and 18% of females aged 60 years and older (Glyn-Jones et al., 2015). OA is a degenerative disease of the entire joint and occurs most commonly in the hip and knee, characterized by a gradual loss of articular cartilage and remodeling of the subchondral bone (Hochberg et al., 2013). The symptoms and signs of OA include pain, stiffness, deformity and disability, which significantly affect the quality of life of patients (Sharma, 2021). It was estimated that from 1990 to 2019, the number of people affected by OA increased by 48% (Hunter et al., 2020). It should also be noted that the incidence of OA is rising even among young and physically active people (Long et al., 2020).

The pathogenesis of OA has not yet been fully elucidated. Old age, female sex and obesity are primary risk factors of OA (Collins et al., 2020). Obesity is one of the modifiable risk factors for OA, and was conventionally believed to cause OA *via* the increased mechanical loading on weight-bearing

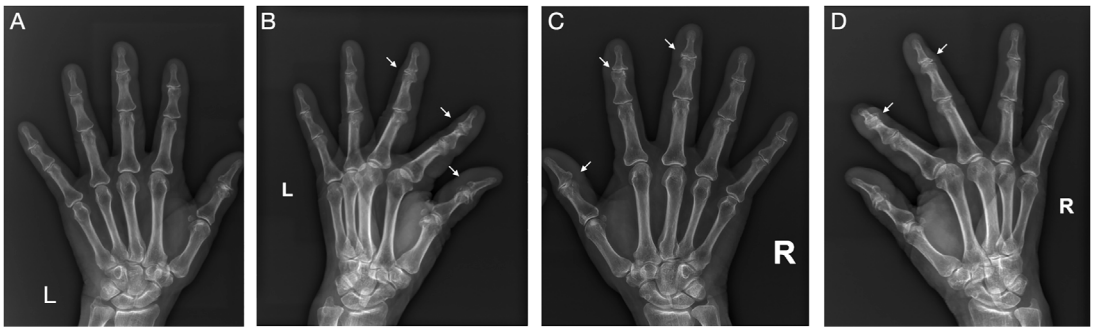

FIGURE 1 | Typical X-ray films of OA in hand. This is the X-ray films of both hands of a 71-year-old obese woman (BMI 28.0). Significant manifestations of OA (narrowing of joint space, osteophyte formation, and subchondral bone sclerosis) can be seen in these non-weight-bearing joints. **(A)** Frontal view of left hand; **(B)** Lateral view of left hand; **(C)** Frontal view of right hand; **(D)** Lateral view of right hand.

joints, leading to "wear and tear" (Zhang et al., 2018). However, recent evidence showed that this might not be the only pathogenesis. Studies have demonstrated that OA is also common in non-weight bearing joints, such as hand joints, and is more prominent in obese patients, which indicates that OA is not simply a "wear and tear" disease (Neumann et al., 2016; Favero et al., 2022) (**Figure 1**). Epidemiology study has shown that hand OA was seen in ~60% of North American and European adults ≥65 years of age—far greater than the ~33% found in the knee and ~5% in the hip (Plotz et al., 2021). Increasing evidence suggests that there are multiple subtypes of OA that reflect a complex and multifactorial nature, in which obesity-induced OA has been proposed as a new phenotype of OA that displays a unique characteristic (Sun et al., 2021).

The low-grade chronic inflammation seen in obesity-induced OA might be the main characteristic which are different from other phenotypes of OA (Sun et al., 2021). In obese patients, the cytokines produced and released by adipose tissues, which are also termed as "adipokines," might play important roles (Conde et al., 2015). In this process, adipose tissue secretes various adipokines (leptin, adiponectin, resistin, visfatin, omentin, vaspin, retinol binding protein 4, etc.) and cytokines [interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, etc.], and contribute to the degeneration of chondrocyte and breakdown of extracellular matrix (Urban and Little, 2018; Giardullo et al., 2021). The synovial adipokines might come from the secretion of the infrapatellar fat pad itself or from the blood circulation system permeating the synovial membrane and entering the joint cavity. It's close association with OA have been widely reported in literature (Collins et al., 2020). Clinical data has shown that there was a positive correlation between synovial adipokine level and OA activity index in elderly women with knee OA (Eldjoudi et al., 2022; Ilia et al., 2022).

Several mechanisms have been proposed. First, studies have shown that the single nucleotide polymorphism rs182052 in the ADIPOQ gene encoding adiponectin may modify individual susceptibility to knee OA (Jiang et al., 2021). In addition, dipokines regulate the metabolic balance of joints by regulating cytokines, chemokines, matrix degrading enzymes, and cell growth/differentiation factors (Xie and Chen, 2019). Many studies have shown that adipokines such as leptin and adiponectin released from the synovium, infrapatellar fat pad and osteophytes can upregulate the levels of inflammatory cytokines such as prostaglandin E2 (PGE2), IL-6, IL-8, vascular cell adhesion molecule (VCAM)-1, and TNF-α in knee synovial fluid, and even infiltrating cartilage and activating the degenerative cascade (Ushiyama et al., 2003; Tang et al., 2007; Tong et al., 2008; Moilanen et al., 2009; Conde et al., 2012; Yang et al., 2013; Tsuchida et al., 2014). During the process, multiple signaling pathways were associated, including AMP-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR), nuclear factor-κB (NF-κB), mitogen-activated phosphokinase (MAPK), etc.

The detailed pathophysiology by which adipokines lead to the onset and progression of OA is not fully understood. To our knowledge, no review has examined the adipokine signaling pathways in OA. In this context, this review aims to summarize the signaling pathways associated with adipokine in OA. We searched for articles containing the key terms "osteoarthritis," "obesity," "adipokine," and "signaling pathway." A total of over 200 publications were reviewed. Specifically, the reports on the signaling pathways involving in adipokine-associated OA were carefully reviewed. Publications on other phenotypes of OA, such as traumatic OA, were excluded to avoid selection bias. In addition, a structured search in the clinicaltrials.gov database was performed, and all phase II and phase III trials with pharmaceuticals against signaling pathways for the treatment of OA were selected. The purpose of this review is to help to reveal the underlying biological mechanism, so as to develop new therapeutic targets or biomarkers, and eventually provide insights for the development of preventive strategies and effective treatments.

2 ADIPOKINE SIGNALING PATHWAYS IN OSTEOARTHRITIS

2.1 Main Signaling Pathways

2.1.1 AMP-Activated Protein Kinase/Mammalian Target of Rapamycin Signaling Pathway

AMPK is a highly conserved cell energy metabolism regulator that plays an important role in the regulation of cell growth and survival and energy metabolism in the body (Hardie, 2014; Garcia

and Shaw, 2018). mTOR belongs to the phosphatidylinositol kinase-related kinase family. It is an atypical serine/threonine protein kinase that is highly evolutionarily conserved and is mainly involved in regulating cell growth, proliferation, apoptosis, and autophagy (Chen and Zhou, 2020). There are two types of mTOR protein complexes: mTORC1 and mTORC2.

AMPK/mTOR was also found to be associated with the pathogenesis of OA. When the AMPK signaling pathway is activated, the phosphorylation of AMPK blocks the phosphorylation of the mTOR signaling pathway, inhibits the IL-1β-stimulated catabolic response, downregulates the expression of MMPs and phospho-NF-κB, decreases the levels of apoptotic markers, and eventually regulates the progression of OA (Zhou et al., 2017). The AMPK/mTOR signaling pathway is involved in cartilage degeneration and chondrocyte aging (Feng et al., 2020; Zheng et al., 2021). The activation of AMPK promotes autophagy of chondrocytes and inhibits the production of inflammatory cytokines, such as IL-6 and TNF-α, in OA (Zhao et al., 2018).

Adiponectin is the most abundant adipocytokine secreted by adipocytes and a critical member in glucose metabolism and energy balance (Chen et al., 2015). It has strong anti-inflammatory and anti-apoptotic properties, which are closely related to the development and progression of OA. Adiponectin receptors 1 and 2 (AdipoR 1 and AdipoR 2) are two major transmembrane receptors that interact with adiponectin (Harasymowicz et al., 2021). Kang et al. (2010) reported that adiponectin increased the expression of matrix metalloproteinase (MMP)-1, MMP-3, MMP-13, and inducible nitric oxide synthase (iNOS) in human OA chondrocytes through the AdipoR1/2 and AMPK signaling pathways, resulting in the degradation of cartilage matrix. Berendoncks et al. (2013) and Tong et al. (2011) also found that adiponectin may activate AMPK through AdipoR1, which affects the MMP-3 promoter and leads to cartilage destruction. In addition to its effects on chondrocytes, adiponectin can stimulate the proliferation, differentiation and mineralization of osteoblasts in autocrine and/or paracrine fashion through the AMPK signaling pathway (Kanazawa et al., 2007). Chen et al. (2015) further demonstrated that adiponectin promotes osteogenic differentiation of human adipose-derived stem cells by activating the AMPK pathway. Furthermore, adiponectin can also induce intercellular adhesion molecule-1 (ICAM-1) expression *via* AMPK to promote the adhesion of monocytes to human OA synovial fibroblasts (Chen et al., 2014). Moreover, AMPK plays an important role in the expression of vascular cell adhesion molecule-1 (VCAM-1) in human and mouse chondrocytes induced by adiponectin and leptin and makes cartilage degradation permanent (Conde et al., 2012).

Leptin is a ubiquitous fat factor produced by fat and other tissues that regulates food intake and energy consumption. The increased expression of leptin and the enhanced effect of leptin on the infrapatellar fat pad, synovium, articular cartilage and bone are also involved in the pathogenesis of OA (Gao et al., 2020). The role of AMPK/mTOR in leptin-induced OA has not been widely reported. Huang et al. (2016) found that the increase in leptin levels in patients with OA significantly stimulated the expression of lysyl oxidase-like 3. Increased lysyl oxidase-like 3 expression induces chondrocyte apoptosis, activates mTORC1 and inhibits chondrocyte autophagy (Huang et al., 2016).

Resistin induces the expression of proinflammatory factors and chemokines in human cartilage, thus inhibiting the synthesis of cartilage matrix. Epidemiological studies have found that resistin levels in serum and synovial fluid are positively correlated with the severity of OA (Song et al., 2016). The role of AMPK/mTOR in resistin-induced OA is also unknown. Su et al. (2017) studied the mechanism by which low shear stress (2 dyn/cm^2) regulates the catabolism of resistin in human OA chondrocytes and found that preshear activated the AMPK/sirtuin 1 (SIRT1) signal, but postshear inhibited this signal and regulated the cyclooxygenase-2 expression in human OA chondrocytes induced by resistin.

2.1.2 Nuclear Factor-κB Signaling Pathway

NF-κB is an ubiquitously expressed transcription factor that plays an important role in cell survival, differentiation, proliferation, aging, inflammation, immune response, and apoptosis (Hayden and Ghosh, 2012). Meanwhile, NF-κB has also been proven to be involved in the occurrence and development of OA, including chondrocyte survival and catabolism, as well as synovial inflammation (Roman-Blas and Jimenez, 2006; Rigoglou and Papavassiliou, 2013; Saito and Tanaka, 2017; Choi et al., 2019).

The destruction of cartilage matrix integrity in OA is caused by the increase in chondrocyte catabolism and apoptosis and the decrease in chondrocyte anabolism (Hwang and Kim, 2015; Zheng et al., 2021). Studies have shown that adipokines can induce the expression of matrix-degrading enzymes and/or proinflammatory mediators in chondrocytes through the NF-κB signaling pathway (Choi et al., 2019). Studies have shown that leptin increases the expression of PGE2, IL-6, IL-8, MMP-1, MMP-3, and MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-4, ADAMTS-5, and ADAMTS-9 genes through the NF-κB signaling pathway (Tong et al., 2008; Moilanen et al., 2009; Koskinen et al., 2011; Yaykasli et al., 2015). The increased production of MMPs and ADAMTSs is a marker of the destruction of cartilage homeostasis and the initiator and promoter of OA (Mobasheri et al., 2017). Additionally, adiponectin activates p38 and AMPK through AdipoR1, thereby activating NF-κB on the MMP-3 promoter and leading to cartilage breakdown (Tong et al., 2011). Huang et al. (2010) and Tang et al. (2007) also found that the NF-κB signaling pathway may play a critical role in adiponectin promoting the expression of bone morphogenetic protein (BMP)-2 in osteoblasts and IL-6 in human synovial fibroblasts. Moreover, in one study by Li et al. (2017), resistin was found to combine with toll-like receptor-4 through the p38 MAPK and NF-κB signaling pathways, increasing the expression of chemokine ligand 4 in nucleus pulposus cells and leading to macrophage infiltration. Su et al. (2017) found that human OA chondrocytes exposed to different low shear stress modes have an opposite effect on resistin-induced catabolic cyclooxygenase-2 expression, which is closely related to the NF-κB signaling pathway. That is, preshearing over a short duration inhibits the NF-κB-p65 subunit and cAMP response element

binding protein to attenuate the resistin effect. However, postshear for a longer duration enhances the resistin effect by activating only the NF-κB-p65 subunit (Su et al., 2017). Furthermore, resistin can mediate osteoclastogenesis by activating NF-κB (Thommesen et al., 2006). Osteoclastogenesis is also an important pathophysiological change of subchondral bone in OA, as OA is usually associated with subchondral bone sclerosis and remodeling. Another common adipokine, visfatin, was found to induce the expression of MMP-3, MMP-12, MMP-13, IL-6, monocyte chemokine protein 1, and keratinocyte chemokine in osteoblasts and chondrocytes *via* NF-κB (Laiguillon et al., 2014; Yang et al., 2015).

Lipocalin 2, an adipokine initially isolated from neutrophil granules, has been considered a factor that may damage chondrocyte phenotype, cartilage homeostasis, and growth-plate development (Conde et al., 2017). Studies have demonstrated that NF-κB is a key member of lipocalin 2-induced OA (Guo et al., 2014; Conde et al., 2016). A novel adipokine, Nesfatin-1, was also found to stimulate NF-κB pathway (Lee et al., 2021).

2.1.3 Janus Kinase/Signal Transducer and Activator of Transcription Signaling Pathway

The Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling pathway is the process by which STAT is phosphorylated and dimerized by JAK and then transported to the nucleus through the nuclear membrane to regulate the expression of related genes (Xin et al., 2020). It is an ubiquitously expressed intracellular signal transduction pathway that participates in many important biological processes, including cell proliferation, differentiation, apoptosis, and immune regulation (Rawlings et al., 2004).

Leptin, in one study, has been proven to induce chondrocyte apoptosis and produce MMP-3, MMP-13, reactive oxygen species, iNOS, NO, and VCAM-1 through the JAK/STAT signaling pathway (Otero et al., 2003, 2005; Koskinen et al., 2011; Conde et al., 2012; Zhang et al., 2016). Ben-Eliezer et al. (2007) also demonstrated that the effects of leptin on growth-plate chondrocytes are specifically mediated by STAT 3. Pallu et al. (2010) further found that 500 ng/ml leptin triggers signal transduction through the STAT signaling pathway and thus induces the expression of insulin growth factor (IGF)-1, type 2 collagen, tissue inhibitors of metalloproteinase (TIMP)-2 and MMP-13. Although leptin at 100 ng/ml failed to activate STAT 3, it could induce STAT 1α phosphorylation in the chondrocytes of obese patients (Pallu et al., 2010). Leptin receptor activation initiated by leptin binding was reported to activate multiple signaling pathways including JAK/STAT (Eldjoudi et al., 2022).

Lipocalin 2 is a novel adipokine that has a negative effect on articular cartilage and triggers the catabolism and inflammatory response of chondrocytes in OA. Guo et al. (2014) suggested that lipocalin 2 is a regulator of macrophage polarization and STAT 3 signaling pathway activation. Studies have also shown that IL-1 induces the expression of lipocalin 2 in chondrocytes through JAK2 (Conde et al., 2017).

2.1.4 Mitogen-Activated Phosphokinase Signaling Pathway

MAPK is an important pathway of eukaryotic signal transduction. It regulates many cell biological processes in OA, including proliferation, differentiation, migration and apoptosis (Lan et al., 2021). MAPK signal transduction is carried out *via* a three-level kinase cascade. First, MAPK kinase (MAPKKK) is activated by mitogen-stimulated phosphorylation. On this basis, MAPKKK phosphorylates and activates MAPK kinase (MAPKK). Last, MAPKK phosphorylates MAPK to activate it and then transfers it into the nucleus. MAPK is a proline-directed protein kinase that can phosphorylate serine or threonine residues adjacent to proline. Thus, MAPKs activate many protein kinases, transcription factors, and nuclear proteins, resulting in downstream signal transduction (Cuschieri and Maier, 2005).

Increased MAPK activity has been found in human OA. Currently, p38, c-Jun N-Terminal Kinase (JNK), and Extracellular Signal-Regulated Kinase (ERK)½ in the MAPK family have been investigated and found to be involved in the pathogenesis of OA (Saklatvala, 2007). The p38 and JNK MAPK signaling pathways are important signals involved in the regulation of the inflammatory response. A variety of external stressors, such as cytokines and hypoxia, can cause the phosphorylation of JNK and p38 and lead to the chain reaction of intracellular protein kinase (Shi et al., 2016; Sun et al., 2017). The ERK½ MAPK signal transduction pathway is the key factor determining cell fate when stimulated extracellularly. Its main function is to promote proliferation and regulate cell terminal differentiation (Cuschieri and Maier, 2005).

2.1.4.1 p38 Mitogen-Activated Phosphokinase Signaling Pathway

p38 MAPK plays a pivotal role in cartilage destruction in adipokine associated OA. Researchers have found that leptin dose-dependently stimulates the proliferation of abnormal OA osteoblasts and increases the levels of phosphorylated p38 and ERK½ MAPK (Ben-Eliezer et al., 2007; Mutabaruka et al., 2010). However, inhibition of the p38 MAPK signaling pathway fails to block the effect of leptin on the expression of type X collagen in ATDC5 chondrogenic cells (Ben-Eliezer et al., 2007). Additionally, studies have shown that leptin can increase the expression of PGE2, IL-6, IL-8, nitric oxide synthase (NOS) type II, ADAMTS-4, ADAMTS-5, ADAMTS-9, MMP-1, and MMP-13 in human chondrocytes through the p38 MAPK signaling pathway (Otero et al., 2005, 2007; Moilanen et al., 2009; Koskinen et al., 2011; Yaykasli et al., 2015). Adiponectin, another key adipokine, increases the expression of BMP-2 in osteoblasts, MMP-3 in human chondrocytes, and IL-6 in human synovial fibroblasts *via* the p38 MAPK signaling pathway (Tang et al., 2007; Huang et al., 2010; Tong et al., 2011).

2.1.4.2 c-Jun N-Terminal Kinase Mitogen-Activated Phosphokinase Signaling Pathway

JNK was initially known as a 54-kDa stress-activated protein kinase (SAPK), which is responsible for immune reactions

and cellular functions, such as cell growth, differentiation, survival, and apoptosis (Chen et al., 2001; Ge et al., 2017). Many studies have indicated that JNK is phosphorylated and activated in OA and is closely associated with cartilage destruction in the pathogenesis of obesity-induced OA. Wang et al. (2016) demonstrated that dual specificity protein phosphatase 19, a downstream target of leptin, inhibited chondrocyte apoptosis by dephosphorylating JNK. In another study, Lee et al. (2015) found that leptin suppresses TNF-α-induced chondrocyte death through the JNK MAPK signaling pathway in rat articular chondrocytes. Additionally, studies have shown that leptin-induced NO, PGE2, IL-6, and IL-8 production was reduced by inhibitors of JNK (Moilanen et al., 2009). On the other hand, adiponectin leads to the degradation of the OA cartilage matrix and increases the expression of MMPs and iNOS in human OA chondrocytes through the JNK MAPK pathway (Kang et al., 2010). Luo et al. (2005) also found that adiponectin could stimulate human osteoblast proliferation and differentiation through p38 and JNK but not the ERK ½ MAPK signaling pathway. Furthermore, the JNK MAPK signaling pathway is related to adiponectin increasing the expression of ICAM-1 in human OA synovial fluid and promoting the adhesion between monocytes and OA synovial fluid (Chen et al., 2014).

2.1.4.3 Extracellular Signal-Regulated Kinase½ Mitogen-Activated Phosphokinase Signaling Pathway

Although ERK½ MAPK has no direct impact on the degradation of the extracellular matrix like p38 MAPK and JNK MAPK, it mainly mediates the proliferation and hypertrophic differentiation of chondrocytes, leading to cartilage calcification and osteophyte formation (Lin et al., 2021). As the only cell of articular cartilage, the fate of chondrocytes determines the formation and development of adipokine-associated OA.

Leptin, as a proinflammatory fat factor, was found to play a key role in cartilage metabolism by inducing the upregulation of MMP1 and MMP13 expression with concomitant activation of the STAT, MAPK (p38, JNK, and ERK), Akt, and NF-κB signaling pathways. Otero et al. (2005), Otero et al. (2007) strongly suggested that ERK½ MAPK and p38 MAPK play pivotal roles in the activation of leptin-mediated NOS type II. Moreover, ERK has also been reported to be involved in activating lipocalin 2 by IL-1α during OA progression (Conde et al., 2017). Collagen II expression was also found to be stimulated by leptin-medicated MAPK/ERK signaling pathway in an *in vivo* study (Wang et al., 2022).

Chemerin is a novel adipokine identified in 2007. It is highly expressed in the synovial fluid and synovial membrane of patients with OA and is positively correlated with the severity of OA (Huang et al., 2012; Ma et al., 2015). Berg et al. (2010) suggested that the stimulation of chondrocytes with chemerin could lead to the phosphorylation of ERK½ MAPK and Atk. Additionally, the levels of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α, MMP-1, MMP-2, MMP-3, MMP-8, and MMP-13, are significantly increased (Berg et al., 2010).

2.1.5 Activating Protein-1 (AP-1) Signaling Pathway

Activating Protein-1 (AP-1) is most often defined as a collective term, which refers to dimeric transcription factors composed of Jun (v-Jun, c-Jun, JunB, and JunD), Fos (v-Fos, c-Fos, FosB, Fral-1, and Fra2), activating transcription factor (ATF-2, ATF-3/LRF1, ATF-4, ATF-5, ATF-6B, ATF-7, BATF, BATF-2, BATF-3, and JDP2), and MAF (c-MAF, MAFA, MAFA-B, MAFA-F, MAFA-G, MAFA-K, and Nrl) subunits, which bind to a common DNA site (Bejjani et al., 2019).

The AP-1 signaling pathway may be the signaling pathway of leptin-induced IL-6 production in OA synovial fibroblasts. Yang et al. (2013) found that leptin activated IRS-1/PI3K/Akt signaling and increased the production of IL-6 by binding with the OBRI receptor, thus enhancing the transcriptional activity of AP-1 and eventually leading to the transcription of IL-6. There are three known cis-regulatory elements in the promoter region of the IL-6 gene, including AP-1, NF-κB, and C/EBP-β. Yang et al. (2013) confirmed that leptin-stimulated luciferase activity was eliminated by AP-1 binding site mutation, and an AP-1 inhibitor (curcumin) and c-jun siRNA could antagonize the increase in IL-6 expression mediated by leptin. In addition, the stimulation of OA synovial fibroblasts by leptin increased c-Jun phosphorylation in a time-dependent manner. Moreover, leptin increased the binding of c-Jun to the AP-1 (-312 to -39) element in the IL-6 promoter (Yang et al., 2013).

AP-1 is a critical transcription factor that controls ICAM-1 production and cell movement during the progression of adiponectin-induced OA. By exploring the effect of adiponectin on the expression of ICAM-1 in synovial fibroblasts, researchers found that adiponectin led to the transactivation of ICAM-1 expression by enhancing the binding of the AP-1 transcription factor and ICAM-1 promoter and promoted the adhesion of monocytes to human OA synovial fibroblasts through the AP-1 pathway (Chen et al., 2014).

2.1.6 Insulin Receptor Substrate-1 Signaling Pathway

Insulin Receptor Substrate-1 (IRS-1) is the main substrate of receptor tyrosine kinase for insulin and insulin-like growth factor 1, and it is also a substrate of IL-4-activated tyrosine kinase and has been associated with adipokine associated OA pathophysiology (Myers et al., 1994). Specifically, leptin in the synovium may play a proinflammatory role in the pathogenesis of OA through the IRS-1 signaling pathway (Gao et al., 2020). Studies have shown that leptin induces the production of IL-8 in OA synovial fibroblasts *via* an IRS-1/PI3K/Akt/NF-κB-dependent pathway (Tong et al., 2008). Yang et al. and Tang et al. found that leptin also increased the production of IL-6 *via* the activation of the IRS-1/PI3K/Akt/NF-κB signaling pathway (Yang et al., 2013).

2.1.7 Phosphatidyl Inositol 3 Kinase-Protein Kinase B Signaling Pathway

The Phosphatidyl Inositol 3 Kinase (PI3K)-Protein Kinase B (Akt) signaling pathway functions in many cellular processes that are essential for homeostasis, including the cell cycle, survival, metabolism, inflammation, and apoptosis (Tang et al.,

2018). Molecules, such as adipokines and cytokines, can activate receptor tyrosine kinases and G protein-coupled receptors and then activate PI3K to generate phospholipase (De Santis et al., 2019). Akt, as a downstream effector of PI3K, is subsequently activated by these signals. Activated Akt is transferred to other cellular compartments to activate various downstream substrates, including metabolic enzymes, protein kinases, small G protein regulators, E3 ubiquitin ligases, and transcription factors (Manning and Toker, 2017). The PI3K-Akt signaling pathway plays an important regulatory role in cartilage homeostasis, subchondral bone dysfunction and synovitis (Sun et al., 2020). A previous study suggested that the PI3K-Akt signaling pathway is downregulated in human cartilage with OA compared with normal cartilage (Rosa et al., 2011). Specifically, inhibition of the PI3K-Akt signaling pathway can promote the proliferation, apoptosis, and autophagy of articular chondrocytes and reduce the inflammatory response in OA (Xue et al., 2017; Sun et al., 2020). Many studies have shown that adipokines can regulate the progression of OA through the PI3K-Akt signaling pathway.

Visfatin is also a newly discovered proinflammatory adipokine produced by visceral white adipose tissue, which can be found in muscle, bone, synovium, and cartilage (Franco-Trepat et al., 2019). Vascular endothelial growth factor and endothelial progenitor cells are critical factors that promote angiogenesis of the pannus in OA (Kiewisz et al., 2016; Patel et al., 2016; MacDonald et al., 2018). Studies have shown that visfatin inhibits the synthesis of miRNA-485-5p through the PI3K-Akt signaling pathway, which affects the expression of vascular endothelial growth factor in OA synovial fluid and the angiogenesis of endothelial progenitor cells (Tsai et al., 2020).

Vaspin (visceral adipose tissue-derived serine protease inhibitor), a novel adipokine, is produced by skeletal muscle and participates in bone metabolism in an obesity-dependent manner (Tarabeih et al., 2020). Researchers have declared that vaspin can inhibit osteogenic differentiation by activating the PI3K-Akt signaling pathway. During this process, PI3K-Akt and miRNA-34c constitute a modulation loop and control the expression of each other (Liu et al., 2016). Another study has also indicated that PI3K-Akt is critical in Vaspin-induced proliferation of BMSc in OA (Wang et al., 2021).

Apelin is an adipokine involved in the pathogenesis and angiogenesis of OA (Wang et al., 2020). It plays a catabolic role in cartilage metabolism. Specifically, apelin could stimulate chondrocyte proliferation and significantly increase the catabolic factors MMP-1, MMP-3, MMP-9, ADAMTS-4, ADAMTS-5, and the proinflammatory cytokine IL-1β as well as reduce the level of type II collagen (Hu et al., 2010). Chang et al. first proved that apelin stimulates IL-1β expression by activating the PI3K and ERK signaling pathways and inhibiting the downstream expression of miRNA-144-3p in OA synovial fibroblasts (Chang et al., 2020).

Omentin-1, also known as intelectin-1, is a newly identified anti-inflammatory adipokine involved in lipid metabolism (Chai et al., 2020). It has been reported that omentin-1 can stimulate the proliferation and inhibit the differentiation of mouse osteoblasts (Xie et al., 2011, 2012). Wu et al. (2013) found that omentin-1 could induce human osteoblast proliferation through the PI3K-Akt signaling pathway.

Furthermore, stimulation of OA synovial fluid with leptin leads to time-dependent phosphorylation of Akt (Yang et al., 2013). Additionally, leptin can promote the expression of IL-6 in human OA synovial fluid and IL-8 in human synovial fibroblasts through the PI3K-Akt signaling pathway (Tong et al., 2008; Yang et al., 2013). In addition, PI3K was demonstrated to be involved in leptin and adiponectin increasing the expression of VCAM-1 in human and mouse chondrocytes (Conde et al., 2012). Moreover, PI3K could induce the expression of lipocalin 2 in cartilage and affect cartilage homeostasis (Guo et al., 2014). Chemerin can also induce Akt phosphorylation in chondrocytes and increase the levels of proinflammatory cytokines, including IL-6, IL-8, IL-1β, TNF-α, and MMPs (Berg et al., 2010).

2.1.8 Peroxisome Proliferator-Activated Receptor Signaling Pathway

Peroxisome Proliferator-Activated Receptor (PPAR) is a ligand-activated transcription factor that plays a key regulatory role in lipid metabolism and energy homeostasis (Huang et al., 2021). There are three PPAR isotypes, namely, α, γ and β/δ (Dubois et al., 2017).

PPARs regulate the homeostasis of articular cartilage *via* various pathways and reduce the inflammatory response of OA cartilage. First, PPAR-γ, known as the main adipogenesis regulator, may affect the deposition of fat in skeletal muscle and connective tissue. Fat deposition is an important risk factor for knee OA. The main adipose tissue of the knee joint is the infrapatellar fat pad, which can produce inflammatory cytokines and adipokines. Therefore, the activation of the PPAR-γ signaling pathway may promote adipogenesis, which could be related to the pathological changes in the infrapatellar fat pad in OA (Cordani et al., 2013; Reggio et al., 2019, 2020; Cerquone Perpetuini et al., 2020). Meanwhile, the lack of PPAR-γ in articular cartilage may accelerate the cartilage destruction and progression of OA by reducing chondrocyte differentiation and proliferation and increasing catabolic activity (Monemdjou et al., 2012; Vasheghani et al., 2013, 2015). Studies have shown that stimulation of adiponectin leads to increased PPAR-α ligand activity, fatty acid oxidation and glucose uptake in skeletal muscle (Kanazawa et al., 2007). It was also reported that the PPAR-γ coactivator (PGC)-1α critically mediates anti-catabolic activity in human knee OA chondrocytes (Wang et al., 2015). In addition, the activation of PPAR was found to significantly reduce the synthesis of key adipokine-associated OA mediators, such as MMPs, ADAMTS-5, TNF-α, PGE2, IL1-β, IL-6 and nitric oxide (NO), and inhibit the activation of the ERK½ MAPK, p38, AP-1, and NF-κB signaling pathways (Boileau et al., 2007; Clockaerts et al., 2011; Fahmi et al., 2011; Scirpo et al., 2015). Ratneswaran et al. (2015) also reported that the activation of PPAR-δ could induce the upregulation of proteolytic active genes in cartilage principal extracellular matrix as well as increase the degradation of aggrecan and the release of glycosaminoglycan in knee joint explants. Cartilage-specific PPAR-δ knockout mice showed a strong protective effect on cartilage degeneration in the destabilization of the medial meniscus model of post-traumatic OA (Ratneswaran et al., 2015). Moreover, activated PPAR can inhibit the activation of

NF-κB induced by adipokines or promote its inactivation through the following mechanisms, leading to the inhibition of inflammation. PPAR interferes with the activation of NF-κB in the inflammatory response mainly by upregulating the expression of IκBα, sirtuin 1, and phosphatase and tensin homolog (Korbecki et al., 2019). A possible mechanism of PPAR promoting NF-κB inactivation is that PPAR, as an E3 ubiquitin ligase, can physically interact with p65 NF-κB, which leads to inactivation and ubiquitination of p65 NF-κB and finally results in the proteolytic degradation of p65 NF-κB (Hou et al., 2012). Furthermore, adiponectin could selectively activate human monocytes into anti-inflammatory M2 macrophages through the PPAR-α/γ signaling pathway, thus controlling the progression of OA (Lovren et al., 2010).

2.1.9 Wnt/β-Catenin Signaling Pathway

Wnt is an extracellularly secreted glycoprotein. Its signal transduction involves 19 Wnt genes and various Wnt receptors that regulate the canonical β-catenin-dependent signaling pathway (Wang et al., 2019). The overactivation of the Wnt/β-catenin signaling pathway is related to the degradation process of OA (Huang et al., 2020; Palma and Nalesso, 2021). Wang et al. (2017) investigated the effects of adiponectin on osteogenic differentiation and bone formation of bone mesenchymal stem cells (BMSCs). They conducted both *in vivo* and *in vitro* studies and found higher gene and protein expression levels of osteogenesis-related genes and Wnt/β-catenin pathway-related factors β-catenin and cyclin D1 in adiponectin transgenic BMSCs and rats. Therefore, adiponectin can facilitate osteogenic differentiation and bone formation of BMSCs *via* the Wnt/β-catenin signaling pathway (Wang et al., 2017).

2.2 Other Pathways

2.2.1 RhoA/ROCK Signaling Pathway

Rho kinase (ROCK), a serine/threonine kinase, is a downstream effector of member A of the Ras homologous gene family (RhoA) and is involved in regulating cell migration, proliferation and survival (Cai et al., 2021). The abnormal activation of RhoA/ROCK signaling is involved in the early response to abnormal mechanical stimulation, which is considered to be a promoter of the progression of OA. RhoA/ROCK interacts with OA pathological factors, including β-catenin, transforming growth factor (TGF), epidermal growth factor receptor (EGFR), IL-1, insulin-like growth factor-1 (IGF-1) and leptin, and induces cartilage degeneration through the degradation of chondrocyte extracellular matrix (Deng et al., 2019). Liang et al. (2011) proved that leptin mediates cartilage cytoskeleton remodeling through the RhoA/ROCK signaling pathway and its downstream mediators LIMK1 and cofilin-2.

2.2.2 Sirtuin-1 Signaling Pathway

SIRT-1 is a histone deacetylase. SIRT-1 regulates gene expression and protein function by deacetylating lysine residues in histones and nonhistones (Matsuzaki et al., 2014). It is reported that SIRT-1 regulates aging and age-related diseases in simple eukaryotes (Chen et al., 2020).

It is widely recognized that SIRT-1 activity plays an anti-catabolic role in chondrocytes in the development of OA (Gabay et al., 2013; Matsuzaki et al., 2014; Goldring and Berenbaum, 2015). Researchers have found that SIRT-1 is involved in the pathogenesis of OA by regulating chondrocyte gene expression and hypertrophy (Fujita et al., 2011). SIRT-1 increases the expression of the gene encoding cartilage extracellular matrix in human chondrocytes and improves the survival rate of human OA chondrocytes by inhibiting apoptosis (Takayama et al., 2009; Gagarina et al., 2010; Goldring and Berenbaum, 2015). Moreover, SIRT-1 could also exert anti-inflammatory effects in different tissues by inhibiting the transcription of pro-inflammatory genes (Michan and Sinclair, 2007). Studies have shown that SIRT-1 inhibits the expression of cartilage-degrading enzymes induced by IL-1β and TNF-α by regulating the NF-κB pathway (Matsushita et al., 2013; Moon et al., 2013). In particular, Su et al. (2017) reported that low shear stress regulates the expression of resistin-induced catabolic cyclooxygenase-2 in human OA chondrocytes *via* the AMPK/SIRT-1 signaling pathway and then NF-κB and cAMP response element binding protein transcription factors. Specifically, AMPK activity and SIRT-1 expression in human OA chondrocytes decrease under resistin-induced cyclooxygenase-2 expression. In addition, the attenuation of the preshear effect and the enhancement of the postshear effect of resistin-induced cyclooxygenase-2 expression are caused by the regulation of AMPK and then SIRT-1 signaling (Su et al., 2017). Furthermore, Patel et al. suggested that omentin-1 reduced the expression of IL-1β-induced senescent factors (including caveolin-1, p21 and PAI-1) and p53 acetylation by ameliorating SIRT1 reduction (Chai et al., 2020).

2.2.3 p53/p21 Signaling Pathway

The p53/p21 signaling pathway is implicated in the progression and severity of OA (Xu et al., 2020). In knee OA cases, the protein and mRNA expression of p53 is significantly higher than that in healthy controls (Zhu et al., 2016). Xu et al. (2020) found that chondrocyte apoptosis and p53 increased during the progression of OA, while the expression of SIRT1 decreased in human cartilage. The deletion of SIRT1 in cartilage leads to the acceleration of OA through the abnormal activation of senescence-associated secretory phenotype, hypertrophy, and apoptosis mediated by p53/p21 (Xu et al., 2020). Zhao et al. (2016) further confirmed that high-dose leptin induces cell cycle arrest and senescence in chondrogenic progenitor cells by activating the p53/p21 signaling pathway and inhibiting the SIRT1 signaling pathway.

2.2.4 Calcium Calmodulin-Dependent Kinase II Signaling Pathway

Calcium Calmodulin-Dependent Kinase II (CaMKII) is a serine/threonine protein kinase and a general integrator of Ca^{2+} signaling (Wei et al., 2018). The upregulation of CaMKII is highly correlated with the pathogenesis and progression of OA and the reactivation of articular cartilage hypertrophy (Saitta et al., 2019; Cai et al., 2021). Studies have demonstrated that activated phosphorylated-CaMKII may play a key role in chondrocyte apoptosis through the MAPK and Akt/mTOR

signaling pathways (Wei et al., 2018). Researchers have proven that adiponectin can promote ICAM-1 expression and monocyte adhesion in synovial fibroblasts through the CaMKII signaling pathway (Chen et al., 2014).

2.2.5 Protein Kinase C and Protein Kinase A Signaling Pathways

Many studies have indicated that Protein Kinase C (PKC) and Protein Kinase A (PKA) have a close relationship with adipokine-induced OA. Thommesen et al. revealed that resistin stimulates the proliferation of preosteoblasts (MC3T3-E1) and the differentiation of preosteoclasts (RAW 264.7) through the PKC and PKA signaling pathways (Thommesen et al., 2006). Studies have also shown that vaspin inhibits TNF-α-induced ICAM-1 expression *via* activation of PKC, thereby inhibiting the inflammatory state of vascular smooth muscle cells during OA progression (Phalitakul et al., 2011). In addition, PKC is involved in leptin-induced MMP production (Koskinen et al., 2011).

2.2.6 ATF4/RANKL Signaling Pathway

ATF4 is an osteoblast-specific member of the cyclic AMP response-binding protein (CREB) family. It has been reported that leptin regulates osteoclast differentiation and osteoblast proliferation by using ATF4 and its target gene RANKL as transcription medium through sympathetic signals (Fu et al., 2005). In addition, adipokine-induced IL-6 also triggers osteoclast formation and bone resorption through the ATF4/RANKL signaling pathway (Xie and Chen, 2019).

2.2.7 Bone Morphogenetic Protein-2 Signaling Pathway

BMP-2 is an important growth factor promoting osteogenesis and plays a pivotal role in osteoblastic differentiation and bone formation. It has been found to be expressed at low levels in the synovial fluid of OA patients (Yang et al., 2018). Studies have shown that adiponectin affects the progression of OA by enhancing the expression level of BMP-2 in osteoblasts (Huang et al., 2010).

2.2.8 CCAAT/Enhancer-Binding Protein-β Signaling Pathway

The CCAAT/Enhancer-Binding Protein (C/EBP-β) pathway is a key signaling pathway that regulates the downstream target genes of osteoblastogenesis. The expression of C/EBP-β is a reliable output for measuring the activity of osteoblastogenesis at the cellular level (Wang et al., 2018). It has been confirmed that resistin can activate the C/EBP-β signaling pathway and thus upregulate proinflammatory cytokines and chemokines (Zhao et al., 2019).

2.2.9 Notch Signaling Pathway

Notch is a single-pass transmembrane cell surface receptor that plays a key role in cell fate by regulating cell differentiation and apoptosis. The direct transcription targets downstream of Notch are Hes1, Hes5, Hes7, Hey1, Hey2, and HeyL, of which only Hes1 is highly expressed in articular chondrocytes (Hosaka et al., 2013; Sugita et al., 2015). Activation of the Notch signaling pathway affects the occurrence of OA by enhancing the production of inflammation-related molecules in OA synovial cells and chondrocytes (Saito and Tanaka, 2017). Leptin can stimulate the expression of IL-6 in serum and OA synovial fluid. The upregulated IL-6 then reduces the synthesis of bone proteoglycans and increases the expression of the decomposition factor MMP13 through the Notch signaling pathway, resulting in the degradation of proteoglycans and the suppression of cartilage formation (Zanotti and Canalis, 2013; Yan et al., 2018).

3 SIGNALING PATHWAYS OF OSTEOARTHRITIS AS DIAGNOSTIC BIOMARKER AND THERAPEUTIC TARGETS

Signaling pathway as a diagnostic biomarker of OA is rarely reported in the literature. Most studies on diagnostic biomarkers still focus on collagenous markers of cartilage decomposition, metabolic mediators (mainly adipokines) and inflammatory mediators (mainly cytokines) (Kumavat et al., 2021). Only a few literatures have reported the potential of signaling pathway as a diagnostic biomarker of OA, mainly by means of high throughout sequencing and bioinformatics analysis (Zhang et al., 2020). Therefore, in the future work, whether the signal pathway or its key components can be used for early diagnosis of OA needs further research.

To date, the key management strategies for OA have included non-pharmacological (e.g., education and self-management, exercises, weight loss if overweight), pharmacological (e.g., NSAIDs and intra-articular injection of corticosteroids), and surgical approaches. (Sun et al., 2020). There are currently no approved disease-modifying treatments for osteoarthritis. Although many preclinical studies have reported various signaling pathways involved in OA, as reviewed above, there are still few drugs applied in clinical trials. (**Table 1**) Moreover, some studies have even been terminated. Our review can provide ideas and directions for more clinical trials in the future.

4 SUMMARY AND FUTURE PERSPECTIVES

In this review, we summarize the possible adipokine signalling pathways in OA reported in the current literature, including the ones that are common and classic, such as AMPK/mTOR and NF-κB, and some novel pathways, such as C/EBP-β and ATF4/RANKL. Various studies have suggested adipokines play important roles in obesity-induced OA, and exert downstream function *via* the activation of these signaling pathways. In addition, based on these findings, some pharmaceuticals targeting these pathways (such as Wnt, NF-κB, and p38 MAPK) have been applied into ongoing clinical trials and showed encouraging results. Inhibitors or antibodies against some novel pathways have demonstrated

TABLE 1 | Ongoing clinical trials investigating inhibitors of signaling pathways as treatment for OA.

Intervention	Mechanism	Targets	Joint	Phase	NCT No.	Recruitment Status
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	knee	Phase 3	NCT04520607	Active, not recruiting
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	knee	Phase 3	NCT04385303	Completed
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	knee	Phase 3	NCT03928184	Completed
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	knee	Phase 2	NCT03706521	Terminated
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	knee	Phase 2	NCT03122860	Completed
Lorecivivint (SM04690)	Wnt signaling pathway inhibitor	Wnt signaling pathway	Not given	Phase 2	NCT02536833	Completed
SAR-113945	I-kappa B kinase inhibitors	NF-κb	knee	Phase 2	NCT01598415	Completed
SAR-113945	I-kappa B kinase inhibitors	NF-κb	Not given	Phase 1	NCT01113333	Completed
SAR-113945	I-kappa B kinase inhibitors	NF-κb	knee	Phase 1	NCT01463488	Completed
SAR-113945	I-kappa B kinase inhibitors	NF-κb	knee	Phase 1	NCT01511549	Completed
PH-797804	p38 MAPK inhibitor	p38 MAPK	Not given	Phase 2	NCT01102660	Completed

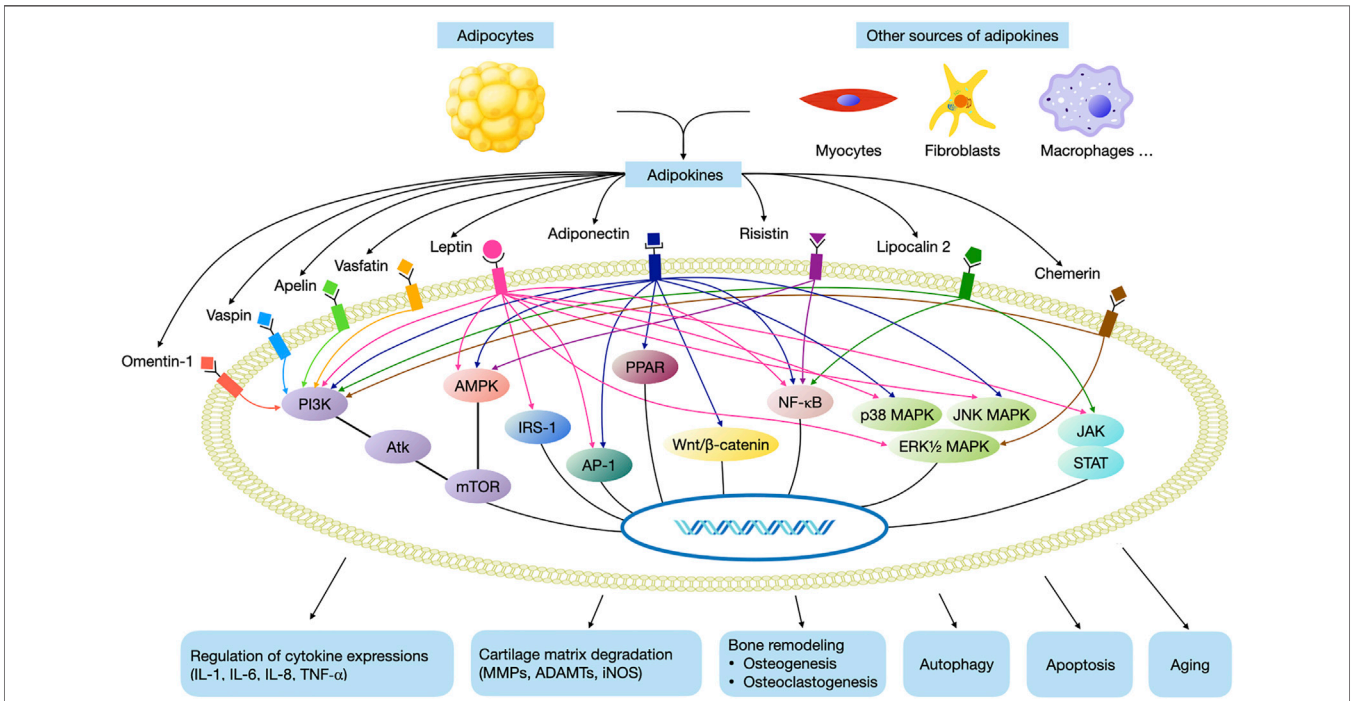

FIGURE 2 | Main adipokine signaling pathways in OA. (AMPK, AMP-activated protein kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB, JAK, janus kinases; STAT, signal transducer and activator of transcription, MAPK, mitogen activated phosphokinase; AP-1, activating protein-1; IRS-1, insulin receptor substrate-1; PI3K, phosphatidyl inositol 3 kinase; Akt, protein kinase B; PPAR, peroxisome proliferator-activated receptor).

excellent results in alleviating OA in preclinical studies. These data might provide novel therapeutic strategies for the treatment of OA, especially obesity-induced OA.

However, these signaling pathways are complex and converge into a common network with each other (**Figure 2**). It has to be noted that none of the adipokines mediates the downstream reaction *via* a single signaling pathway, and a specific signaling pathway is also the common target of several different adipokines. Therefore, in the future work, more research is warranted to further investigate how this network works. Moreover, more high quality randomised controlled trials are needed in order to investigate the therapeutic effects of pharmaceuticals against these pathways for the treatment of OA, as these drugs may serve as disease-modifying drugs. Our review may help researchers to better understand the pathogenesis of OA, so as to provide new insight for future clinical practices and translational research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial and direct contribution to the work, and approved it for publication.

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REVIEW
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Advances in Stem Cell Therapies for Rotator Cuff Injuries

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Rotator cuff injury is a common upper extremity musculoskeletal disease that may lead to persistent pain and functional impairment. Despite the clinical outcomes of the surgical procedures being satisfactory, the repair of the rotator cuff remains problematic, such as through failure of healing, adhesion formation, and fatty infiltration. Stem cells have high proliferation, strong paracrine action, and multiple differentiation potential, which promote tendon remodeling and fibrocartilage formation and increase biomechanical strength. Additionally, stem cell-derived extracellular vesicles (EVs) can increase collagen synthesis and inhibit inflammation and adhesion formation by carrying regulatory proteins and microRNAs. Therefore, stem cell-based therapy is a promising therapeutic strategy that has great potential for rotator cuff healing. In this review, we summarize the advances of stem cells and stem cell-derived EVs in rotator cuff repair and highlight the underlying mechanism of stem cells and stem cell-derived EVs and biomaterial delivery systems. Future studies need to explore stem cell therapy in combination with cellular factors, gene therapy, and novel biomaterial delivery systems.

Keywords: rotator cuff, stem cell, extracellular vesicle, exosome, biologic, regenerative medicine

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1 INTRODUCTION

Rotator cuff injury is one of the leading musculoskeletal diseases worldwide and the most common condition that leads to the complaint of shoulder pain (Picavet and Schouten, 2003). It is estimated that the prevalence of shoulder problems in primary care is 2.4% in the UK (Linsell et al., 2006), and 30%–70% of shoulder pain results from rotator cuff diseases (Mitchell et al., 2005). Intrinsic factors contribute to rotator cuff disease, including age, obesity, smoking, diabetes mellitus, genetics, and narrow anatomical subacromial spaces (Titchener et al., 2014). Among these factors, age-related degeneration is considered the main reason for rotator cuff disease, and the prevalence of rotator cuff tears increases with age in the general population. It was found that the prevalence of sonographic full-thickness rotator cuff tears was 10.7% in the 50s, 15.2% in the 60s, 26.5% in the 70s, and 36.6% in the 80s (Minagawa et al., 2013). While the intrinsic risks decrease the structural resilience of rotator cuff, the extrinsic risks, such as occupations and sports activities, cause excessive mechanical loading on it, involving rotator cuff injury (Whittle and Buchbinder, 2015). According to the continuum of the tendon pathology model, mechanical loading plays an important role in pathological changes (Lewis, 2010), and a repeated and biomechanical loading on the rotator cuff tendon increases the risk of rotator cuff injury (Edmonds and Dengerink, 2014). Rotator cuff injuries may start from tendinopathy and progressively develop into partial or complete tendon tears (Lewis, 2010), which typically result in pain, loss of motion, and functional impairment of the shoulder (Craig et al., 2017). Generally, tendinopathy does not cause substantial problems; therefore, patients with

tendinopathy are initially recommended for a course of conservative management, such as physiotherapy and analgesia (Seida et al., 2010). In some cases, patients with tendinopathy may have an increased risk of tendon rupture, especially among those in the older population (Yasui et al., 2017). Nevertheless, acute shoulder trauma may cause partial or complete tendon tears, which require surgical treatment to repair the continuity of the structure or surgery to reattach the tendon back to its bony insertion.

A previous systematic review that included 15 studies and 371 patients after rotator cuff injury demonstrated improved clinical outcomes with an earlier time of receiving surgery (Mukovozov et al., 2013). Unfortunately, the clinical outcomes remained after surgery, and the overall failure rate of healing was 43% at 12 months postsurgical repair (Rashid et al., 2017), and even up to 90% in the elderly (Galatz et al., 2004). The rehabilitation process following rotator cuff arthroscopic repair usually lasts for a few months, and athletes take over 6 months to return to sports (Thigpen et al., 2016). Additionally, the formation of scar tissue at the injury site can cause tissue adhesion and joint stiffness, as well as poor mechanical properties, which increase the risk of retear (Thomopoulos et al., 2010). Due to these issues, there has been a growing interest in the past decade in preparing stem cells to enhance rotator cuff repair and regeneration. Mesenchymal stem cells (MSCs) are the most popular stem cells because of their accessibility to multiple tissues, anti-inflammatory properties, secretion of trophic factors, and differentiation ability into tenocytes to recellularize the regenerating tissue (Lim et al., 2019). In this review, we summarize the advances of stem cells and stem cell-derived extracellular vesicles in rotator cuff repair, gene therapy, and their biomaterial delivery systems.

2 ROTATOR CUFF STRUCTURE AND HEALING

The rotator cuff comprises four muscles, namely, supraspinatus, infraspinatus, subscapularis, and teres minor muscles, which envelop the shoulder joint and attach closely to the humeral head *via* their tendons (Escamilla and Andrews, 2009; Oliva et al., 2015). These muscles play a critical role in both movement and dynamic stabilization during the locomotion of the shoulder joint (Lin et al., 2018). The particular anatomy of rotator cuff and lack of blood vessels can lead to injuries that cannot be healed easily or effectively (Hegedus et al., 2010). Rotator cuff injury often involves the entire muscle–tendon–bone complex, of which the tendon and tendon–bone interface are the most frequently injured and concerned sections.

The tendon is a unique form of connective tissue that transmits muscle-contraction force to the skeleton to maintain posture or produce motion. It comprises resident cells and the extracellular matrix (ECM). Tenocytes, the main type of cell located inside collagen fibers, produce collagen I and ECM molecules (Nourissat et al., 2015). There are tendon stem/progenitor cells (TSPCs), also commonly termed tendon-derived stem cells (TDSCs), which are capable of renewing tenocytes through differentiation and proliferation to maintain homeostasis (Bi et al., 2007). The ECM contains multitudinous molecules, including collagen, elastin, proteoglycans, and glycoproteins, which are involved in tendon-specific collagen I. The triple-helical collagen I molecules are assembled into fibrils that, in turn, form fibers, fascicles, and, ultimately, tendons. Of these, the collagen fibrils are considered to be the basis for force transmission (Kannus, 2000).

The tendon–bone unit is a specialized structure called an enthesis, which represents a transition between soft tendinous and hard bony tissue (Yang and Temenoff, 2009; Andarawis-Puri et al., 2015) (**Figure 1**). Various resident specialized cell types are found in this tissue, including osteoblasts, osteocytes, osteoclasts, fibrochondrocytes, and tenocytes. The enthesis has been divided into four continuous but distinct zones: tendon, non-mineralized fibrocartilage, mineralized fibrocartilage, and bone (Thomopoulos et al., 2010). Zone 1 (tendon area) mainly consists of collagen I fibers together with a small amount of decorin. Zone 2 (non-mineralized fibrocartilage) predominantly contains collagen II and III fibers, as well as small amounts of collagen I, IX, and X–collagen fibers. The decorin and aggregates also exist. In Zone 3 (mineralized fibrocartilage), the fibrocartilage is mineralized and inserted into the subchondral bone layer. Both aggrecan and mineral components are present in the extracellular matrix composition. Zone 4 (bone area) is mainly a bone-like composition that contains collagen I fiber mineralized in osteoblasts, osteocytes, and osteoclasts. The gradual changes in microstructure allow for mechanical strain, stress distribution, and efficient energy transition (Rossetti et al., 2017; Takahashi et al., 2017).

Following a rotator cuff tear, the injured site undergoes a natural healing process involving three overlapping stages—inflammatory, proliferation, and remodeling (Docheva et al., 2015). In the inflammatory stage, inflammatory cells are attracted to the injury site by pro-inflammatory cytokines, such as neutrophils, monocytes, and macrophages and they yield inflammatory cytokines, including interleukin (IL)-6 and IL-1β (Lin et al., 2004). Additionally, various growth factors are released by cells to promote tissue repair, such as basic fibroblast growth factor (bFGF), bone morphogenetic proteins (BMPs), transforming growth factor-beta (TGF-β), and vascular endothelial growth factor (VEGF) (Docheva et al., 2015). The angiogenic factors induce the formation of a neovascular network that handles the blood supply for newly formed fibrous tissue (Fenwick et al., 2002; Hegedus et al., 2010). Meanwhile, the tenocytes are recruited to the wounded site and induced to proliferate. The second stage is characterized by the abundant synthetic activity of the ECM component; predominately collagen III, directed by recruited fibroblasts, which leads to disorganized alignment of the tendon and mechanical weakness. In the remodeling stage, the density of cells and the synthesis of ECM components both decrease. At the same time, collagen III is gradually replaced by collagen I, which induces the ECM of the tendon to become more aligned; meanwhile, tendon stiffness and tensile strength are restored to the pre-injury level (Voleti et al., 2012).

The healing process involves both intrinsic and extrinsic healing processes (Longo et al., 2011). The extrinsic healing

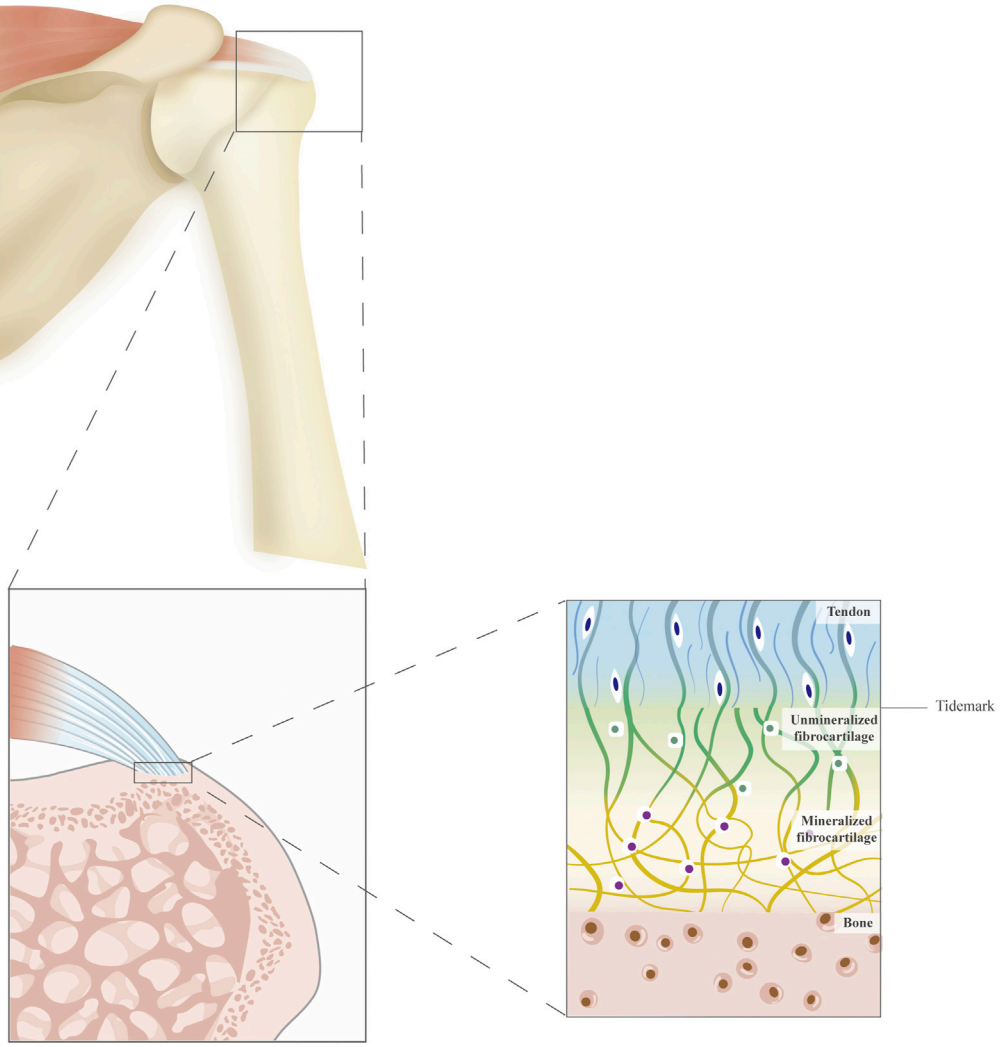

FIGURE 1 | A schematic diagram of the supraspinatus tendon and the structure of the tendon–bone interface. The tendon–bone interface is divided into four continuous but distinct zones: tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone.

process initially occurs, which involves the invasion of extrinsic cells from the surrounding sheath and synovium, resulting in scar tissue formation to substitute for the native enthesis. The formation of scar tissue and the absence of fibrocartilage lead to the secretion of collagen III fibers rather than collagen I fibers. Then, intrinsic healing is activated and simultaneously induces the tenocyte recruitment, proliferation, and secretion of collagen I fibers, which can strengthen the mechanical property of the tendon (Docheva et al., 2015). The scar tissue lacks the gradient of mineral distribution, and the diameter of collagen III fibers is smaller than that of collagen I fibers (Hexter et al., 2017). For this reason, the structure will exhibit weak mechanical properties. Although collagen III fibers can be replaced by collagen I fibers, it usually takes up to 12 months to complete the healing process, which may lead to a higher chance of re-tearing (Lee et al., 2019; Haleem et al., 2021).

3 STEM CELLS FOR ROTATOR CUFF INJURY

3.1 Stem Cell-Based Therapy

The use of MSCs is a promising and outstanding therapeutic efficacy in regenerative medicine. The stem cells have been successfully derived from multiple tissues, including bone marrow, adipose tissue, tendon, umbilical cord blood, bursa, and urine. In this review, we summarize the animal and clinical studies of these stem cells for rotator cuff injuries. The therapeutic effects of MSCs for rotator cuff injuries are summarized in **Figure 2**.

3.1.1 Bone Marrow-Derived Mesenchymal Stem Cells

Bone marrow-derived mesenchymal stem cells (BMSCs) are the first-discovered mesenchymal stem cells, which act as pluripotent

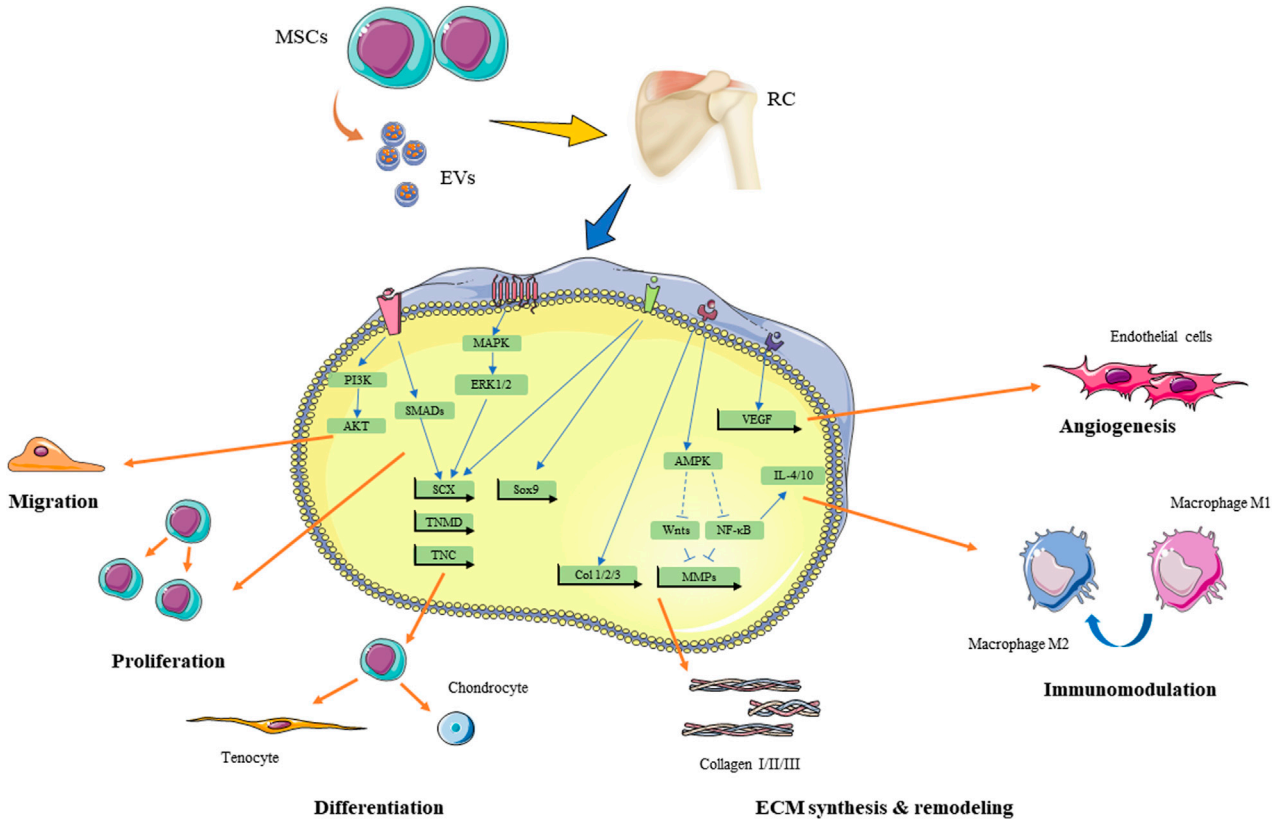

FIGURE 2 | Theraputic effect of MSCs and MSC-EVs for rotator cuff injuries and underlying signaling pathways. RC, rotator cuff; MSCs, mesenchymal stem cells; EVs, extracellular vesicles; MAKP, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinases; PI3K, phosphoinositide 3-kinase; SCX, scleraxis; TNMD, tenomodulin; TNC, tenascin C, Sox9, SRY-Box transcription factor 9; Runx2, runt-related transcription factor 2; AMPK, 5′ AMP-activated protein kinase; NF-κB, nuclear factor kappa B; MMPs,matrix metalloproteinases; IL, interleukin. ECM, extracellular matrix.

cells (Heo et al., 2016) with multilineage differentiation ability (Docheva et al., 2007; Dai et al., 2015; Perucca Orfei et al., 2019) into adipocytes, osteoblasts and chondrocytes, and tenocytes. Therefore, they have been used in various tissue repairs and regeneration procedures. The principal source of BMSCs in rotator cuff injury is autologous cells that can be harvested from the iliac crest and proximal humerus.

Kida et al. (2013) found that drilled holes in the humerus footprint could stimulate autologous BMSCs to infiltrate into the repair site to promote tendon–bone healing by enhancing the ultimate force-to-failure. Exogenous BMSC can be delivered to the repair site by various carriers (Chen P. et al., 2020). The untreated BMSCs increased the early formation of fibrocartilage and collagen orientation as well as biomechanical strength at 2 weeks. The enhancement of fibrocartilage formation is due to the higher chondrogenesis expression, such as *SRY-Box Transcription Factor 9* (*Sox9*), *COL2A1*, and *aggrecan*, during tendon–bone healing (Alves de Araújo et al., 2012). However, it seemed that the effect augmented with BMSCs dissipated by 4 weeks (Degen et al., 2016). In contrast, Gulotta et al. (2009) found no differences amounting to new cartilage formation, collagen fiber organization, or biomechanical strength at either two or four weeks. Additionally, bioactive factor-induced BMSCs could achieve better efficiency in promoting tissue regeneration than BMSCs alone. For instance, BMSCs endowed with platelet-rich plasma (PRP) enhanced the production of growth factors, the ability of osteogenic differentiation, and the resistance of cell death *in vitro*, and they promoted bone formation and the biomechanical property of the newly generated bone *in vivo* (Han et al., 2019). The therapeutic effects of BMSCs for rotator cuff injuries are summarized in **Table 1**.

Some clinical studies have investigated the effectiveness of BMSCs for patients with arthroscopic rotator cuff repair. The procedure of multiple channeling for rotator cuff repair creates holes in the greater tuberosity to promote endogenous BMSCs of the proximal humerus infiltrating into the repair site. A cohort study found no difference between groups that underwent arthroscopic rotator cuff repair with multiple channeling and those without channeling in clinical and structural outcomes at a follow-up of 2 years (Jo et al., 2013). However, another study suggested that patients who accepted the multiple channeling procedures reported a significantly lower retear rate, indicating that BMSCs may improve structural integrity for arthroscopic rotator cuff repair (Hernigou et al., 2014). Taniguchi et al. (2015) reported that applying bone marrow stimulation to the footprint during arthroscopic surface-holding (ASH) repair resulted in

TABLE 1 | Summary of bone marrow mesenchymal stem cells for rotator cuff injuries.

	Animal	Injury model	Type of cells	Method of delivery	Time of observation	Results
Gulotta et al., (2009)	Rat	Acute tear and repair of supraspinatus tendon	BMSC	Fibrin glue carrier	2 and 4 weeks	No improvement on the structure, composition, or strength of the healing tendon attachment
Kida et al., (2013)	Rat	Acute tear and repair of supraspinatus tendon	BMSC	Transosseous drilling	2, 4, and 8 weeks	BMSC infiltrated the repaired tendon, and improved biomechanical property
Degen et al., (2016)	Rat	Acute tear and repair of supraspinatus tendon	BMSC	Fibrin glue carrier	2 and 4 weeks	Improved early histologic appearance and biomechanical strength
Thangarajah et al., (2018)	Rat	Chronic tear and repair of supraspinatus tendon	BMSC	A demineralized bone matrix with Fibrin glue carrier	6 weeks	Enhanced rotator cuff healing and restore bone mineral density at the enthesis
Liu et al., (2019)	Dog	Acute tear and repair of infraspinatus tendon	BMSC	Engineered tendon–fibrocartilage–bone composite with BMSC cell sheet	6 weeks	Enhanced rotator cuff anatomic structure, collagen organization and biomechanical strength
Han et al., (2019)	Rat	Acute tear and repair of supraspinatus tendon	BMSC	Not mentioned	4 and 8 weeks	Enhanced biomechanical property of the newly generated bone
Chen P. et al., (2020)	Rabbit	Acute tear and repair of supraspinatus tendon	BMSC	3D-printed PLGA Scaffolds	4, 8 and 12 weeks	Enhanced collagen formation and increased collagen diameter in the tendon–bone interface, and improved the biomechanical properties
Gulotta et al., (2010)	Rat	Acute tear and repair of supraspinatus tendon	BMSC transfected with MT1-MMP	Fibrin glue carrier	2 and 4 weeks	Presence of more fibrocartilage at the insertion and improved biomechanical strength
Gulotta et al., (2011b)	Rat	Acute tear and repair of supraspinatus tendon	BMSC transfected with BMP-13	Fibrin glue carrier	2 and 4 weeks	No differences in the histologic appearance and biomechanical strength of the repairs
Gulotta et al., (2011a)	Rat	Acute tear and repair of supraspinatus tendon	BMSC transfected with sleraxis	Fibrin glue carrier	4 and 8 weeks	Improved histologic appearance with more fibrocartilage and higher biomechanical strength

improved cuff repair integrity based on Sugaya's classification by postoperative magnetic resonance imaging, particularly in large-massive tears. In a case-control study, the healing rates of BMSC augmented repair and repair only during arthroscopy were 100% and 67%, respectively. Furthermore, the augmentation of BMSCs prevents further tears after a follow-up of 10 years (Hernigou et al., 2014).

3.1.2 Adipose-Derived Stem Cells

In recent years, it has been attractive to use adipose-derived stem cells (ADSCs) to enhance rotator cuff repair because of their easy acquisition and ability to inhibit osteogenic differentiation by modulating the microenvironment and anti-inflammatory properties (Bunnell et al., 2008; Kokubu et al., 2020). ADSCs are an ideal source of stem cells in regeneration therapy due to their accessibility; they can be isolated in large quantities from subcutaneous adipose tissue (Bunnell et al., 2008) and liposuction aspirates (De Francesco et al., 2015). As undifferentiated stem cells, they have high proliferation rates and potentially differentiate into tenocytes with growth factors and mechanical stress (Dai et al., 2015; Rinella et al., 2018).

In addition, ADSCs have shown similar therapeutic effects to BMSCs in rotator cuff regeneration. It showed that ADSCs mediated acute inflammation with diminished presence of edema and neutrophils but did not improve the biomechanical properties of tendon–bone healing from two to eight weeks after repair in a rat acute rotator cuff repair model (Mora et al., 2014; Barco et al., 2015). Lipner et al. (2015) reported that ADSCs could reverse the dominated fibrovascular scar response in acute tendon–bone healing. Compared to acute rotator cuff injury, chronic rotator cuff injury causes bone loss and reduced structural properties. ADSCs transplanted to the injured site can increase the bone mineral density of the proximal humerus to promote tendon–bone healing in repairs of chronic tears (Kaizawa et al., 2019; Rothrauff et al., 2019; Shin et al., 2020). Additionally, injection of ADSCs into the musculotendinous junction area of the subscapularis can improve muscle function by electromyographic evaluation and decrease fatty infiltration of the muscle, and it tends to enhance the load-to-failure in chronic rotator cuff tears (Oh et al., 2014). The therapeutic effects of ADSCs for rotator cuff injuries are summarized in **Table 2**.

ADSCs are some of the most commonly used stem cells in clinical research on rotator cuff injuries. The cohort study by Kim et al. (2017) revealed that 182 patients treated with an injection of ADSCs loaded in fibrin glue (4.46×10^6 cells) during arthroscopic rotator cuff repair could significantly improve structural outcomes assessed in terms of the retear rate, and MRI results indicated that the retear rate of the ADSC group was less than that of the control group (14.3% and 28.5%, respectively) at a

TABLE 2 | Summary of adipose stem cells for rotator cuff injuries.

	Animal	Injury model	Type of cells	Method of delivery	Time of observation	Results
Oh et al., (2014)	Rabbit	Chronic tear and repair of subscapularis tendon	ADSC	Balanced salt solution	6 and 12 weeks	Improved muscle function and decreased fatty infiltration, but no significant different biomechanical strength after cuff repair
Mora et al., (2014)	Rat	Acute tear and repair of supraspinatus tendon	ADSC	Collagen carrier	2 and 4 weeks	Less inflammation, but no improvement of biomechanical property
Barco et al., (2015)	Rat	Acute tear and repair of supraspinatus tendon	ADSC	Fibrin sealant	4 and 8 weeks	Less presence of neutrophils and more presence of plasma cells without improving histologic appearance and biomechanical strength
Lipner et al., (2015)	Rat	Acute tear and repair of supraspinatus tendon	ADSC/ ADSC transfected with BMP-12	PLGA nanofibers with gradients in mineral with fibrin hydrogel	2, 4 and 8 weeks	ADSC transfected with BMP-12 decrease mechanical properties, strength, and modulus in the repair site
Rothrauff et al., (2019)	Rat	Acute tear and repair of the supraspinatus and infraspinatus Tendons/ Chronic Intramuscular injection of botulinum toxin A and repair	ADSC	GelMA/fibrin hydrogel	4 weeks	Higher bone mineral density of the proximal humerus in chronic model with both GelMA/Fibrin hydrogel delivery system
Kaizawa et al., (2019)	Rat	Chronic tear and repair of supraspinatus tendon	ADSC	Human tendon hydrogel	8 weeks	Tendon hydrogel augmentation with ADSC improves biomechanical properties and fibrocartilage area than no treatment, but no improvement of tendon–bone interface than tendon hydrogel alone
Shin et al., (2020)	Rat	Chronic tear and repair of supraspinatus tendon	ADSC	Engineered cell sheets	2 and 4 weeks	Larger fibrocartilage area, higher bone volume/total volume values, and biomechanical property

minimum of 12 months after surgery. However, there were no significant differences in pain intensity, range of motion, or self-reported function at 28 months of follow-up.

For patients with symptomatic partial-thickness rotator cuff tears (sPTRCT), surgery may not be the first-choice therapy; thus, many studies have used ADSC therapy as a regeneration strategy. In a pilot RCT study, patients with sPTRCT who did not respond to physical therapy treatments for at least 6 weeks were randomly assigned to receive a single injection of unmodified, autologous adipose-derived regenerative cells (UA-ADRC) (11.4×10^6 cells) or a single injection of 80 mg of methylprednisolone (40 mg/ml; 2 ml) plus 3 ml of 0.25% bupivacaine. The patients in the UA-ADRC group showed significantly higher total scores on the American Shoulder and Elbow Surgeons Standardized Shoulder Assessment Form (ASES) at 3 and 12 months post-treatment compared to those in the corticosteroid group. No severe adverse events related to the injection of UA-ADRCs were reported at the 12-month post-treatment follow-up (Hurd et al., 2020). A retrospective comparative study showed that a high-dose (1.0×10^8 cells) intratendinous injection of ADSCs for patients with sPTRCT can improve shoulder function scores and rotator cuff strength for up to 2 years post-treatment. The results of the MRI showed that the bursal-sided defects nearly disappeared at 1 year and did not recur for up to 2 years. Importantly, there were no treatment-related adverse events at a minimum 2-year follow-up (Jo et al., 2020).

3.1.3 Tendon Stem/Progenitor Cells

Progenitor/tendon stem cells (TSPCs), also known as tendon-derived stem cells (TDSCs), were isolated and identified from humans and mice in 2007 (Bi et al., 2007). TSPCs are so named because they can be harvested and isolated from the tendon of the supraspinatus and the long head of the biceps during arthroscopic rotator cuff repair procedures (Tsai et al., 2013; Dei Giudici and Castricini, 2020). Like other MSCs, TPSCs have characteristics of high clonogenicity, self-renewal capacity (Al-Ani et al., 2015), and multi-differentiation potential, including tenocytes, chondrocytes, osteocytes, and adipocytes (Zhang and Wang, 2010; Leonardi et al., 2021). They have highly expressed tendon-related genes, including *COL1A1*, *tenascin C* (*TNC*), *Scleraxis* (*Scx*), and *Tenomodulin* (*TNMD*), which may contribute to spontaneous tenogenic differentiation (Guo et al., 2016). Therefore, TPSCs are a promising source of tendon regeneration. However, owing to their abundance in the tendon, it is challenging to obtain autologous TSPCs, which could limit their application in clinical studies.

Several studies have investigated the utilization of TPSCs to treat tendon disorders in pre-clinical studies (Song et al., 2018). Shen et al. (2012) used allogenic TSPC-seeded scaffolds to augment rotator cuff repair in a rabbit model. There was no elicited immune response, with decreasing lymphocytic infiltration at early repair and improving histological and biomechanical properties compared to non-TSPC treatment

control repairs at 12 weeks post-surgery. Furthermore, it showed that transplanting the cell sheet that was derived from the rotator cuff promoted cartilage regeneration and angiogenesis at the enthesis and upregulated the expression of genes *VEGF* and *COL2A1* at 4 weeks and a greater ultimate failure load at 8 weeks after surgery (Harada et al., 2017).

3.1.4 Umbilical Cord-Derived Mesenchymal Stem Cells

Umbilical cord-derived mesenchymal stem cells (UCB-MSCs) are a promising source of human cells because of their easy availability, high proliferation capacity, and low immunogenicity (Wang et al., 2009; Bai et al., 2016). The advantage of UCB-MSCs is that allogeneic stem cells do not require autologous tissues, such as bone marrow aspiration and adipose tissue (Kasper et al., 2009). Therefore, UCB-MSCs can be prepared early before treatment, and the function of stem cells is not affected by the age of patients or disease. Park et al. (2015) introduced the UCB-MSCs injection under ultrasound guidance to rabbits with acute full-thickness subscapularis tendon tears and revealed that UCB-MSCs promoted the partial regeneration of rotator cuff tendon tears with improved histologic appearance, tendon size, and walking capacity. Then, they investigated the efficacy of UCB-MSCs for chronic full-thickness rotator cuff tendon tears without repair and found that the injection of UCB-MSCs had a similar therapeutic effect in histological examination and motion analysis of walking 4 weeks after treatment (Rak Kwon et al., 2020). However, there was no difference between the high-dose and low-dose (2×10^6 and 1×10^6 cells, respectively) of UCB-MSCs, indicating that the benefits of UCB-MSCs were not in a dose-dependent manner (Kwon et al., 2019). In another study, UCB-MSCs–seeded biomimetic hydroxyapatite-gradient scaffold regenerated the tendon–bone interface of the rotator cuff in a rat repair model in terms of improving collagen organization, cartilage formation, and similar biomechanical properties as the normal tendon–bone interface at 8 weeks (Yea et al., 2020).

3.1.5 Bursa-Derived Cells

Previous studies have suggested that the subacromial bursa is an important source of pluripotent stem cell potency for tendon healing (Utsunomiya et al., 2013; Baldino et al., 2020). Bursa-derived stem cells (B-MSCs) are easily accessible stem cells that can be harvested from routine rotator cuff repair surgery (Baldino et al., 2020). Like other stem cells, B-MSCs demonstrate high proliferation ability and multipotential differentiation *in vitro* (Utsunomiya et al., 2013). Recently, Muench et al. (2020) proved that B-MSCs consistently exhibited high cellular proliferation regardless of patient demographics (age, sex, body mass index, smoking status, and presence of systemic comorbidities), characteristics of rotator cuff tear (size, tendon retraction, fatty infiltration, and muscle atrophy), and the severity of glenohumeral joint degeneration.

Another study found that B-MSCs isolated from human bursae were characterized by multilineage differentiation, including osteoblastic, adipogenic, chondrogenic, and tenogenic lineages *in vitro* and *in vivo*. Additionally, they were induced into the bone, fibrocartilage, and tendon under different environments or pretreatments (Song et al., 2014). Compared with B-MSCs, cells isolated from bursae displayed superior engraftment and survival in tendon tissue and increased the thickness of the healing tissue compared with tissue that did not receive cells (Dyrna et al., 2018). Thus, it is suggested that B-MSCs are potent promising cells in rotator cuff injury; further studies should confirm their therapeutic effect for rotator cuff injury in pre-clinical and clinical studies.

3.1.6 Urine-Derived Stem Cells

Currently, studies pay more attention to stem cells isolated from urine (USCs) due to their robust proliferation ability and multipotential differentiation into osteocytes, chondrocytes, adipocytes, neurocytes, and myocytes (Bharadwaj et al., 2013; Ji et al., 2017). The obvious advantage of USCs is that the harvest method is noninvasive and accessible. As autologous stem cells, USCs exhibit low immunogenicity, which may cause a low rejection response during treatment. Therefore, USCs are considered an attractive source of stem cells for rotator cuff healing. Chen Y. et al. (2020) implanted an autogenous, TGF-β3-induced USC sheet to the injured site of rotator cuff repair, evident by increased bone volume and trabecular thickness, which yielded enthesis-like tissue with more proteoglycan and collagen, as well as higher failure load and stiffness in comparison to the control group only at 12 weeks post repair. Nevertheless, more studies are required to evaluate the efficacy of untreated USCs on both acute and chronic rotator cuff injuries to offer a better research basis for future clinical transplantation.

3.2 Extracellular Vesicle-Based Therapy

MSCs are thought to mediate therapeutic functions in a paracrine manner in addition to their multipotent differentiation capacity and direct intercellular interactions. In the example of a rotator cuff injury model, conditioned medium (CM) of human bone marrow-derived stem cells promotes rotator cuff healing by increasing histologic score, bone mineral density and biomechanical tensile after surgery (Reiner et al., 2017; He et al., 2021). To search for the therapeutically active components, Bruno and colleagues successfully fractioned CM of MSCs by ultracentrifugation and discovered the therapeutic vesicular structures (Bruno et al., 2009). Collectively, these nano-sized particles with a lipid bilayer, naturally released by cells, are called extracellular vesicles (EVs) (Théry et al., 2018). They can be categorized into exosomes, microvesicles, and apoptotic bodies according to their cellular origins. Exosomes are the smallest vesicle types (40–120 nm), and they originate from the inward budding of late multivesicular endosomes (MVEs) and are released upon fusion of MVEs with the plasma membrane. Meanwhile, microvesicles are formed by budding from the plasma membrane, and their size can vary from 50 nm to 1 μm. Unlike exosomes and microvesicles, which are released by all cells, apoptotic bodies are vesicles (50 nm–5 μm) produced by cells undergoing apoptosis. EVs have become an attractive approach in regenerative medicine because they exert biological activities like those of stem cells and overcome the shortages of cell-based therapy, such as cell expansion, low survival rate, and potential immunological rejection (Keshtkar et al., 2018; Woo

TABLE 3 | Summary of extracellular vesicles from mesenchymal stem cells for the repair of rotary cuff injuries.

	Animal	Injury model	Source of EVs/extraction/dose/frequency	Delivery method/site	Time of observation	Results
Wang C. et al., (2020)	Rabbit	Acute tear and repair of supraspinatus tendon	human ADSC-derived EVs/ultracentrifugation/1 $\times 10^{11}$ particles/once	Saline/distal site of the supraspinatusmuscle	6 and 18 weeks	ASC-Evs showed significantly lower fatty infiltration, a higher histological score and more newly regenerated fibrocartilage at the repair site and biomechanical properties than the saline group
Huang et al., (2020)	Rat	Chronic tear and repair of supraspinatus tendon	Rat BMSC-derived EVs/ultracentrifugation/200 μg/once	PBS/tail vein	4 and 8 weeks	BMSC-Evs increased the breaking load and stiffness of the rotator cuff after repair, induced angiogenesis around the rotator cuff endpoint, and promoted growth of the tendon–bone interface
Wang et al., (2021)	Mice	Chronic supraspinatus tendinopathy	Human ADSC-derived EVs/ultracentrifugation/1 $\times 10^{11}$ particles/once	Saline/enthesis site of the supraspinatusmuscle	4 weeks	Mice in the ASC-Evs group showed less cellular infiltration, disorganization of collagen, and ground substance deposition, and higher maximum failure load and stiffness, than that of the saline group
Fu et al., (2021)	Rat	Chronic tear and repair of supraspinatus tendon	Human ADSC-derived EVs/ultracentrifugation/300 μg/once	Commercial hydrogel/shoulder site (no detailed information)	4 and 8 weeks	The ADSC-EVs have less inflammation and mire regular alignment and greater the expression of *RUNX2*, *Sox9*, *TNMD*, *TNC* and Scx, and higher mechanical properties compared with other groups
Kim et al., (2022)	Rabbit	Chronic tear and repair of supraspinatus tendon	Human umbilical cord-derived EVs/ultracentrifugation/2.9 $\times 10^{9}$ particles/once	Collagen/bursal side of the repaired site	12 weeks	The umbilical cord-derived EVs showed greater histomorphometric total score of collagen maturation in bone–tendon interface, lower fatty degeneration, and growing trends in mechanical properties as compared with applying collagen only or repair only

et al., 2020). It is believed that MSCs cultured in chemically defined serum-free media may be more suitable for the manufacture of EVs. The methods of separation and concentration may vary depending on the size of EVs and the purpose of end-use. According to a worldwide ISEV survey in 2015, ultracentrifugation was the most widely used primary EV separation and concentration technique (Gardiner et al., 2016). A variety of techniques have also been developed to achieve better specificity of separation, such as density gradients and size-exclusion chromatography (Théry et al., 2018). However, there are currently no accepted metrics for assessing the purity or degree of purity in EV preparation (Reiner et al., 2017). Although the mechanism of EVs is not fully understood, it is believed that EVs secreted by stem cells can promote tissue repair and regeneration by inducing the proliferation of cells, promoting angiogenesis, modulating the inflammation process, and affecting cell apoptosis (He et al., 2021). The therapeutic effects of MSC-EVs for rotator cuff injuries are summarized in **Figure 2** and **Table 3**.

Notably, the most important impact of EVs on tissue regeneration is their immunomodulatory properties at both humoral and cellular levels. They can reduce injury-induced inflammation by dampening but not inhibiting complement activation through CD59, and they involve the promotion of anti-inflammatory and pro-regenerative (M2) macrophages over pro-inflammatory M1 macrophages and concomitantly enhance the expression of anti-inflammatory cytokines such as IL-10 instead of pro-inflammatory cytokines such as IL-1β and TNF-α (Toh et al., 2018). Additionally, EVs contain a large amount of biological information, including biologically mRNAs, miRNAs, and lncRNAs, which are important for modulating the signaling of the endogenous and exogenous cells of the injured site (Forsberg et al., 2020). Because of this advantage, engineered EVs are also regarded as candidate cargo to realize gene therapy for injuries. Additionally, the different resources of human MSC-derived EVs can exhibit distinct characteristics that reveal their potential applications in different fields. Bone marrow MSC-derived EVs have shown superior regeneration ability, and adipose tissue MSC-derived EVs have played a significant role in immune regulation, whereas umbilical cord MSC-derived EVs are prominent in tissue damage repair (He et al., 2021).

BMSC-derived EVs (BMSC-EVs) are widely used in the musculoskeletal regeneration field. Recently, one study solely injected BMSC-EVs into the vein to promote rotator cuff repair (Huang et al., 2020). The results showed that BMSC-EVs promoted angiogenesis around the tendon–bone interface, histologic histological appearance, and biomechanical strength, and they inhibited the secretion of pro-inflammatory factors in rat rotator cuff reconstruction. They believed that the mechanism by which BMSC-EVs achieve the healing process may be through the proliferation, migration, and angiogenic tube formation of

human umbilical vein endothelial cells (HUVECs) by regulating the angiogenic signaling pathway, inhibiting the polarization of M1 macrophages, and also inhibiting the secretion of pro-inflammatory factors by M1 macrophages (Huang et al., 2020). A previous study reported BMSC-EVs can also suppress inflammation by increasing the expression of anti-inflammatory mediators IL-10 and IL-4 at an early phase of healing (Shi et al., 2019). Moreover, local delivery of BMSC-EVs can promote tendon regeneration by facilitating the proliferation, migration, and tenogenesis differentiation of endogenous TPSCs (Shi et al., 2019; Yu et al., 2020).

ADSC-derived EVs (ADSC-EVs) have regeneration and immunomodulation capacities (Chen et al., 2021; Wang et al., 2021). To investigate the effect of ADSC-EVs on chronic rotator cuff tears, Wang C. et al. (2020) established a bilateral rotator cuff chronic tear model and injected isolated ADSC-EVs at the injury site of the supraspinatus muscle at the time of repair. This demonstrated that the local injection of ADSC-EVs inhibited fatty infiltration, regenerated fibrocartilage, and increased biomechanical strength. Similarly, Fu et al. (2021) reported that rotator cuff injected with ADSC-EV-loaded hydrogel exhibited aligned collagen fiber and muscle bundles and enhanced mechanical properties. A variety of mechanisms may contribute to ADSC-EVs in rotator cuff repair. TPSCs regulate the proliferation, migration, and tenogenic differentiation of TPSCs. Studies have reported that ADSC-EVs can upregulate the expression of the tenogenesis genes *TNMD*, *TNC*, and *Scx in vivo* (Liu H. et al., 2021; Fu et al., 2021). Smad signaling pathways play vital roles in regulating stem cell activity. This effect may be related to activated SMAD2/3 and SMAD1/5/9 signaling pathways, which play vital roles in regulating stem cell activity (Liu H. et al., 2021). In addition, ADSC-EVs regulate the early inflammatory response in rotator cuff healing by decreasing the M1 macrophage, enhancing the M2 macrophage, and reducing the secretion of pro-inflammatory cytokines, such as IL-1β, IL-6, IL-8, and MMP-9 (Liu H. et al., 2021). In a human supraspinatus explant experiment, ADSC-EVs maintained homeostasis of the impaired tendon by increasing expression of *COL1A1*, *COL3A1*, and an elevated type I/III ratio and by decreasing expression of *MMP-9* and *MMP-13* (Zhang et al., 2021). The underlying mechanism might be enhancing AMPK signaling to suppress Wnt/β-catenin activity or NF-κB pathway (Ma et al., 2019; Zhang et al., 2021).

Few research observations of TPSC-derived EVs (TPSC-EVs) exist on rotator cuff repair. Recently, a study reported that TPSC-EVs suppressed inflammation and apoptosis at 1 week after surgery; the tendon exhibited a more continuous and regular arrangement and a larger collagen fiber diameter in the TPSC-EV-treated group compared to the non-TPSC-EV-treated group at two and eight weeks after surgery (Zhang et al., 2020a). The augment of TPSC-EVs can be partially explained by promoting the proliferation and migration of tenocytes in a dose-dependent manner in an *in vitro* study, which was related to the activation of the PI3K/AKT and MAPK/ERK1/2 signaling pathways (Zhang et al., 2020a). Nevertheless, the detailed mechanism of TPSC-EVs is still poorly understood and needs further investigation.

Many studies have reported the potential of human umbilical cord mesenchymal stem cell-derived EVs (HUMSC-EVs) in tendon repair. Kim et al. (2022) demonstrated that the HUMSC-EVs laden injectable collagen could effectively promote bone-to-tendon healing *via* collagen maturation in the bone–tendon interface and prevent fatty degeneration of the rotator muscle at 4 weeks after rotator cuff repair. In a rat Achilles tendon injury model, treatment with HUMSC-EVs improved the histological structure, enhanced tendon-specific matrix components, and optimized biomechanical properties of the Achilles tendon, which was related to the overexpression of miR-29a-3p regulated by PTEN/mTOR/TGF-β1 signaling (Yao et al., 2021).

3.3 Gene Therapy

An increasing number of studies have utilized gene therapy to enhance and expand the therapeutic effectiveness of stem cells in tendon repair. In comparison to conventional stem cell therapy, modified stem cells or their EVs yield more production of a gene in the local injured site, with greater biological activity and lower immune response. There are two main methods of transferring the gene to target cells: viral and non-viral vector methods. Viral vectors have been widely used in gene therapy due to their high efficiency in gene delivery into the cells they infect. Commonly, recombinant viruses include adenovirus, lentivirus, retrovirus, and adeno-associated virus in tendon repair. However, the use of viral vectors may also meet the challenges of high-cost expenses and safety issues. Thus, non-viral vectors, such as plasmids, increase the interest of researchers in gene delivery because of their safety, simple manufacture, and lower immunogenicity.

Concerning rotator cuff regeneration, many studies have focused on facilitating the tenogenic differentiation of stem cells to promote rotator cuff repair. Gulotta et al. (2011a) showed that BMSCs transfected to overexpress *Scx* promoted the formation of fibrocartilage at the tendon insertion and improved biomechanical strength at 4 weeks for rats who underwent unilateral detachment and repair of the supraspinatus tendon. Owing to the tear of enthesis (Zones 3 and 4) in rotator cuff injuries, it was meaningful to transfer osteogenic and chondrogenic genes to enhance tendon–bone healing. It was demonstrated that, when transfected to overexpress developmental genes, membrane type 1 matrix metalloproteinase (MT1-MMP), which is thought to direct the process of ossification, promotes the formation of fibrocartilage at the tendon insertion and improves biomechanical strength (Gulotta et al., 2010). However, ADSC transduced with the osteogenic factor bone morphogenetic protein 2 (BMP-2) led to impaired healing by losing bone mass and decreasing biomechanical properties (Lipner et al., 2015).

In terms of chondrogenic genes, the genetically modified BMSCs with overexpressing BMP-13 showed no improvement in either biomechanical parameters or histological appearance in acute rotator cuff repair (Gulotta et al., 2011b). Additionally, gene-modified stem cells can inhibit inflammation during the healing process. Cheng et al. (2014) discovered that the silent TNF-α stimulated gene/protein 6 (TSG-6) of TPSCs reduced biomechanical strength, indicating that TPSCs might promote

rotator cuff healing through regulating anti-inflammatory response by TSG-6 signaling. Schnabel et al. (2009) explored using the insulin-like growth factor-I (IGF-1) gene enhanced BMSCs significantly improved tendon histological scores and reduced ECM degradation in collagenase-induced bilateral tendinitis lesions, but the benefit of IGF-1 gene enhancement was not obvious compared to untreated BMSCs.

EVs contain miRNAs from donor cells that can be transferred to recipient cells, thereby promoting the expression of specific proteins. Therefore, an increasing number of studies pay attention to utilizing engineered EVs to transfer genes in musculoskeletal disorders such as osteoporosis (Yang et al., 2020) and osteoarthritis (Tao et al., 2017). Several studies have demonstrated the promising results of engineered EVs in tendon repair and regeneration. Early tendon remodeling plays a vital role in tendon regeneration. Yao et al. (2021) revealed that miRNA-29a-3p loaded HUMSC-EVs reduced the area of the lesion and improved histological scores in a tendinopathy model. The underlying pathway was reducing transcript levels of collagen III *via* the PTEN/mTOR/TGF-β1 signaling pathway (Millar et al., 2015; Watts et al., 2017).

Another strategy of gene therapy for tendon healing is to inhibit the fibrous process of the tendon and surrounding tissues. The EVs derived from antagonists targeting miR-21a-3p treatment of HUMSC, which expressed low levels of miR-21a-3p, expanded the inhibition of tendon adhesion by manipulating p65 activity, suggesting that delivering low-abundance miR-21a-3p may inhibit tendon adhesion. Furthermore, some miRNAs participate in tenogenic differentiation and prevent chondro-osteogenic differentiation, including miR-124, miR-135a, miR-140, and miR-337-3p (Chen et al., 2015; Wang et al., 2016; Geng et al., 2020; Liu Y. J. et al., 2021). However, the effectiveness of the delivery of these genes by EVs has not been confirmed.

4 BIOMATERIALS

Innovation in the field of biomaterials has driven the development of regenerative medicine and tissue engineering. Biomaterials are bioresorbable and gradually degraded so that tissues have sufficient space for regeneration as well as negligible immunogenicity and side effects locally and systematically (Garg et al., 2012). In rotator cuff repair, biomaterials used for stem cell or EV delivery can be divided into two categories: implantable and injectable delivery systems (Chen et al., 2019; Liu et al., 2020). Commonly, an implantable delivery system is composed of tissue engineering scaffold biomaterials, which should have a three-dimensional structure that allows for cell attachment, growth, and proliferation. In addition, biomaterials used for injectable delivery systems are supposed to carry bioactive factors and cells to the target injury site while minimizing the spread of drugs. Injectable deliveries have the advantage of a minimally invasive nature, but they cannot provide sufficient support for cells and impaired tissues. The delivery system in rotator cuff repair includes, decellularized tissues, electrospun nanofiber scaffolds, hydrogels, and patterned scaffolds, but is not limited to these (Longo et al., 2012; Saveh-Shemshaki, 2019).

4.1 Biomaterial Polymers

To date, various natural and synthetic materials have been developed to promote stem cells in rotator cuff repair and regeneration. In our review, natural polymers are most widely used in stem cell therapies for rotator cuff injuries (Gulotta et al., 2010; Gulotta et al., 2011a; Degen et al., 2016; Liu et al., 2019). They have the advantages of non-toxicity, biocompatibility, and biodegradation, as well as cell proliferation and cell adhesion. Nevertheless, it is difficult to modify their physical and chemical properties, which remains a potential immunogenicity problem (Garg et al., 2012). Commonly used natural materials include ECM-derived biomaterials, hyaluronic acid (HA), chondroitin sulfate (CS), and fibrin. As the main component of the ECM, ECM-based biomaterials provide a biomimetic environment suited for tissue remodeling. For instance, Liu et al. (2019) reported a novel biomaterial that uses engineered tendon–fibrocartilage–bone composite (TFBC) augmentation with BMSCs to form a "sandwich" structure that can enhance rotator cuff healing in terms of anatomic structure, collagen organization, and biomechanical strength. Decellularized matrices have been explored for their regenerative effects on tendon repair; however, tissue resources should be considered. While the tendon-derived decellularized matrix promoted the tendinous phenotype in TSPCs and inhibited their osteogenesis, the dermal skin-derived collagen matrix had no apparent effect on TSPC differentiation (Yin et al., 2013). Hyaluronic acid (HA) is an anionic, non-sulfated glycosaminoglycan that is distributed in the intercellular matrix of most connective tissues. It has been reported that HA decreases the cell proliferation and expression level of procollagen α1 (III) mRNA of tendon-derived fibroblasts (Yamada et al., 2007). Moreover, CS is a natural polymer and a major ECM component that has the ability to reduce inflammation by diminishing NF-κB activation and nuclear translocation (Vallières and Du Souich, 2010). Human mesenchymal stem cells cultured within the decellularized amniotic matrix wrapped around the collagen-chondroitin sulfate scaffold could maintain metabolic activity and down-regulate the pro-inflammatory cytokines (Hortensius et al., 2018). Fibrin is formed following the cleavage of fibrinogen and thrombin and can be processed into hydrogels or fibrous scaffolds. Tissue-engineered construction based on fibrin hydrogel has better extracellular matrix organization and biomechanical properties compared to collagen-based hydrogels (Breidenbach et al., 2015; Thangarajah et al., 2018).

Synthetic materials are also used extensively in tendon regeneration since their molecular weight, hydrophobicity, and degradation speed can be easily modified, and due to their low cost of fabrication (Ruiz-Alonso et al., 2021). Notably, it is possible to achieve similar mechanical properties with tendon tissue and good structural integrity, which are important in the regeneration of tendon repair (Pina et al., 2019). Compared to natural biomaterials, synthetic materials have a low risk of disease transmission because they are not obtained from biological organisms or tissues. For this reason, they have a high risk of immune response (Won et al., 2014). Since synthetic materials are hydrophobic in nature, they may also cause poor cell adherence, low proliferation rates, and altered phenotypes of stem cells

(Theisen et al., 2010). Numerous synthetic materials are used for tendon tissue repairs, such as poly-ε-caprolactone (PCL), poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (ethylene glycol) (PEG), and poly (lactic-co-glycolic acid) (PLGA). These synthetic polymers can be electrospun into nano- and microfibrous scaffolds, which mimic aligned collagen fibers in tendon tissue and promote tenogenic differentiation (Vuornos et al., 2016; Laranjeira et al., 2017; Calejo et al., 2019; El Khatib et al., 2020). The rate of degradation determines its usage. For example, polymers with a low degradation rate, such as PCL, are suitable for building longer-term tendon scaffolds (Laranjeira et al., 2017; Calejo et al., 2019), while polymers with faster degradation rates are less suitable since they may increase the inflammation response, including PLA, PGA, and PLGA (Yokoya et al., 2008; Vuornos et al., 2016; Chen et al., 2019; Chen P. et al., 2020; Araque-Monrós et al., 2020; El Khatib et al., 2020). According to this characteristic, a PEG-based hydrogel system with a range of degradation rates can control the timing of MSC delivery to the target site of tendinopathy (Qiu et al., 2011).

4.2 Interactions Between Biomaterials and Stem Cells

The topographical and mechanical properties of biomaterials impact the proliferation and tenogenic differentiation of stem cells, including fiber diameter, pore size, alignment, surface roughness, and matrix stiffness. Fiber with a large diameter promotes the expression of tenogenic genes, such as *Scx*, in stem cells. This suggests that large-diameter fibers (e.g., >2 μm) may be more suitable for MSC differentiation into tendon lineage than small-diameter fibers (Cardwell et al., 2014). Generally, the pore size of the scaffold plays an important role in migration ability (Zheng et al., 2017); a larger pore size of PLGA scaffolds significantly enhances the migration of BMSCs *in vitro* (Dai et al., 2018). Fiber alignment provides tissue-specific biomechanical cues to resident cells in the native tendon. Studies have indicated that scaffolds with aligned fibers enhance cell infiltration, ECM deposition, collagen alignment, and tendon-related gene expression of stem cells when compared to nonaligned fibers (Orr et al., 2015; Zheng et al., 2017). In the massive rotator cuff repair model, scaffolds with aligned fibers exhibit more conspicuous native microstructures, better alignment, and better mechanical properties at 12 weeks post-implantation (Zheng et al., 2017). Due to the mechanical microenvironment of the tendon, matrix stiffness impacts stem cells during tendon repair. The proliferation of TPSCs increases and more stress fibers form with increasing matrix stiffness. Furthermore, the differentiation of TPSCs into tenogenic lineages is inhibited on stiff hydrogel with reduced expression of tendon-specific genes *THBS4*, *TNMD*, and *SCX* by regulating FAK and ERK1/2 pathways (Liu et al., 2018).

Concerning rotator cuff repair, the scaffold is an effective tool for transmitting mechanical stimulation to delivered cells; thus, the mechanical environment provided by biomaterials should be considered in cell delivery. The mechanical stimulation of stem cells is vital in tendon tissue repair and has been shown to influence the differentiation and proliferation of stem cells (Wang H.-N. et al., 2020). The magnitude of stretching could lead to different cell fates. Studies indicated that 4% stretching promoted the differentiation of TPSCs into tenocytes with increased gene expression of *COL1A1*; 8% stretching, however, promoted the differentiation of TPSCs into non-tenocytes, including adipocytes, chondrocytes, and osteocytes, aside from differentiation into tenocytes, as evidenced by higher expression levels of genes such as *PPARγ*, *COL2A1*, *Sox9*, and *Runx2 in vitro* (Wang H.-N. et al., 2020).

Furthermore, mechanical stimulation of BMSCs significantly increased the expression of tenogenic genes and anti-inflammatory cytokines (Ciardulli et al., 2020). In addition, biomaterials containing magnetic elements have been developed to mechanically stimulate stem cells in tendon regeneration. Human adipose stem cells cultured on the magneto-mechanical actuation scaffold increased the expression of tendon-related genes *Scx* and *Tnmd* when compared to static culture and steered the mechanosensitive YAP/TAZ signaling pathway. This magneto-mechanical stimulation also modulated the inflammation response by upregulating the expression of anti-inflammatory cytokines IL-4 and IL-10 while reducing the expression of pro-inflammatory cytokines COX-2 and IL-6 (Tomás et al., 2019).

4.3 Responsive Biomaterial Strategies for Rotator Cuff Injury

To achieve the different requirements of delivery, "smart" delivery systems, such as stimulation-responsive hydrogel, can provide possibilities for precise treatment for different stages of healing (Bawa et al., 2009; Yun et al., 2015). These hydrogels respond to visible or UV light and release drugs for tissue regeneration. A gelatin methacryloyl hydrogel loaded with TPSC-EVs was placed in the Achilles tendon defect to promote tendon healing. By using a 405 nm blue light source at a distance, the carrier is converted to the gel state by irradiation for 10–20 s. After delivering TPSC-EVs, tendon repair is promoted by suppressing inflammation and apoptosis and regulating ECM balance (Zhang et al., 2020b). Additionally, matrix metalloproteinase 2 (MMP-2) is a valuable endogenous trigger for responsive release systems, achieving localized and on-demand drug delivery. MMP-2 is a member of the zinc endopeptidase family and has the ability to cleave ECM components, which is upregulated in the peritendinous area where the adhesion tissue forms after tendon injury. Cai et al. (2021) have designed an innovative anti-adhesion electrospun nanofiber scaffold system for the on-demand and unidirectional release of polyplexes to inhibit fibroblast proliferation and collagen deposition by gene therapy. The MMP-2 degradable hydrogel is fabricated by crosslinking allyl glycidyl ether (AGE) modified carboxymethyl chitosan (CMCS-AGE) and the MMP-2 substrate peptide CPLGLAGC (MMP-2 sp).

5 DISCUSSION

Rotator cuff injuries cause persistent symptoms, and they greatly impair movement ability and quality of life. Currently, the clinical

options of surgery and conventional therapies for treating rotator injuries are unsatisfactory. Due to the special structures of the rotator cuff, several clinical problems have not been solved, such as the delayed healing process, poor biomechanical strength of newly formed tissue, and scar adhesion. Therefore, stem cell therapies are attractive because they activate the self-potential of the body to repair injured tissues. According to present pre-clinical and clinical studies, several stem cells have been successfully isolated and have shown promising potential in rotator cuff repair due to their strong capacity for regeneration, tenogenic differentiation, and paracrine activity. As a primary effector in stem cell therapy, EVs can promote the healing process by reducing inflammation and fatty infiltration, stimulating cell proliferation and tenogenic differentiation, and maintaining homeostasis. Moreover, gene therapy and biomaterials expand the effectiveness of the application of stem cell therapy by regulating the environment, stimulating directional differentiation, and ensuring high efficacy of delivery. Therefore, stem cell therapy is a promising strategy for rotator cuff repair.

Nevertheless, numerous issues still need to be investigated in future studies. Although more stem cells and their EVs, such as Bursa-derived cells, have been successfully discovered and isolated, the lack of pre-clinical and clinical studies limits their further application. Moreover, with the deepening research of gene therapy, the efficient, safe, and targeted gene vector and therapeutic genes need to be addressed and verified in large animal models before beginning clinical trials. Innovation in biomaterials is evolving rapidly; thus, the translation of safe and valid carriers is the key to advancing the clinical application of stem cell therapy. Finally, to fully understand the safety, effectiveness, and mechanism of stem cell therapy, basic clinical research is still required.

AUTHOR CONTRIBUTIONS

H-NW, XR, and G-XN drafted the manuscript. L-MY and W-ZH searched for some manuscripts and figures and contributed to the final version of the manuscript. H-NW and G-XN supervised the whole project and reviewed the manuscript. All authors discussed and provided ideas to publish the manuscript.

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REVIEW
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The Hunt Is On! In Pursuit of the Ideal Stem Cell Population for Cartilage Regeneration

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Cartilage injury and degeneration are hallmarks of osteoarthritis (OA), the most common joint disease. OA is a major contributor to pain, loss of function, and reduced quality of life. Over the last decade, considerable research efforts have focused on cell-based therapies, including several stem cell-derived approaches to reverse the cartilage alterations associated with OA. Although several tissue sources for deriving cell-based therapies have been identified, none of the resident stem cell populations have adequately fulfilled the promise of curing OA. Indeed, many cell products do not contain true stem cells. As well, issues with aggressive marketing efforts, combined with a lack of evidence regarding efficacy, lead the several national regulatory bodies to discontinue the use of stem cell therapy for OA until more robust evidence becomes available. A review of the evidence is timely to address the status of cell-based cartilage regeneration. The promise of stem cell therapy is not new and has been used successfully to treat non-arthritic diseases, such as hematopoietic and muscle disorders. These fields of regenerative therapy have the advantage of a considerable foundation of knowledge in the area of stem cell repair mechanisms, the role of the stem cell niche, and niche-supporting cells. This foundation is lacking in the field of cartilage repair. So, where should we look for the ideal stem cell to regenerate cartilage? It has recently been discovered that cartilage itself may contain a population of SC-like progenitors. Other potential tissues include stem cell-rich dental pulp and the adolescent growth plate, the latter of which contains chondrocyte progenitors essential for producing the cartilage scaffold needed for bone growth. In this article, we review the progress on stem cell therapies for arthritic disorders, focusing on the various stem cell populations previously used for cartilage regeneration, successful cases of stem cell therapies in muscle and hemopoietic disorders, some of the reasons why these other fields have been successful (i.e., "lessons learned" to be applied to OA stem cell therapy), and finally, novel potential sources of stem cells for regenerating damaged cartilage *in vivo*.

Keywords: cartilage, regenerative medicine, growth plate, osteoarthritis, musculoskeletal health, stem cells

INTRODUCTION

Osteoarthritis (OA) is a major health concern, affecting more than 50% of adults over the age of 65 (Loeser, 2010). OA contributes significantly to pain, disability, and rising healthcare costs (Cross et al., 2014). The average annual cost per person afflicted with OA is as high as €23,000, a massive sum considering the millions of individuals affected with OA worldwide. In the United States, the annual cost of OA is >$16.5 billion, accounting for >4% of the combined costs for all hospitalizations (CDC Prevention, 2021). OA of the hip and knee contribute the most to OA burden, often resulting in joint replacement surgery, including >1 million annual joint replacements in the United States and roughly 1,60,000 in the United Kingdom (Centers for Disease Control and Prevention, 2010; Cross et al., 2014; Registry, 2020). It is predicted that OA will soon be the fourth most disabling chronic disease in the world, and OA is the fastest growing major health condition (Silverwood et al., 2015). The burden of OA has, therefore, become an urgent international healthcare issue.

OA is a total joint disease, but the end result is the complete loss of articular cartilage. The presence of early cartilage defects is a strong risk factor for OA progression (Guermazi et al., 2017; Everhart et al., 2019). In the OA-affected joint, the products of cartilage breakdown that are released into the synovial fluid are phagocytosed by synovial cells, amplifying synovial inflammation (Sellam and Berenbaum, 2010). In turn, activated synovial cells in the inflamed synovium produce catabolic and pro-inflammatory mediators, such as interleukins 1 and 6 and tumor necrosis factor-α (Sellam and Berenbaum, 2010). This inflammatory response is amplified by activated synovial T cells, B cells, and infiltrating macrophages (Sellam and Berenbaum, 2010). The resulting inflammatory milieu leads to the secretion of matrix-degrading enzymes from chondrocytes, further propagating tissue breakdown and creating a positive feedback loop as joint degeneration continues and progresses (Sellam and Berenbaum, 2010; Mata et al., 2017). To date, there are no accepted disease-modifying OA drugs (DMOADs) to slow OA progression; therefore, treatment has been aimed at reducing symptoms (McAlindon et al., 2014). Analgesic medications such as acetaminophen and nonsteroidal anti-inflammatory drugs do not alter OA-related degeneration. Though some studies evaluating cartilage-based treatment with nutritional supplements such as glucosamine and chondroitin sulfate have suggested these treatments reduce pain and delay in structural progression, other studies have shown equivocal results (Towheed et al., 2009; Bruyère et al., 2016), generating equipoise as to whether these nutritional supplements should be recommended (Hochberg et al., 2012; McAlindon et al., 2014). Antibodies targeting pain pathways, such as tanezumab, have shown some benefits in clinical trials but are believed to contribute to rapidly-progressive OA in a notable proportion of treated individuals, thus they are presently excluded from OA treatment guidelines (Schnitzer et al., 2019; Berenbaum et al., 2020). In addition to changes in cartilage, other articular tissues are affected by OA, including the synovium, ligament, and bone (Barr et al., 2015). Treatments, such as strontium ranelate, directed at preventing pathologic bone alterations have shown some positive effects on clinical outcomes such as pain and disability (Reginster et al., 2013; Bruyère et al., 2016), and possibly structural progression (Roubille et al., 2015); however, these results have not been widely accepted, nor has strontium ranelate been approved for OA treatment (Hochberg et al., 2012; McAlindon et al., 2014).

Surgical strategies to halt the progression from cartilage defect to the development of OA have been limited and prone to failure (Palmer et al., 2019; Zamborsky and Danisovic, 2020). Marrow-stimulation procedures, such as microfracture, rely on the development of a primitive mesenchymal blood clot that often forms fibrous tissue with variable patient outcomes (Haleem et al., 2010). Osteochondral grafting limitations include donor site availability, morbidity, and fibrocartilage formation between osteochondral plugs (Redman et al., 2005; Haleem et al., 2010). Once OA is established, treatment is essentially palliative (McAlindon et al., 2014). As such, research has turned to stem cell-based therapies to slow and/or reverse OA, an area of research that has expanded dramatically over the last decade (Koelling and Miosge, 2009; Yubo et al., 2017; Whittle et al., 2019; Zhang et al., 2019).

RECRUITING AND STIMULATING ENDOGENOUS STEM CELLS TO TREAT OSTEOARTHRITIS

The development of OA in the joint represents a failure of the endogenous articular repair system to maintain healthy osteochondral units (Goldring and Goldring, 2016). Specific to articular cartilage, native chondrocytes are unable to maintain the extracellular matrix, resulting in cartilage fibrillation, fissuring, and thinning (Pritzker et al., 2006; Sulzbacher, 2013). Over time, cartilage may erode completely, exposing the subchondral bone (Moisio et al., 2009). A complete understanding of the mechanism(s) by which cartilage regeneration fails is lacking; however, the reparative function of the chondrocyte may be disrupted by pathologic changes in the OA joint microenvironment (Sellam and Berenbaum, 2010; Jayasuriya et al., 2016). These changes include increased inflammation along with the production of reactive oxygen species and pro-degradation proteins, including matrix metalloproteinases and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) (Sellam and Berenbaum, 2010; Jayasuriya et al., 2016). In addition, the subchondral bone becomes more permeable, allowing bone morphogenetic proteins (BMPs) and transforming growth factor β (TGF-β) to diffuse into cartilage from the bone, favoring the terminal differentiation of chondrocytes and osteophyte formation (Zhen et al., 2013; Jayasuriya et al., 2016). As a result, the OA joint microenvironment becomes catabolic, with little support for endogenous cartilage repair. Biomechanical influences also play a role, with overloaded joint compartments experiencing accelerated OA-related structural changes such as osteophyte formation, bone attrition, and deformity, as well as microenvironmental OA alterations and greater overall

susceptibility to cartilage injury and loss (Sharma et al., 2001; Guilak, 2011; Chen et al., 2013). Under such circumstances, additional aid must arrive at the site of damage to assist the failing chondrocytes. Mesenchymal stem cells (MSCs) have been proposed as strong candidates for enhancing the articular repair process (McGonagle et al., 2017). MSCs are clonogenic progenitor cells capable of differentiating into mesoderm-derived cells such as osteoblasts, chondrocytes, and adipocytes (Prockop, 1997). One attractive feature of MSCs is that they are found in many tissues of the synovial joints including the bone, synovium, and adipose, representing about 1% of the total cell population (Jones et al., 2010a). As well, endogenous MSCs play a supportive role in the immune system, providing immunomodulation that can either enhance or dampen the inflammatory cascade of the OA articular milieu by adapting their immunoregulatory properties to the local immunological environment (Hoogduijn, 2015; Song et al., 2020a). Through the excretion of cytokines, growth factors, chemokines, and cell–cell contact, MSCs exert their immunomodulatory effect on immune cells, such as T and B cells, natural killer (NK) cells, macrophages, monocytes, dendritic cells (DCs), and neutrophils, thus exerting a potentially potent effect on the local immune response (Song et al., 2020a). With this in mind, exogenous MSCs have been used to treat inflammatory conditions such as graft-versus-host disease, graft rejection, and autoimmune diseases (Müller et al., 2021). To our knowledge, however, capitalizing on the immunomodulatory capabilities of joint-resident MSCs has not yet been attempted for the treatment of OA.

MSCs were shown to accumulate in greater numbers in the regions of the damaged OA bone (Campbell et al., 2016). Directing such subchondral MSC populations to effectively restore the joint microenvironment and repair OA-associated cartilage damage would present a powerful therapeutic target to slow or halt OA progression, particularly if initiated early in the disease (McGonagle et al., 2017). McGonagle and Jones reviewed the potential origins of such reparative MSCs, noting that the MSC native environment of origin (niche) is critical to their function (McGonagle et al., 2017). As an example, synovium-derived MSCs showed superior chondrogenic potential as compared to those derived from bone or subcutaneous fat (Sakaguchi et al., 2005; Mochizuki et al., 2006; Koga et al., 2008). Another attractive characteristic of synovial-resident MSCs is that they have direct access to the synovial fluid, which in turn gives facile migratory access to the superficial layer of cartilage. Early OA-associated damage occurring in the superficial cartilage layers create an anatomic challenge for bone marrow-resident MSCs to reach the site of injury; as it requires their migration through the deeper, undamaged cartilage layers to reach the site in need of repair (McGonagle et al., 2017). Synovial MSCs, on the other hand, would have direct access to the site of injury *via* the articular space in order to initiate repair. This repair pathway was supported by experiments in a canine model showing that synovium-derived MSCs are able to adhere to the areas of cartilage injury (Wood et al., 2012).

Perhaps the most obvious endogenous progenitor cell population for cartilage repair is that which resides within cartilage itself, as recently reviewed by Rikkers et al.(2022). Termed articular cartilage-derived progenitor cells (ACPCs), these cells most likely reside in the superficial zone in healthy cartilage and will migrate toward the sites of cartilage injury (Grogan et al., 2009; Williams et al., 2010). These cells show similar markers to those of MSCs (CD90, CD105, CD73, and CD166) (Rikkers et al., 2022), possibly distinguished phenotypically by an increased expression of CD44 and enhanced expression of fibronectin and integrin-α5β1 (Dowthwaite et al., 2004; Levato et al., 2017), suggesting a unique progenitor cell population. Correspondingly, OA joint-derived ACPCs were shown to form more colonies *in vitro* compared to those from healthy human cartilage, suggesting greater proliferation capacity with increasing OA severity (Wang et al., 2020; Rikkers et al., 2022). An increase in progenitor markers, such as CD271 (Wang et al., 2020), CD105 (Zhang et al., 2016), and VCAM (Grogan et al., 2009) at sites of trauma or in OA cartilage were also observed. Though these data suggest the involvement of ACPCs in cartilage repair, *in vivo* models outlining corresponding mechanisms are lacking, leaving the role(s) of these progenitor cells in cartilage repair and homeostasis unclear at this time (Rikkers et al., 2022).

One major hurdle faced by relying on endogenous MSCs to repair cartilage is enhancing their repair capacity. Clearly, the phenotype/reparative potential of these cells in OA patients is inadequate to halt disease progression. Causative factors include irreversible factors such as age. Bone marrow-resident MSCs decline functionally with age (Sethe et al., 2006; Oh et al., 2014), a distinct disadvantage for those with OA, which tends to occur later in life (Felson, 2004). As well, cultured MSCs derived from an inflammatory joint environment have reduced chondrogenic potential *in vitro* (Jones et al., 2010b). *In vivo*, the number of MSCs in magnetic resonance imaging (MRI)-determined bone marrow lesions is five-fold greater than non-bone marrow lesion (Campbell et al., 2016); from a functional perspective; however, these MSCs demonstrated reduced proliferative and osteogenic functional capacity, possibly due to cellular fatigue while residing in a chronically damaged trabecular bone niche (Campbell et al., 2016). Restoring the functional capacity of MSCs or ACPCs at the site of tissue damage could provide a large number of repair cells at the site of injury. With respect to cartilage, regrowth following procedures such as microfracture or bone drilling (albeit often suboptimal fibrocartilage) indicates that a population of bone marrow-resident cells exist within the bone marrow that can repair damaged cartilage (Palmer et al., 2019; Zamborsky and Danisovic, 2020). Enhancing the repair capacity of these cells through the intra-osseous application of growth factors or other molecular interventions may represent a viable treatment opportunity (Delgado et al., 2019) (**Figure 1**). The application of chemotactic factors to the site of injury to augment the recruitment of endogenous MSCs, as well as other cells involved in the cartilage repair and local immune suppression, could be combined with approaches that enhance the chondrogenic function (Song et al., 2020a). Intra-articular treatment targeting synovium-resident MSCs to enhance their chondrogenic capacity and improve their ability to repair cartilage in an inflammatory microenvironment, or the image-guided placement of intra-defect biomaterials that release these

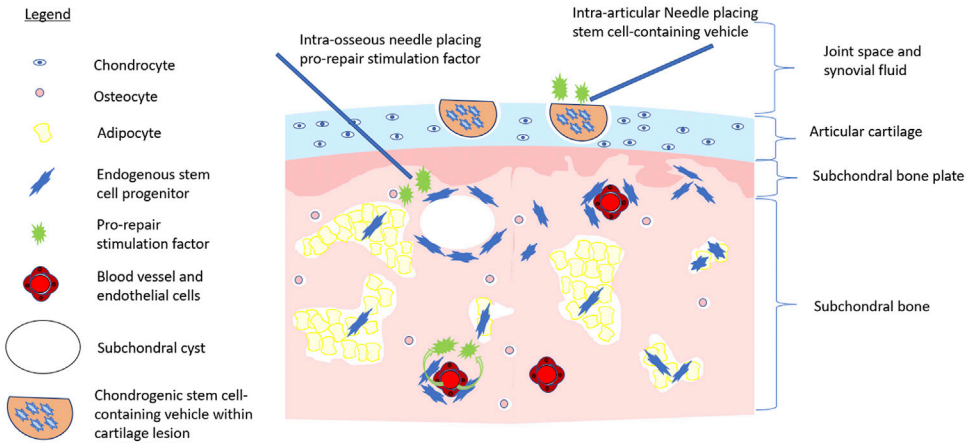

FIGURE 1 | Multistep approach to stem cell-initiated cartilage repair. Image depicts the application of chondrogenic stem cell-containing vehicle (e.g., hyaluronic acid injectate) to a cartilage lesion *via* intra-articular injection while pro-repair stimulation factors are applied to intra-articular space, *via* intra-osseous application, or systemically *via* the bloodstream to activate endogenous repair of the OA joint.

factors to reparative cells upon their migration to the cartilage lesion, may be a less invasive option than the intra-osseous treatment of bone marrow-resident MSCs (**Figure 1**). Strategies such as arthrocentesis that evacuate the pro-inflammatory synovial fluid of the OA joint, followed by the replacement of the synovial fluid with a pro-chondrogenic cellular and molecular cocktail would be ideal. These treatments, in combination with biomechanical interventions that unload the affected joint compartments such as joint distraction or tibial osteotomy, may further help reduce the hostile microenvironment of the OA joint and prevent further direct mechanical injury (McGonagle et al., 2017; Jansen et al., 2021). Such a combination of interventions could provide a better opportunity for tissue-resident MSCs to repair the cartilage damage, than simply relying on cellular function alone. In summary, our understanding of how endogenous MSCs repair or support the repair of damaged tissue, the effect of the local niche, and potential supporting roles of other cell populations in the OA joint is lacking. As a result, strategies utilizing the recruitment and stimulation of endogenous MSCs to repair OA-associated cartilage injury have yet to emerge. Consequentially, many have turned toward the use of exogenous or transplanted MSCs for cartilage repair.

CELL-BASED THERAPIES FOR OSTEOARTHRITIS

Although DMOADs for OA are lacking, cell-based therapies have shown promise in reversing the symptoms and structural alterations of OA (Koelling and Miosge, 2009). Given the chondrogenic potential of MSCs, these cells quickly emerged as candidates. Their enhanced ability to differentiate toward chondrogenic lineages under low oxygen tension (Merceron et al., 2010) is also an advantage, due to the avascular and hypoxic nature of cartilage tissue (Goldring and Goldring, 2016). Cell-based therapies currently proposed for OA include autologous cultured chondrocyte transplantation, co-culture and transplantation of MSCs with chondrocytes or hematopoietic-lineage cells, 3D-MSC cultures, or transplantation of MSC-laden scaffolds made of hyaluronic acid, or other synthetic derivatives (Mamidi et al., 2016; Brittberg et al., 1994). In an effort to reduce heterogeneity in therapeutic MSC populations, the International Society for Cell and Gene Therapy (ISCT) developed a set of minimal criteria to define the MSC phenotype, including adherence to plastic, specific antigen expression (e.g., CD73, CD90, and CD105), and multipotent differentiation potential (Dominici et al., 2006). Early phase I–II clinical trials showed improvement in pain and function following the intra-articular application of MSCs into OA-affected knees (Soler et al., 2016; Jo et al., 2014); however, despite these encouraging findings, exogenous MSCs have yet to emerge as a mainstream player for OA treatment. The drawbacks of MSC therapies include the necessity for cell culture, loss of differentiation capacity *ex vivo* or with multiple culture passages, and reduced or halted cellular division after multiple population doublings (Mamidi et al., 2016; Jones and Yang, 2011). Such heterogeneity exists in studies using various animal models of OA in preclinical studies (Cope et al., 2019; Wang et al., 2022), as well as clinical trials. Despite these limitations, several clinical trials have recently reported on the efficacy of exogenous MSCs to regenerate cartilage in OA (**Table 1**) (Emadedin et al., 2018; Kuah et al., 2018; Freitag et al., 2019; Khalifeh Soltani et al., 2019; Lee et al., 2019; Lu et al., 2019; Matas et al., 2019; Shapiro et al., 2019; Anz et al., 2020; Yang et al., 2022). Across these trials, methodologic heterogeneity has hindered a standardized approach to MSC-based therapies, including the use of host source (allogeneic vs. autologous), tissue source (bone marrow, adipose, umbilical cord, and placenta), injectate (tissue concentrates vs. isolated MSCs), whether they were expanded *in vitro* prior to injection, the dosage used, and the delivery method (e.g., image-guided or not). In addition, not all clinical studies characterized the stem cells being injected, for

TABLE 1 | Summary of described clinical trials.

Trial (country)	Sample size	Stem cell source	MSC characterization and laboratory processing	Control	Clinical outcome(s)	Cartilage recovery outcome
Emadedin 2018 (Iran)	47	Autologous BM	ISCT criteria tissue culture expansion	Saline	Pain (VAS) function (WOMAC)	NI
Kuah 2018 (Aus)	21	Allogeneic adipose	No characterization tissue culture expansion	Culture media	Pain (VAS) function (WOMAC)	MRI (MOAKS)
Freitag 2019 (Aus)	30	Autologous adipose	ISCT criteria tissue culture expansion under hypoxic conditions	Usual care	Pain (NRS) function (KOOS)	MRI (MOAKS)
Khalifeh soltani 2019 (Iran)	20	Placenta	No characterization tissue culture expansion	Saline	Pain (VAS) function (KOOS)	MRI (cartilage thickness)
Lee 2019 (Korea)	24	Autologous adipose	Code of federal regulations Characterization tissue culture expansion	Saline	Pain (VAS) function (WOMAC)	MRI (cartilage depth—Noyes grading)
Lu 2019 (China)	53	Autologous adipose	ISCT criteria tissue culture expansion	HA	Pain (VAS) function (WOMAC)	MRI (cartilage volume)
Matas 2019 (Chile)	29	Umbilical cord	ISCT criteria tissue culture expansion	HA	Pain (VAS) function (WOMAC)	MRI (WORMS)
Shapiro 2019 (USA)	25	Autologous BM	ISCT criteria no processing	Saline	Pain (VAS)	MRI (Mean T2 values)
Anz 2020 (USA)	90	Autologous bone marrow	No characterization no processing	PRP	Pain (WOMAC) function (WOMAC)	NI
Yang 2022 (Korea)	176	Umbilical cord	Code of federal regulations	BMAC	Pain (IKDC) function (KOOS)	Arthroscopy (ICRS)

Aus, Australia; BM, bone marrow; BMAC, bone marrow aspirate concentrate; HA, hyaluronic acid; IKDC, International Knee Documentation Committee questionnaire; ICRS, International Cartilage Repair Society score; KOOS, knee injury and osteoarthritis outcome score; MOAKS, MRI osteoarthritis knee score; MRI, magnetic resonance imaging; NI, not included; NRS, numeric rating scale; PRP, platelet-rich plasma; USA, United States of America; VAS, visual analogue scale; WOMAC, Western Ontario and McMaster Universities osteoarthritis index; WORMS, whole-organ magnetic resonance imaging score.

example, using the ISCT minimal criteria for MSCs (Dominici et al., 2006), making it difficult to ensure what had actually been injected into participants' joints and impeding the reproducibility of results across studies. Several systematic reviews evaluating the overall efficacy of MSCs for the treatment of OA have been conducted (Kim et al., 2019a; Ha et al., 2019; Song et al., 2020b; Kim et al., 2020) or are underway (Whittle et al., 2019). Although meta-analyses have shown a benefit in pain reduction following MSC intra-articular injection in OA, there is little data supporting their effectiveness for cartilage regeneration. The strength of the evidence supporting their use in clinical practice is, therefore, limited (Kim et al., 2019a; Ha et al., 2019; Song et al., 2020b; Kim et al., 2020). Indeed, many regulatory and scientific bodies, including the United States Food and Drug Administration and Health Canada, have issued position statement warning against the clinical use of these unproven stem cell (SC) therapies for cartilage repair (Marks et al., 2017; CDA, 2019; ISSCR, 2021).

Despite the aforementioned caveats, clinical studies evaluating MSCs for cartilage repair continue (Clinicaltrial.gov, 2022). Factors such as ease of access, low likelihood of side effects, and potential immune-suppressing characteristics are attractive (Whittle et al., 2019). MSCs also present the opportunity to use allogenic sources, suggesting the possibility of an "off-the-shelf" formulation produced through a standardized good manufacturing process (Sanz-Nogués and O'Brien, 2021). With these attractive features in mind, researchers have sought to enhance MSC function *in vivo*. Tissue engineering strategies began in the 1990s using scaffolds and matrices to maintain MSCs at the site of injury and potentiate their repair capacity (Mandrycky et al., 2016; Kim et al., 2019b). Three-dimensional encapsulating matrices could safely deliver MSCs to the site of cartilage injury and provide a biologically optimal milieu for MSCs to repair tissue, protecting them from the hostile inflammatory OA joint environment (Kim et al., 2019b). Growth and differentiation factors could be encapsulated within the matrix, providing stimulus toward chondrogenic differentiation (Kim et al., 2019b). Biomaterials using the proteins fibrin and collagen, or polysaccharides such as hyaluronic acid and agarose have the advantage of being derived from endogenous materials, having good biocompatibility and being biodegradable (Li et al., 2015a). Alternatively, synthetic biomaterials such as polylactic acid, polyglycolide, and polyethylene glycol have the advantage of improved mechanical strength and are easier to mold; for example, to match the shape of a cartilage lesion (Matai et al., 2020). In addition, synthetic biomaterials are immunologically-neutral, are not associated with the risk of transmitting pathogens, and have modifiable chemical and mechanical properties as well as the rate of degradation (Mandrycky et al., 2016; Kim et al., 2019b; Matai et al., 2020). Clinical trials have mainly utilized natural biomaterials such as fibrin, and most studies using synthetic materials were performed in animal models. Like MSCs, the ideal scaffold would be available in a standardized off-the-shelf format.

Culture-expanded MSCs may exert a therapeutic effect through immune modulation and *via* trophic actions on local joint cells through secreted factors without directly participating in new cartilage formation (Mak et al., 2016; Zhang et al., 2022). Consequently, non-cellular biologically-based strategies harnessing the MSC secretome have been explored for cartilage and bone repair (Kalluri and LeBleu, 2020; To et al., 2020; Zhang et al., 2022). MSC-

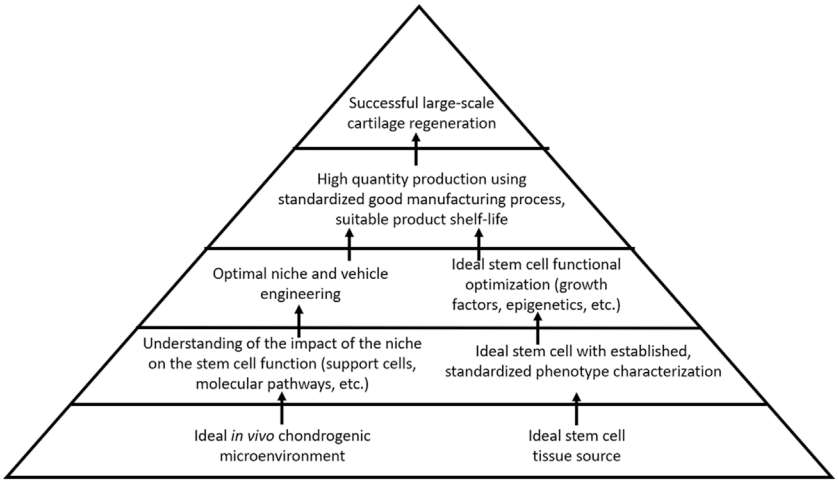

FIGURE 2 | Suggested directions for cell-based therapy research for cartilage regeneration.

derived extracellular vesicles (MSC-EVs) are MSC-produced nanovesicles ranging from 10 nm to several μm in diameter that contain components such as messenger RNA, microRNA, lipids, and bioactive proteins that produce regenerative paracrine effects within damaged tissue (To et al., 2020). Compared to MSCs, MSC-EVs have the advantage of low toxicity and immunogenicity with repeated transplantation and can be stored for potential off-the-shelf applications (To et al., 2020). A systematic review evaluating the use of MSC-derived extracellular vesicles (MSC-EVs) in preclinical studies for cartilage regeneration showed reduced cartilage loss across a variety of animal models of OA (To et al., 2020). MSC-EVs have been evaluated in preclinical studies for their potential toward bone healing (Kirkham et al., 2021), and a systematic review by Kirkham et al. (2021) reported a promising potential (Kirkham et al., 2021). Though the review focused on fracture healing, pathologic bone changes in the osteochondral unit are well-characterized as a part of OA progression, suggesting that this type of treatment could someday have a role in treating both cartilage and bone-related OA alterations.

In summary, despite important advances in MSC therapy for cartilage regeneration over the last decade, the hunt is still on for the optimal regenerative cell-based approach to repair the cartilage damage associated with OA. In pursuit of the ideal approach, we look to lessons learned from the successes seen in other fields of regenerative medicine, as well as at emerging discoveries of novel chondrogenic stem cell populations, that can be applied to the treatment of OA.

HEMATOPOIETIC STEM CELL THERAPY: THE IMPORTANCE OF TISSUE MICROENVIRONMENTS AND PARALLELS FOR CARTILAGE REGENERATION

Transplantation of donor-derived hematopoietic stem and progenitor cells to regenerate the hematopoietic system has a long and established history that provided a starting point for future generations of cell therapies (Passweg et al., 2021). Cell product characterization ensured an adequate dose and viability of blood-forming stem cells for the recipient (Gauntner et al., 2021). Extensive regulations and standards protect donors and patients and ensure optimal outcomes following allogeneic transplantation. The processes, standards, and regulations that guide the procurement and transplantation of hematopoietic cell products can be leveraged for other cell-based therapies. The use of autologous cells can also rescue hematopoiesis in recipients following the high-dose chemotherapy with lower risks of transmission of infections, and avoids issues such as graft-versus-host disease. Whether allogeneic or autologous cells are used is an important aspect of cell collection and characterization. The cells from young allogeneic donors can reduce transplant complications and improve survival compared with cells from older allogeneic donors (Shaw et al., 2018). The more robust regenerative capacity of younger cells likely accounts for the improved outcomes. Autologous cells in patients who suffer from disease and/or its treatment may yield cell products with compromised function (Choudhery et al., 2012; Yang et al., 2019) and this should be considered in applications such as cartilage regeneration for OA.

A critical aspect of successful hematopoietic engraftment following transplantation relates to the function and status of the bone marrow microenvironment (Morrison and Scadden, 2014). Recipient age, prior therapy, and disease-induced changes in the marrow microenvironment can impair hematopoiesis. MSCs, a chief component of the marrow microenvironment, that are derived from patients with acute myeloid leukemia are abnormal with skewed differentiation potential and reduced ability to support normal hematopoiesis (Chandran et al., 2015; Le et al., 2016). Understanding the tissue microenvironment will be paramount in cell therapies for cartilage regeneration. Strategies restoring the health of the tissue microenvironment may augment the success of cell-based therapies. Exercise, for instance, was shown in a mouse

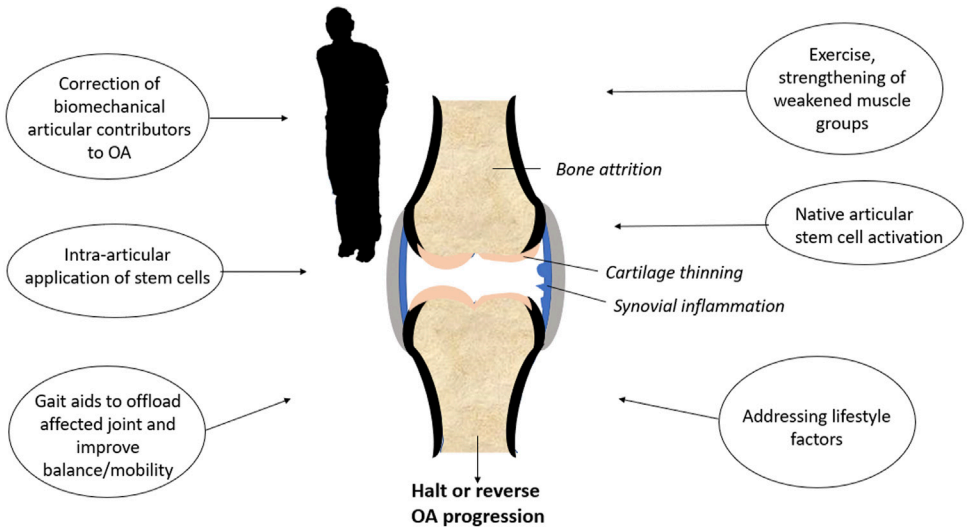

FIGURE 3 | Multimodal treatment of the osteoarthritis-affected knee.

model to accelerate hematopoietic engraftment following transplantation (De Lisio et al., 2013) and nutritional status including the essential amino acids is crucial for robust hematopoiesis (Wilkinson et al., 2018).

A global network of registries of healthy volunteer donors exists to facilitate the collection of blood stem cells to support unrelated allogeneic hematopoietic transplantation. The process of identifying HLA-compatible donors who are healthy and free of transmissible disease is well-established through the World Marrow Donor Association and its connected network of international registries and collection centers (Bochtler et al., 2011). Whether these donors could provide cells and tissues to support other forms of cellular therapy is intriguing. In a recent survey of registrants on the Stem Cell Registry at Canadian Blood Services, many registrants were willing to donate cells for uses other than blood cell transplants (Liao et al., 2020).

Leveraging the clinical experience from hematopoietic transplantation in the areas of product characterization and donor cell procurement may accelerate the translation of stem cell therapy to cartilage degeneration in OA.

MUSCLE STEM CELL THERAPY

The development of cell-replacement therapies for the treatment of muscle wasting diseases has received much attention (Dao et al., 2020). Most efforts focused on the muscle-resident stem cell, termed satellite cells, juxtaposed between the basal membrane, and muscle fiber (Aziz et al., 2012). Since their discovery in the early 1960s (Katz, 1961; Mauro, 1961), considerable knowledge was gained on the role of satellite cells in muscle repair, the influence of the niche in which they reside, as well as the role of other niche-supporting cells (Sousa-Victor et al., 2021). These critical aspects of the repair process are less-well described for cartilage repair (McGonagle et al., 2017). For example, in normal resting muscle, the majority of satellite cells are maintained in a long-lived Pax7-expressing quiescent (G0 reversible arrest) state (Aziz et al., 2012; García-Prat et al., 2016). To ensure tissue homeostasis, satellite cells will repair small myofiber defects by re-entering the cell cycle, undergoing a single asymmetric cell division that generates one differentiating daughter cell that will contribute to the myofiber and one daughter stem cell to replenish the stem cell pool (Aziz et al., 2012; Sousa-Victor et al., 2021). Only after enough progenitor cells have been made to repair the myofiber will a small number of progenitors return to the quiescent state to repopulate the stem cell niche (Cutler et al., 2021; Robinson et al., 2021).

Myogenic regulatory factors (MRFs) contribute to establishing the myogenic cell identity, to the subsequent differentiation and formation of muscle fibers during muscle regeneration in postnatal life (Sousa-Victor et al., 2021). Molecular mechanisms governing the transition between quiescence and activation have been studied and include transcriptional and post-translational, epigenetic (Sousa-Victor et al., 2021), as well as metabolic and proteostatic regulation (Sousa-Victor et al., 2021). In addition, several extrinsic factors, including epidermal growth factor (Roe et al., 1989), hepatocyte growth factor (Miller et al., 2000), angiopoietin 1 (Abou-Khalil et al., 2009), nitric oxide (Wehling et al., 2001), fibroblast growth factor (Bischoff, 1986), and insulin-like growth factor (Allen and Boxhorn, 1989), are known to modulate satellite cell quiescence, activation, expansion, self-renewal, and differentiation (Kuang et al., 2008; Aziz et al., 2012). Further modulation of satellite cells occurs through other cells within the satellite cell niche, including macrophages, neutrophils and other white blood cells, fibroadipogenic progenitors (FAPs), and endothelial cells (Sousa-Victor et al., 2021). The satellite cell crosstalks with other constituents of the niche through signaling pathways such as TGFβ, Notch, and Wnt further

refines the satellite cell function and fate (Sousa-Victor et al., 2021).

This breadth of data regarding the phenotype, function, and niche of satellite cells has provided a foundation of knowledge toward stem cell therapy for muscle-related conditions, including age-related sarcopenia (García-Prat et al., 2016; Lukjanenko et al., 2019) and muscular dystrophies (Périé et al., 2014; Campbell and Puymirat, 2021). Like those faced by MSC therapies, ongoing challenges with the use of satellite cell-derived myoblasts include cell culture-passaging limitations that restrict their expansion potential *in vitro*, and the development of specific cell differentiation protocols (Chal and Pourquié, 2017; Magli et al., 2017; Xi et al., 2017). The continuously evolving knowledge of satellite cell repair mechanisms, the role of their niche, and niche-supporting cells provides great optimism for their therapeutic use for muscle disease in the future. Such a foundation of knowledge may contribute to the stem cell regenerative approach to cartilage repair in OA.

FUTURE AVENUES FOR STEM CELL THERAPY FOR CARTILAGE REGENERATION

Stimulating native articular stem cells to enhance their ability to repair cartilage *in vivo* constitutes a viable avenue for future research. Among MSCs, a significant amount of heterogeneity exists (Jones and Schafer, 2015). Factors such as culture age, donor sex, and health status play a role in the MSC function and surface receptor expression (Murphy et al., 2002; Baxter et al., 2004; Astudillo et al., 2008; Zhen et al., 2013; Jones and Schafer, 2015). Of these factors, the niche from which MSCs are isolated is believed to have a decisive influence on their function and differentiation capacity (Risbud et al., 2006; Jones and McGonagle, 2011; de Sousa et al., 2014; Jones and Schafer, 2015). Bone marrow-derived MSCs are believed to have a more optimal capacity for chondrogenic differentiation than those derived from adipose tissue, while adipose-derived MSCs have a higher proliferative capacity (Li et al., 2015b). Even among bone-derived MSCs, the particular bone and the topographic region, therein, can influence differentiation capacity and surface marker expression (Risbud et al., 2006; Ackema and Charite, 2008; Tormin et al., 2011; Jones and Schafer, 2015). Therefore, selecting an appropriate endogenous stem cell target and niche to assist in OA joint repair will be a crucial factor toward positive outcomes. Similarly, the tissue of origin of exogenous MSCs transplanted into the site of cartilage injury will also be a primary factor toward successful cartilage regeneration. Interestingly, while studies have compared stem cell chondrogenic capacity from different niches *in vitro* (Mochizuki et al., 2006; Koga et al., 2008), and studies have compared stem cells to implanted chondrocytes (Nejadnik et al., 2010) and bone marrow concentrate (Yang et al., 2022) *in vivo*, we are not aware of any *in vivo* studies directly comparing chondrogenic repair capacities of different stem cell populations derived from different tissue sources. To date, clinical trials evaluating MSCs have shown some evidence of reducing OA-associated pain and can produce cartilage *in vitro*, but there is little evidence that current MSC treatment regenerates damaged cartilage *in vivo*. In sum, the hunt is still on for the most potent stem cell population capable of mediating cartilage repair *in vivo*. This begs the question: where would such a population exist?

Beyond MSCs derived from adipose sources and articular tissues, other potential candidates for cartilage repair are also being pursued. Dental-derived MSCs, such as dental pulp stem cells (DPSCs) originating from neural crest mesenchyme in the dental pulp, can be extracted with minimal donor site morbidity (Ibarretxe et al., 2012; Lo Monaco et al., 2020; Li et al., 2021). The dental pulp provides a protective environment for DPSCs during a person's lifetime, preserving their stem cell capacity (Fernandes et al., 2018). *In vitro*, DPSCs can be differentiated into cartilage-producing cells and can secrete several chondrogenic growth factors (Bronckaers et al., 2013; Ahmed Nel et al., 2016). Like bone-derived MSCs, they also have immunomodulatory capabilities that may be beneficial in the inflamed OA joint (Li et al., 2014; Lo Monaco et al., 2020). Preclinical models evaluated DPSC chondrogenic capacity. Lei et al. (2014) transplanted human DPSC cell pellets into the dorsal surface of immunodeficient mice where they maintained their chondrogenic capacity (Lei et al., 2014). Lo Monaco et al. (2020) showed an improved *in vitro* pro-survival effect when immature murine chondrocytes were cultured with DPSC-conditioned media (Lo Monaco et al., 2020). Mata et al. (2017) evaluated the ability of DPSCs to repair osteochondral defects in rabbits using alginate matrix-embedded DPSCs and alginate-embedded rabbit chondrocytes. Compared to controls, both rabbit chondrocytes and human DPSCs showed an improved quantity of *in vivo* cartilage regeneration and collagen fiber alignment (Mata et al., 2017). Fernandes et al. (2018) demonstrated that the DPSCs in a biomaterial scaffold showed a thicker deep layer of cartilage with less fibroblastic tissue, as compared to scaffold-alone (Fernandes et al., 2018). Overall, though DPSCs remain in the preclinical stage of experimentation for cartilage regeneration, they have shown potential to repair cartilage lesions *in vivo* and to promote native chondrocyte survival. These encouraging data may eventually benefit patients with OA.

The growth plate is the site of long bone growth in youth, through the process of endochondral bone growth (Kronenberg, 2003; Berendsen and Olsen, 2015). This process involves the longitudinal growth and ossification of a cartilage matrix, which is initiated by the action of chondrocytes in the *proliferative zone* of the growth plate (Koelling et al., 2009). These, in turn, are derived from small and relatively inactive stem cells located in the *reserve zone* close to the secondary ossification center (Koelling et al., 2009). Endochondral ossification and bone growth, therefore, rely on a yet-uncharacterized population of undifferentiated skeletal stem cells (SSCs) with highly robust chondrogenic functional capacity that resides in a protected niche within the growth plate (Chan et al., 2015). In 2015, Chan isolated SSCs from the femoral head growth plate of mice (Yin et al., 2013). The characterization of these skeletal stem cells showed a robust ability to differentiate *in vitro* to

chondrocytes, osteoblasts, or bone marrow stromal cells. Although the chondrogenic potential of these murine SSCs was not directly compared to that of MSCs, studies from muscle (and other tissues) suggest that tissue-embedded stem cell populations are more efficient at repairing damaged tissue compared to MSCs (i.e., MSCs sacrifice efficiency for versatility while tissue-specific stem cells are more efficient at repairing, but show limited versatility) (Jankowski et al., 2002; Chan et al., 2018). In 2018, a human SSC population demonstrating self-renewal and multilineage differentiation to the bone, cartilage, and stroma was isolated from the growth plate of human embryonic femoral bones (Murphy et al., 2020). Within this growth plate population, several subpopulations were described that were able to produce cartilage *in vitro*, identified by surface marker expression $PDPN^{+}CD146^{-}$. The cells that were $CD146^{+}$, a marker commonly associated with MSCs, displayed reduced colony size and frequency compared to $PDPN^{+}CD146^{-}$ SSCs, as observed by light microscopy and flow cytometry (Murphy et al., 2020). As well, two of the three chondrogenic SSC subpopulations did not express CD73, a marker included in the ISCT MSC definition, further suggesting these cells are not MSCs and are indeed a distinct and functionally unique stem cell population. The SSC with greatest functional diversity, including high chondrogenic potential, was identified as $PDPN^{+}CD146^{-}CD73^{+}CD164^{+}$ with the highest expression of these marker transcripts isolated from the proliferative and pre-hypertrophic zone of fetal bones (Murphy et al., 2020). The authors developed a monocyte-derived induced pluripotent stem cell (iPSC) line that also expressed $PDPN^{+}CD146^{-}CD73^{+}CD164^{+}$ and produced cartilage *in vivo,* an important finding that would allow laboratory-based production of a cartilage-generating cell line not requiring access to fetal tissue (Murphy et al., 2020). We are unaware of clinical trials evaluating SSCs applied exogenously for cartilage repair.

In line with enhancing the endogenous stem cell population toward repairing cartilage defects *in vivo*, Murphy et al. evaluated the response of SSCs to microfracture in both the distal femur of mice that had undergone the destabilization of the medial meniscus (a well-described model of OA), as well as human fetal phalangeal bone (Csobonyeiova et al., 2021). Following the articular surface microfracture in mice, the authors identified a proliferation of SSCs; however, the resulting regenerative tissue appeared to be morphologically heterogeneous, containing both fibrotic and chondrogenic tissue (Csobonyeiova et al., 2021). The addition of a hydrogel containing BMP2 and a VEGF antagonist enhanced cartilage tissue formation that approximated the structural properties of native cartilage. Similar results were achieved following the microfracture of the human phalangeal articular surface with BMP2 and anti-VEGF treatment, suggesting that endogenous SSCs could be stimulated toward cartilage regeneration following microfracture intervention. Whether such potent cartilage regeneration can be achieved in adult OA-affected articular remains to be determined.

Recently, research has emerged evaluating the differentiation of iPSCs toward chondrocytes for the purpose of treating OA (Takahashi et al., 2007). iPSCs were initially generated from somatic cells by the viral transfection of key reprogramming factors (Oct3/4, Sox2, c-myc, and Klf4) into the donor cells (Koyama et al., 2013). Like embryonic stem cells, iPSCs are pluripotent and have similar cell morphology, gene expression, and proliferation capability; however, iPSCs are derived from somatic cells, avoiding the ethical issues related to collecting cells from embryos (Takahashi et al., 2007). iPSCs may be differentiated toward the chondrocyte lineage using media supplemented with growth factors such as TGF-β, BMP, WNT3A, and FGF-2 (Takahashi et al., 2007). The four main chondrogenic differentiation approaches used to date include 1) the generation of MSC-like iPSCs with further differentiation into chondrocytes (Takahashi et al., 2007; Qu et al., 2013; Nejadnik et al., 2015); 2) co-culture of iPSC-derived MSCs with primary chondrocytes (Takahashi et al., 2007; Rim et al., 2018); 3) through the formation of three-dimensional cellular aggregates (Takahashi et al., 2007; Yamashita et al., 2015); and 4) culturing of iPSCs in a series of media which mimics physiological developmental pathways (Takahashi et al., 2007; Murphy et al., 2018). Using the appropriate cell culture conditions, iPSCs can also be differentiated towards potent chondrogenic progenitors, such as SSCs (Murphy et al., 2020), suggesting that they could generate a pool of precursors mimicking the functionality of a chondrogenic stem cell population. The drawbacks of iPSCs, however, includes genomic instability, difficulties in obtaining uniform mature cell populations, and tumor formation, owing to the risks of insertional mutagenesis and reactivation of transgenes caused by the integration of the viral genome used to create these cells (Takahashi et al., 2007; Martel-Pelletier et al., 2016). Strategies for overcoming these issues, as well as improving differentiation protocol efficiency of the phenotype of the ideal stem cell population for cartilage regeneration is an ongoing and exciting area of research (Takahashi et al., 2007; Martel-Pelletier et al., 2016).

CONCLUSION

Current strategies to regenerate cartilage in the OA joint are limited to surgical interventions that may fail or yield fibrocartilaginous tissue with insufficient biomechanical properties for joint load distribution. Furthermore, such surgical treatments are less ideal for larger, non-focal areas of cartilage loss, as seen in the later stages of OA. Regenerative medicine thus remains an attractive option for OA treatment. Although stem cell therapy holds promise for cartilage regeneration in OA, safe and reliable strategies to either optimize endogenous stem cells toward cartilage repair, to apply exogenous stem cells into damaged joints, or both simultaneously, have yet to emerge. The research study has emphasized the importance of the stem cell source niche, which proffers the characteristics that stem cells bring to the table with respect to their regenerative capacity. Despite this, however, there remains a lack of standardization across clinical studies regarding stem cell tissue source and phenotypic characterization. A well-characterized cell product that can be

reproducibly isolated or manufactured, and that can reliably produce healthy cartilage in the avascular cartilage microenvironment—that is, standardization of stem cells used for treatment and evidence-based outcome measures—will be an essential factor for moving the field of cartilage regeneration forward. The effective means of contending with the catabolic microenvironment of the OA joint and maintaining cellular chondrogenic functional capacity in a region primed toward cartilage destruction will also be an important challenge to overcome. The lessons from other areas of regenerative medicine can be applied to the field of cartilage regeneration. Hematopoietic therapies benefit from a deep understanding of the impact of the microenvironment on stem cell activity, not only the stem cells, but also the intricate role played by other support cells within the hematopoietic niche. A well-established international infrastructure responsible for thoroughly characterizing, standardizing, and distributing hematopoietic stem cells represents a major advantage. The field of muscle regeneration also takes the advantage of decades of data describing the mechanism of regeneration as well as the impact on the microenvironment on the satellite cells, additionally benefiting from a strong understanding of molecular pathways by which satellite cells develop, replicate, and respond to external function-altering stimuli. As compared to these other areas of regenerative medicine, our current understanding of the optimal cartilage-producing stem cell, its niche, the identity and role of niche-supporting cells, and the molecular mechanisms governing functional potential remains limited. Further research is needed to address these fundamental factors (**Figure 2**).

Even though stem cell therapy will likely 1 day become a valuable tool for cartilage regeneration, OA remains a whole-joint disease (Martel-Pelletier et al., 2016). Articular tissues including bone, synovium, and the joint capsule are all affected by OA, as well as peri-articular tissues such as muscle (Goldring and Goldring, 2016; Martel-Pelletier et al., 2016). Protocols, such as stem cell injections used in isolation, are unlikely to halt or reverse OA if the numerous factors contributing to joint degeneration are not addressed. Multimodal approaches (**Figure 3**), such as compartmental or total joint unloading to remove excess biomechanical stress, physiotherapy for muscle reconditioning, and lifestyle changes such as weight control and regular exercise will give the ideal stem cell population a fighting chance to regenerate the cartilage in people suffering from OA.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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One Novel Phantom-Less Quantitative Computed Tomography System for Auto-Diagnosis of Osteoporosis Utilizes Low-Dose Chest Computed Tomography Obtained for COVID-19 Screening

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Background: The diagnosis of osteoporosis is still one of the most critical topics for orthopedic surgeons worldwide. One research direction is to use existing clinical imaging data for accurate measurements of bone mineral density (BMD) without additional radiation.

Methods: A novel phantom-less quantitative computed tomography (PL-QCT) system was developed to measure BMD and diagnose osteoporosis, as our previous study reported. Compared with traditional phantom-less QCT, this tool can conduct an automatic selection of body tissues and complete the BMD calibration with high efficacy and precision. The function has great advantages in big data screening and thus expands the scope of use of this novel PL-QCT. In this study, we utilized lung cancer or COVID-19 screening low-dose computed tomography (LDCT) of 649 patients for BMD calibration by the novel PL-QCT, and we made the BMD changes with age based on this PL-QCT.

Results: The results show that the novel PL-QCT can predict osteoporosis with relatively high accuracy and precision using LDCT, and the AUC values range from 0.68 to 0.88 with DXA results as diagnosis reference. The relationship between PL-QCT BMD with age is close to the real trend population (from ~160 mg/cc in less than 30 years old to ~70 mg/cc in greater than 80 years old for both female and male groups). Additionally, the calculation results of Pearson's r-values for correlation between CT values with BMD in different CT devices were 0.85–0.99.

Conclusion: To our knowledge, it is the first time for automatic PL-QCT to evaluate the performance against dual-energy X-ray absorptiometry (DXA) in LDCT images. The results

indicate that it may be a promising tool for individuals screened for low-dose chest computed tomography.

Keywords: osteoporosis, phantom-less QCT, dual-energy X-ray, low-dose CT, COVID-19

INTRODUCTION

Osteoporosis is a complex disease in which the quantity and quality of bone are diminished, causing an increase in bone fragility (Johnell and Kanis, 2006). Osteoporosis and osteoporotic fractures have become global health issues of major concern with the growth in the aging population (Alejandro and Constantinescu, 2018). About 200 million people suffer from osteoporosis, and 89 million fractures occur worldwide every year, with considerable health, societal, and economic burden (Pisani et al., 2016). The prevalence of osteoporosis and the incidence of fragility fracture in china have increased markedly over the last decades. Recent data report an osteoporosis prevalence of 29.1% in women and 6.5% in men aged >50 years, equating to an estimated population prevalence of 49.3 million and 10.9 million, respectively. Approximately 50% of women will have at least one fracture after the age of 50 years (Reid, 2020). It is estimated that by 2050, there will be 5.99 (95% CI, 5.44–6.55) million fractures annually in China, costing $25.43 (95% CI, $23.92 to $26.95) billion, reflecting a 2.7-fold increase since 2010 (Chen et al., 2016). The increase in osteoporosis and fracture rates reflects in part the rapidly aging population of China, and therefore, reliable early screening and timely monitoring of osteoporosis will be critical for individuals and care providers.

Osteoporosis is diagnosed clinically or radiographically. Biochemical markers of bone turnover in the serum or urine are not currently recommended for diagnosis (Mauck and Clarke, 2006). Bone mineral density (BMD) is a surrogate indicator directly related to bone strength, plays an important role, and is widely used to monitor and diagnose osteoporosis in clinical practice (Engelke, 2012). Currently, dual-energy X-ray absorptiometry (DXA), quantitative computed tomography (QCT), and quantitative ultrasound (QUS) are commonly used tools for evaluating osteoporosis (Malekzadeh et al., 2019). Areal BMD testing *via* DXA in the proximal femur, lumbar spine, and the forearm is the gold standard method for diagnosing osteoporosis, but this does not capture the important contributions of clinical risk factors or other bone measures (e.g., trabecular bone score and geometry) and is susceptible to confounding factors (e.g., osteophyte aortic calcification and body mass index) (Salzmann et al., 2019) (Smets et al., 2021). As defined by the World Health Organization (WHO), for osteoporosis, the DXA BMD criterion requires a T-score of less than −2.5; a normal BMD T-score is higher than −1.0, and osteopenia is anything in-between T-scores −1 and −2.5 (World Health Organization, 1994). Different from areal bone mineral density computed by DXA, BMD derived from QCT is a volumetric measure of the vertebral trabecular bone. Given the high turnover rate of trabecular bone compared to cortical bone (Samelson et al., 2019), BMD calculated from QCT offers substantially higher sensitivity and can also be used for diagnosis based on thresholds published by the American College of Radiology of 120 mg/cc and 80 mg/cc to define osteopenia and osteoporosis, respectively (Cheon et al., 2012). Yet, radiation doses associated with CT and frequent manual operations before QCT image analysis limit the application of QCT in osteoporosis screening.

Quantitative computed tomography can be classified into two main kinds, phantom-based QCT (PB-QCT), which includes synchronously calibrated QCT and asynchronously calibrated QCT, and phantom-less QCT (PL-QCT). The asynchronously calibrated QCT provides results comparable to the established synchronously calibrated QCT. Cheng XG et al. have validated the accuracy and short-term reproducibility of asynchronous QCT and carried out research about asynchronous QCT in population-based clinical studies (Cheng et al., 2014; Wang et al., 2017; Wu et al., 2019). However, the phantom-based QCT needs to deploy a reference calibration phantom during the patient scan, which means the beam hardening and scatter effect cannot be avoided. Although the precision is inferior to phantom-based BMD systems, the mean absolute standardized differences and accuracy deviations between the two methods were small (Habashy et al., 2011; Mueller et al., 2011). PL-QCT has been proved a robust clinical utility for the detection of lowered BMD in a large patient population, which can be easily integrated into the CT workflow for non-dedicated quantitative CT (QCT) BMD measurements in thoracic and abdominal scans and achieved without additional radiation exposure from non-contracted CT scans, to perform an ancillary diagnosis of osteopenia or osteoporosis (Mueller et al., 2011).

Coronavirus disease 2019 (COVID-19) outbreak has rapidly swept around the world, causing a global public health emergency. In diagnosis, chest computed tomography (CT) is used in COVID-19 and is an important complement to the real-time reverse transcription-polymerase chain reaction (RT-PCR) test (Ai et al., 2020). Low-dose chest computed tomography (LDCT), popularly used for early lung cancer screening (National Lung Screening Trial Research Team et al., 2011), can also offer a high specificity for distinguishing COVID-19 from other diseases associated with similar clinical symptoms and has become an indispensable image examination for hospitalized patients in China (Schulze-Hagen et al., 2020). As been confirmed, LDCT can be utilized to measure volumetric bone mineral density (vBMD) (Kim et al., 2017) and shows the feasibility of osteoporotic fracture prevention (Cheng et al., 2021). The combination of LDCT and QCT allows further application of imaging data used for COVID-19 or lung cancer screening to provide an accurate diagnosis of osteoporosis without additional radiation and cost for patients (Pan et al., 2020; Cheng et al., 2021). Cheng XG et al. and Lu Y et al. have validated the efficiency of PB-QCT combined with

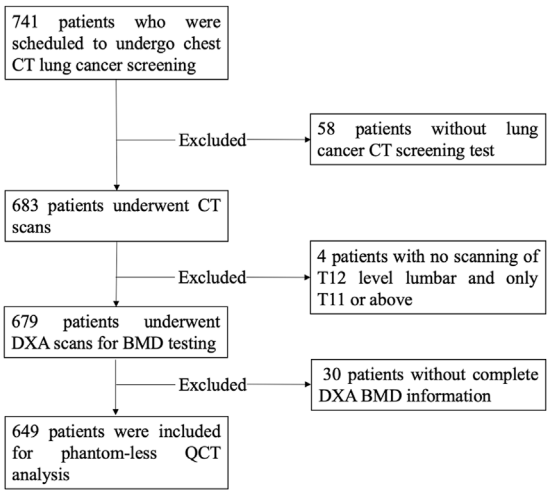


FIGURE 1 | Flow chart of the inclusion process.

LDCT through conventional and deep learning methods (Pan et al., 2020; Cheng et al., 2021). Nevertheless, to the best of our knowledge, clinical validation of PL-QCT with LDCT has not been published in a peer-reviewed journal. The purpose of this study was to determine the accuracy and precision of our newly developed automatic PL-QCT system for BMD measurement and osteoporosis assessment for the hospitalized patients in the COVID-19 period based on low-dose chest computed tomography.

MATERIAL AND METHODS

Patient Population

The retrospective study was approved by the Institutional Board, informed patient consent was waived, and all information and imaging data were under the control of authors throughout the study. All exams were collected from the patients in The Second Hospital of Jilin University with informed consent and reviewed by the Internal Review Board. A total of 741 patients were scheduled for the DXA and PL-QCT analysis. After the screening process shown in **Figure 1**, 58 patients were found to have no low-dose CT screening data for lung cancer, and four patients had only T11 and above levels included in the CT image and without T12 level screening. In addition, there were 30 patients whose DXA bone mineral density information was not complete for analysis. A total of 92 patients were excluded, and the remaining 649 patients (**Table 1**) were included in this study. The average time interval between DXA and QCT scanning of the same patient is 1–3 days.

DXA tests were performed for all patients, including spinal and hip scans and results. At the same time, the newly developed bone density instrument was used to verify. The average DXA BMD results of the total hip and spine were taken as the gold standard. Meanwhile, low-dose chest CT scanning images were used for the analysis and diagnosis by the new phantom-less QCT. The 80 mg/cc and 120 mg/cc were taken as the important criteria for diagnosing osteoporosis and osteopenia in QCT analysis, respectively.

DXA and CT Acquisition

Dual Energy X-Ray Absorptiometry

All patients are performed with DXA on the spine (L1–L4) and hip (femoral neck and total hip). The DXA measurements have been performed on the Hologic device (DXA, Discovery WI, Hologic Inc., USA). The trained technicians and physicians supervised the whole testing process. Since both the spine and hip DXA results were detected, the osteoporosis was diagnosed by the lower T-score of the spine or hip measurement results. According to the international standard, osteoporosis was defined as T-score ≤ -2.5 SD (standard deviation), and osteopenia was defined as $-2.5 <$ T-score ≤ -1.0 SD.

Computed Tomography

The CT images were acquired from several different CT devices, including Philips iCT 256, SCENARIA, NeuViz epoch, and Revolution CT. The scanning parameters of CT are listed in **Table 2**. These CT images were originally scanned for the lung cancer or COVID-19 screening in the endocrinology department of the hospital.

Automatic Phantom-Less QCT BMD Analysis

We developed one automatic phantom-less QCT software, which can be applied in the spine and hip BMD measurements. This novel PL-QCT has the automatic function of selecting the vertebrae, hip, fat, and muscle ROI and calibrating the BMD with high precision. A detailed phantom-less QCT technology development process can be found in our last study (Liu et al., 2021). Fat and muscle ROI CT values have been used to calibrate the BMD results (**Figure 2**). Localized BMD can also be accurately measured, including cancellous and cortical bone. Compared with phantom-based QCT, phantom-less QCT can be utilized to measure BMD without simultaneous scanning of the external phantom. There were many reports on the phantom-less QCT development and relative bone mineral density of fat and muscle.

Statistical Analysis

Osteoporosis Analysis Results by DXA and QCT

Consistency analysis was performed on the BMD results of DXA and QCT. The diagnosis rates of osteoporosis, osteopenia, and normally detected by DXA and QCT were compared and analyzed. Receiver operating characteristic curve (ROC) analysis and confusion matrix analysis were conducted, respectively. The results calculated by DXA were used as the gold standard for the diagnosis of osteopenia and osteoporosis. The diagnostic efficacy of QCT in female and male subgroups was also analyzed by ROC (area under curve: AUC value).

BMD Changes With Age

The enrolled patients were divided into seven subgroups by age. The mean value and standard deviation of different subgroups were calculated, respectively, and the correlation between the

TABLE 1 | Basic information of included subjects.

Basic information	Male (n = 266)	Female (n = 383)	Total subjects (n = 649)
Age (years)	55.06 ± 12.37	60.02 ± 10.47	57.99 ± 11.54
Height (cm)	171.80 ± 5.71	159.76 ± 5.21	164.69 ± 8.03
Weight (kg)	76.68 ± 12.29	63.74 ± 10.08	69.04 ± 12.73
BMI (kg/m^2)	25.96 ± 3.82	24.94 ± 3.54	25.36 ± 3.69

TABLE 2 | Low-dose CT scanning parameters.

Manufacturer	NeuViz epoch	Philips-iCT 256	GE-Revolution CT	SCENARIA
Voltage (kV)	120	120	120	120
mA	345	225	254	254
SFOV (mm)	500	500	500	500
Matrix	512*512	512*512	512*512	512*512
Table height (cm)	130.4	150	132.4	122
Slice thickness (mm)	3	1	5	5
Reconstruction kernal	Standard	Standard	Standard	Standard

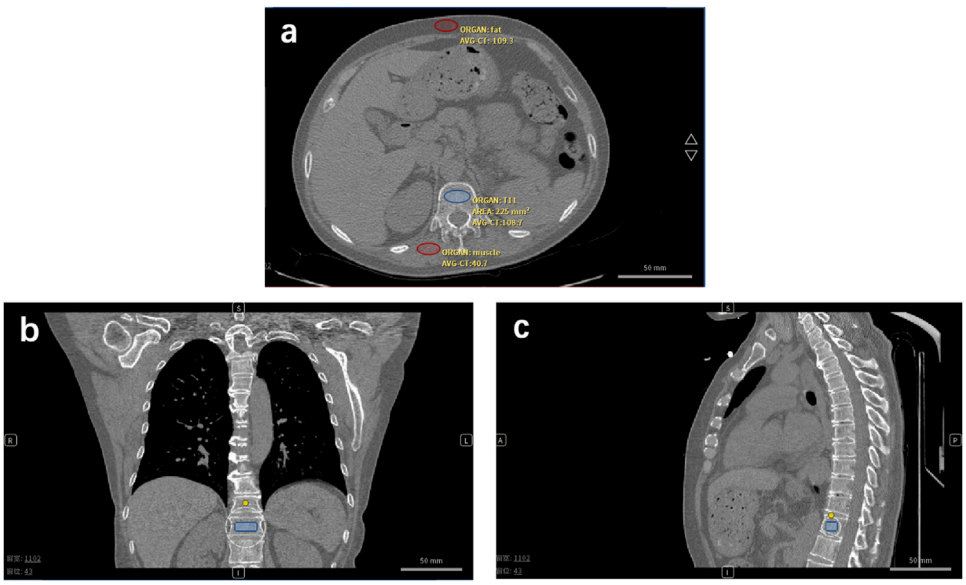


FIGURE 2 | Phantom-less QCT analysis low-dose CT of lung cancer or COVID-19 screening is used for BMD testing and osteoporosis diagnosis. **(A)** Transversal plane, **(B)** coronal plane, and **(C)** sagittal plane of CT image of one enrolled patient. Red ovals represent the muscle and fat ROI. The blue symbol represents the trabecular ROI.

DXA and phantom-less QCT methods was analyzed. The whole research step is shown in **Figure 3**.

BMD Measured by Different CT Devices

The patients were scanned by four main types of CT devices. In some studies, Hounsfield unit (HU) values were used to represent BMD and diagnose osteoporosis. To investigate the influence of the CT devices on the HU value, we have studied the relationship between the CT value and BMD calculated by phantom-less QCT for different CT devices (**Table 3** and **Figure 4**).

RESULTS

Patient Population

After the patient enrollment screening, the data of 649 patients meeting the conditions were retained for validation analysis, and the basic information of patients was collected. The average age of the whole cohort of patients is 57.99 (±11.54) years. The height is 164.69 (± 8.03) cm, and the weight is 69.04 (± 12.73) kg. The body mass index (BMI) of these patients is 25.36 (±3.69) kg/m^2.

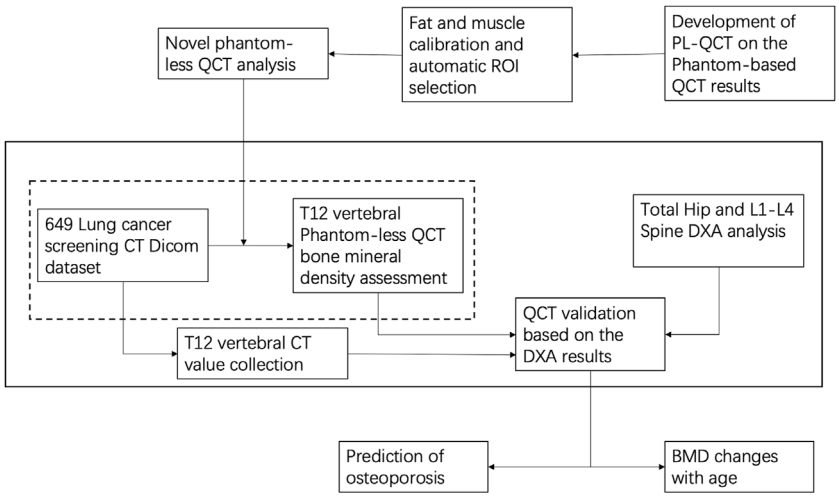
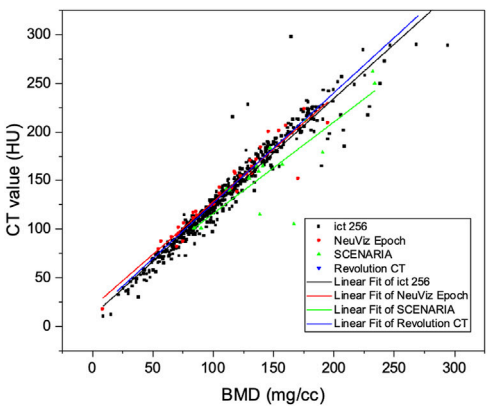


FIGURE 3 | Flow chart of the testing process in the whole project.

TABLE 3 | Comparison between the precision of different QCT studies (Liu et al., 2021).

Result and reference	Phantom-less QCT result			Phantom-based QCT result
	Automatic PL-QCT	Philips	Other study	Mindways
Precision in SD[mg/cm^3]	0.87	3.1	—	—
Precision as CV[%]	0.89	4.0	1–2	1.4–3.6



Equation	y = a + b*x			
Weight	No Weighting			
Residual Sum of Squares	90190.54623	5712.02434	9725.02369	2997.31116
Pearson's r	0.96347	0.95765	0.84901	0.98929
Adj. R-Square	0.92814	0.91458	0.70088	0.97823
		Value	Standard Error	
ict 256	Intercept	10.77034	1.71326	
	Slope	1.11854	0.01405	
NeuViz Epoch	Intercept	19.84524	6.48871	
	Slope	1.08226	0.05665	
SCENARIA	Intercept	21.55972	24.32236	
	Slope	0.94359	0.15694	
Revolution CT	Intercept	12.74061	3.19365	
	Slope	1.13744	0.02474	

FIGURE 4 | Linear relationship between CT attenuation values and vBMD (mg/cc) of different CT devices.

Comparison Between the Diagnosis Rate of Osteoporosis and Osteopenia of DXA and QCT

The different diagnosis rates of osteoporosis, osteopenia, and normal patients for spine DXA result, hip DXA result, and phantom-less QCT results are shown in **Figure 5**. Hip and spine DXA results have been, respectively, settled as the golden standards for the analysis of QCT. Due to surgeons using the lower value of the hip and spine DXA result to diagnose osteoporosis in clinical practice, we also set this lower value as another reference in the ROC analysis (**Table 4**). According to the results of ROC analysis, the AUC index basically remained above 0.7, indicating that bone mineral density calculated by phantom-less QCT can predict bone loss and osteoporosis. However, the BMD results measured by DXA are often higher due to vascular calcification and osteophytes. This leads to a relatively higher false-negative rate in diagnosing osteoporosis for DXA. Thus, a difference exists between the

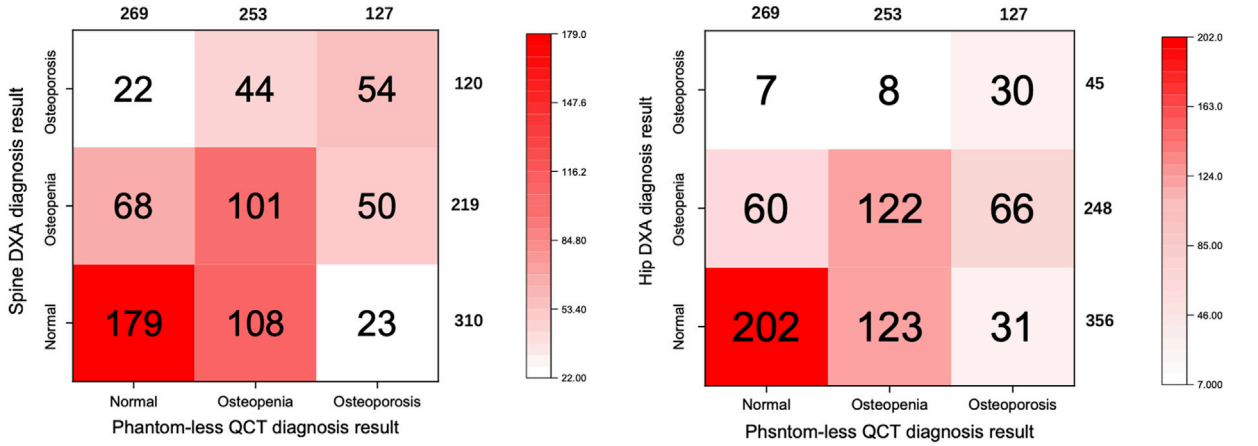


FIGURE 5 | Confusion matrix of QCT and spine DXA diagnosis result comparison.

TABLE 4 | ROC analysis of QCT results with spine and hip DXA as the golden standard.

		Diagnosis	AUC (95%CI)	Sensitivity (%)	Specificity (%)	Youden index J	Associated criterion
Hip DXA result	Women	Osteoporosis	0.74 (0.69–0.78)	67.5	81.9	0.49	≤77.8
	(*n* = 383)	Osteopenia	0.71 (0.66–0.75)	67.1	68.2	0.35	≤105.9
	Men (*n* = 266)	Osteoporosis	0.88 (0.84–0.92)	100	68.2	0.68	≤102.3
		Osteopenia	0.68 (0.62–0.74)	82.5	51.1	0.36	≤129.0
	Total (*n* = 649)	Osteoporosis	0.77 (0.74–0.80)	66.7	85.8	0.52	≤77.8
		Osteopenia	0.71 (0.67–0.74)	64.5	69.1	0.34	≤106.1
Spine DXA result	Women	Osteoporosis	0.72 (0.67–0.76)	68.7	69.0	0.38	≤97.2
	(*n* = 383)	Osteopenia	0.72 (0.67–0.76)	58.0	82.1	0.40	≤98.4
	Men (*n* = 266)	Osteoporosis	0.71 (0.66–0.77)	76.2	65.3	0.42	≤107.2
		Osteopenia	0.63 (0.57–0.69)	77.2	49.7	0.27	≤130.7
	Total (*n* = 649)	Osteoporosis	0.73 (0.69–0.76)	62.5	75.6	0.38	≤92.5
		Osteopenia	0.69 (0.65–0.73)	54.9	75.8	0.31	≤101
Lower value of spine and hip DXA result	Women	Osteoporosis	0.74 (0.69–0.78)	70.1	69.9	0.40	≤97.2
	(*n* = 383)	Osteopenia	0.74 (0.69–0.78)	68.4	71.2	0.40	≤112.4
	Men (*n* = 266)	Osteoporosis	0.76 (0.71–0.81)	81.0	66.9	0.48	≤107.2
		Osteopenia	0.70 (0.64–0.75)	78.7	58.3	0.37	≤130.7
	Total (*n* = 649)	Osteoporosis	0.76 (0.71–0.79)	66.2	74.6	0.41	≤95.6
		Osteopenia	0.73 (0.69–0.76)	74.7	62.0	0.37	≤122.2

diagnosis rates of the two methods (as shown in **Figure 6**), and this can partly explain why the AUC values in the ROC analysis are not so high. In this study, we aim to explore the clinical application potential of the automatic phantom-less QCT, and the results in **Figure 5** and **Figure 6** are able to demonstrate the effectiveness of the new method to some extent, but further validation involving comparison with other accurate devices still needs to be conducted.

BMD Changes Are Associated With Age for Males and Females

The BMD changes have been measured by the QCT and DXA results, relatively absolute BMD value, and T-score of the DXA. The result in **Figure 7** shows that BMD decreases significantly after 40–49 years old, especially for female patients. This result is similar to other studies (Cheng et al., 2021). However, no study has utilized the phantom-less QCT to do the large data screening based on the lung cancer or COVID-19 screening LDCT images. From the DXA BMD results, the change of T-score in the female group has a similar trend (**Figure 7**), but the male groups have a large difference among the spine DXA, femoral neck DXA, and total hip DXA (**Supplementary Figures S3**).

BMD Measured by Different CT Devices

Four main CT devices were used for CT scanning in this study. CT values are correlated with BMD values, but different CT devices and scanning parameters have an impact on the specific relationship between CT and BMD. Therefore, CT values cannot

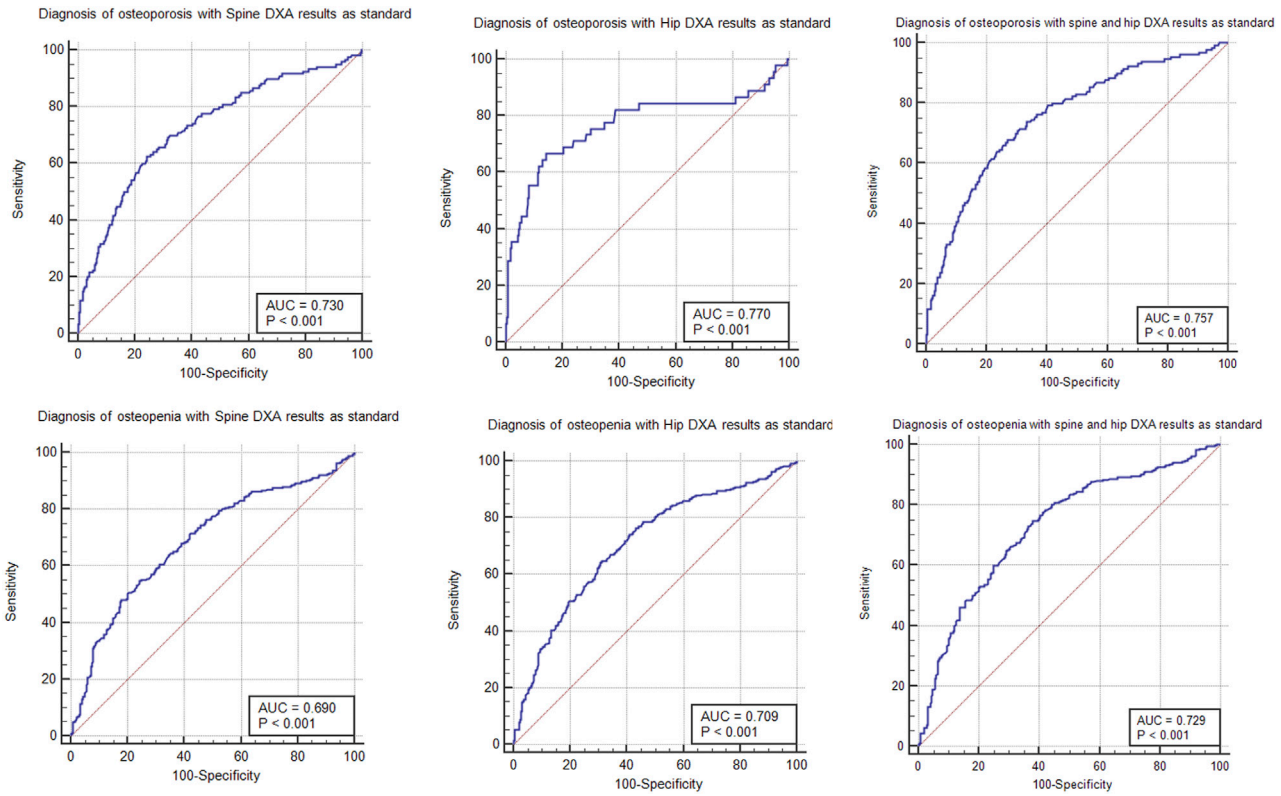


FIGURE 6 | ROC analysis of PL-QCT and DXA diagnosis result comparison.

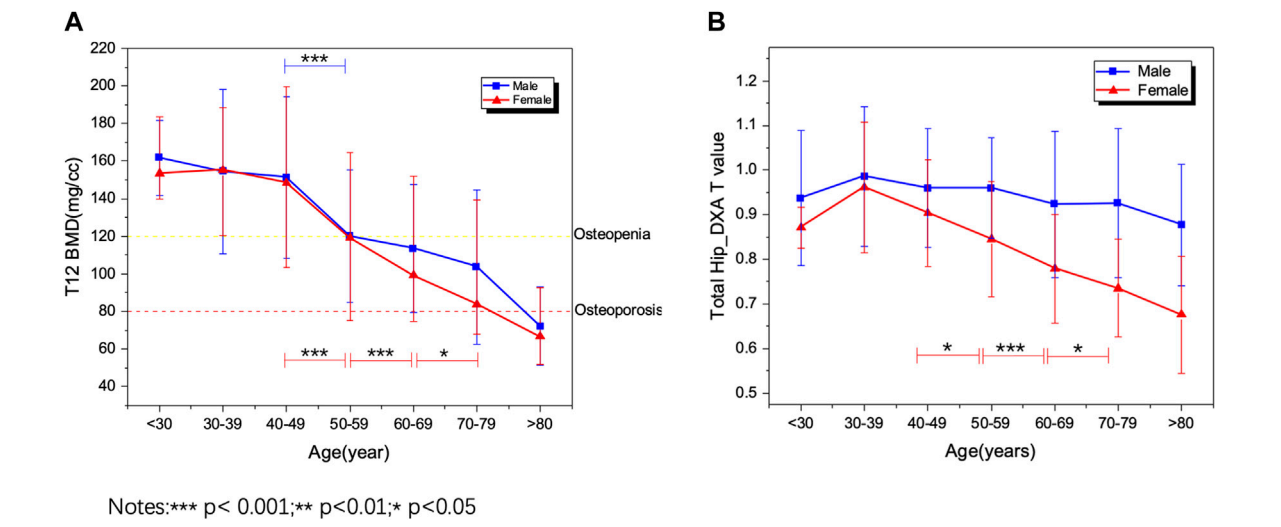


FIGURE 7 | PL-QCT and DXA BMD changes with age. **(A)** PL-QCT; **(B)** DXA.

be directly used as a diagnostic method of osteoporosis in clinical applications. It can be found from the results that the linear regression relationship between CT and BMD is not exactly the same for analysis in different CT images scanned by different CT machines.

DISCUSSION

In this study, we determined the accuracy and precision of our newly developed automatic PL-QCT in BMD measurement and osteoporosis detection based on the retrospectively collected

LOCCT scans for COVID-19 diagnosis, lung cancer, or other indications. To our knowledge, it is the first time for automatic PL-QCT to evaluate the performance against DXA in LDCT images.

Sensitive detection of bone mineral density (BMD) change is a key issue in monitoring and evaluating the individual bone health status, as well as bone metabolism and bone mineral status. Matthew J Budoff et al. have validated that the thoracic and the lumbar QCT provides a similar and much sensitive method for detecting bone mineral loss when compared to DXA (Mao et al., 2017). The accuracy and short-term reproducibility of asynchronous PB-QCT have been verified in a nationwide multicenter study carried out by Cheng et al. (2014), Wang et al. (2017), and Wu et al. (2019), and the lumbar CT has been recommended as analogous to central DXA technologies in assessing or monitoring ages and disease- and treatment-related BMD changes in PB-QCT. PL-QCT does not need to deploy a reference calibration phantom during the patient scan compared with PB-QCT, which uses surrounding tissue like fat and muscle as calibration so that the beam hardening and scatter effect can be avoided (Budoff et al., 2013; Michalski et al., 2020). Nevertheless, conventional QCT analysis, whichever the phantom-based or -less, requires manual localization of vertebral bodies and region of interest (ROI) (National Lung Screening Trial Research Team et al., 2011). Hence, it is necessary to develop an automatic QCT to localize vertebral bodies and select suitable fat or muscle ROI, as well as calculate bone density with high precision. Lu Yet al. developed useful automatic QCT image analysis software based on the deep learning method in LDCT images, which eliminate the heavy manual operation in BMD measurement and liberate the radiologist from reduplicative tasks (Pan et al., 2020). In a previous study, our group also developed an automatic phantom-less QCT system based on traditional machine learning methods in lumbar CT images, which shows high BMD measurement precision with the automatic selection of fat and muscle ROI (Liu et al., 2021). In this study, we further validated the capability and precision of our automatic PL-QCT system in LDCT so as to enhance its possibility of being integrated into the CT workflow in large-scale osteoporosis screening.

DXA is the most common method for the estimation of BMD and fracture risk in the clinical setting. Therefore, the DXA spine and hip BMD standards were utilized as the reference in the diagnosis rate and ROC analysis of the comparison between DXA and PL-QCT. According to the results of ROC analysis, the average AUC index basically remained above 0.75, especially in the situation of the lower value of the hip and spine DXA, indicating that bone mineral density calculated by phantom-less QCT can predict bone loss and osteoporosis. Compared to DXA, the automatic PL-QCT detected a relatively higher proportion of osteoporosis patients, and this may be due to the false-negative cases caused by the osteophyte and vascular calcification in DXA diagnosis. Many studies have also reported similar results regarding the comparison between DXA and QCT (Li et al., 2013). The associated criterion is that BMD is less than 77.8 mg/cc and 92.5 mg/cc in the hip and spine DXA result group, respectively, for this automatic PL-QCT system, which is different from the common standard of 80 mg/cc. Several studies have shown that BMD is higher in the thoracic spine than the lumbar spine (Weishaupt et al., 2001). Due to the low sensitivity of DXA, some patients with osteoporosis may be misjudged, especially the elderly, and may not receive timely treatment, which increases the risk of osteoporotic fractures. Therefore, the current clinical guidelines do not recommend DXA for screening in the United Kingdom, which also explains the relatively lower sensitivity, specificity, and Youden index of this PL-QCT.

After validating the potential function of this PL-QCT in distinguishing osteoporosis and measuring BMD, we also measured the mean and S.D. of BMD variation with age by QCT and compared the trend measured by DXA. **Figure 3** shows the age-dependent mean vBMD for each 10-year interval. Thoracic spine BMD was decreased progressively with age, varying in women from 155.19 mg/cc at age 30–39 years to 66.59 mg/cc at age 80+ years and in men from 161.7 to 72.2 mg/cc. There was a greater rate of bone loss in women than men after the age of 49 years, suggesting the influence of menopause on bone loss. All these results and the tendency are similar to the lumbar spine or low-dose chest CT measured by PB-QCT (Ghildiyal et al., 2018; Cheng et al., 2021). The reliability and accuracy of HU to BMD measurement and determining osteoporosis have been proven in the literature with many reports (Lee et al., 2013; Park et al., 2020), but in its current state, it is not ready for clinical implementation. There is a lack of exchangeability among different machines that limits its broad applicability (Gausden et al., 2017). In our study, we included four main CT devices for BMD measurement, and it can be found that the results between CT value and BMD are not exactly the same for analysis in different CT images scanned by CT machines. However, the similar linear regression relationship between these four machines indirectly indicates the robustness of our PL-QCT.

There were a few limitations to this study. First, the retrospective study used DXA of the lumbar spine instead of the QCT, which could provide a more reliable evaluation of the performance of our developed system as a reference standard for BMD measurement. It is difficult to find any individuals who underwent LDCT and QCT within a short time, which may cause more radiation and high cost. Second, all LDCT scans were obtained at a single center in this study. Further confirmation of the consistency, robustness, and transferability of this system in LDCT scans using scanners from multi-center institutions will be implemented.

CONCLUSION

In order to achieve fully automated BMD measurement and osteoporosis detection on LDCT scans, a newly automatic PL-QCT system was developed in company with auto-location and detection function-based traditional machine learning methods. The performance of the system was evaluated by using DXA as the reference standard. To our knowledge, it is the first time for

automatic PL-QCT to evaluate the performance against DXA in LDCT images. The accuracy and precision of the system for BMD measurement and osteoporosis indicate that it may be a promising tool for individuals screened for low-dose chest computed tomography.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the data presented in this study are available on request from the corresponding author. The data are not publicly available due to the restriction of IRB. Requests to access the datasets should be directed to qinyg@jlu.edu.cn.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Jilin University Second Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization: TX, ZC, QY, and LW. Methodology: ZC, MC, QH, YK, and WT-M. Validation: HM, GD, CB, and SX. Formal analysis: TX, ZC, MC, QH, YK, and WT-M. Investigation: HM, GD, and CB. Resources: HM and TX. Data curation: HM and TX. Writing original draft preparation: ZC and TX. Writing review and editing: MC, QH, YK, and WT-M. Supervision: QY and LW. Project administration: TX, ZC, MC, QY, and LW. Funding acquisition: QY and LW.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.856753/full#supplementary-material

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The role of microenvironment in stem cell-based regeneration of intervertebral disc

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Regenerative medicine for intervertebral disc (IVD) disease, by utilizing chondrocytes, IVD cells, and stem cells, has progressed to clinical trials in the treatment of back pain, and has been studied in various animal models of disc degeneration in the past decade. Stem cells exist in their natural microenvironment, which provides vital dynamic physical and chemical signals for their survival, proliferation and function. Long-term survival, function and fate of mesenchymal stem cells (MSCs) depend on the microenvironment in which they are transplanted. However, the transplanted MSCs and the endogenous disc cells were influenced by the complicated microenvironment in the degenerating disc with the changes of biochemical and biophysical components. It is important to understand how the MSCs and endogenous disc cells survive and thrive in the harsh microenvironment of the degenerative disc. Furthermore, materials containing stem cells and their natural microenvironment have good clinical effects. However, the implantation of tissue engineering IVD (TE-IVD) cannot provide a complete and dynamic microenvironment for MSCs. IVD graft substitutes may need further improvement to provide the best engineered MSC microenvironment. Additionally, the IVD progenitor cells inside the stem cell niches have been regarded as popular graft cells for IVD regeneration. However, it is still unclear whether actual IVD progenitor cells exist in degenerative spinal conditions. Therefore, the purpose of this review is fourfold: to discuss the presence of endogenous stem cells; to review and summarize the effects of the microenvironment in biological characteristics of MSC, especially those from IVD; to explore the feasibility and prospects of IVD graft substitutes and to elaborate state of the art in the use of MSC transplantation for IVD degeneration *in vivo* as well as their clinical application.

KEYWORDS

intervertebral disc, microenvironment, stem cell, tissue regeneration, regenerative medicine

1 Introduction

The intervertebral disc (IVD) is the fibrocartilage connection of the adjacent vertebrae, like an elastic cushion, which can encounter the vibration of the spine from external forces and increase the amplitude of the spinal motion. As the senescence or other injury elements caused by multiple stresses occur and progressively develop, the degeneration of the IVD may happen, eventually leading to low back pain, disc herniation, etc., which impose a tremendous burden on global health. The IVD is avascular and can barely not repair itself (Chen et al., 2022). Currently, the molecular and pathogenesis mechanism of the degenerative disc disease is not totally understood. Surgery such as interbody fusion could relieve pain, but it restricted by the degeneration of the adjacent disc postoperation, so the present treatment is mainly physical therapies, anti-inflammatory medications and analgesic, all of which can not reverse the pathophysiological function of the degenerative IVD (Wu et al., 2020). Therefore, developing a new treatment to facilitate IVD regeneration is necessary.

Stem cells have multipotential to differentiate into many types of cells, which is a promising therapy and has already been applied to treating many diseases. Nowadays, many researchers have been studying on the stem cell-based treatment for IVD regeneration, such as mesenchymal stem cells (MSCs) (Yang et al., 2010; Sakai and Andersson, 2015). In recent years, more and more researchers have been focusing on the endogenous multipotent cells in the IVD. They reside in the IVD stem cell niche quiescently and can differentiate into chondrogenic cells, orthogenic cells and adipogenic cells after injuries or degeneration. However, when the IVD degeneration is ongoing, the disturbed catabolic-anabolic balance and the increasingly harsh microenvironment in the IVD might be unfavourable to progenitor cell activity (Chen et al., 2022). All above is a big challenge for stem cell-based treatments. Moreover, the properties of the IVD graft substitutes, like viscoelasticity, elasticity and microstructure, can determine the fate of stem cells (Chu et al., 2018; Gan et al., 2022). Hence, it is critical to figure out the effects of the materials-related microenvironment on the stem cell for IVD regeneration.

In this review, firstly, we will introduce the native IVD progenitors and the microenvironment changes in the progress of IVD degeneration. And then, the influence of the IVD microenvironment and IVD graft substitutes on the stem cells would be elucidated. At last, we will discuss the clinical applications and future perspectives based on the existing knowledge. We hope that this review can offer new insight for the repair and regeneration of IVD.

2 Tissue-specific progenitor cells in the IVD

Stem cells usually stay quiescent in a region of the IVD, so-called stem cell niches, where they reside. Studies have suggested that IVD stem cell niches might exist in the outer zone of the AF as well as the perichondrium region adjacent to the epiphyseal plate (Figure 1) (Huang et al., 2011). Considering the ratio between the disc volume and the IVD cell number, it is reasonable to suppose the IVD as acellular, so the quantity of the progenitor cells is very small. Stem or progenitor cells have been proven to exist in the IVD in many species with the following supporting reasons. First, despite the avascular and aneural in IVD, the blood vessel and nerve growth, calcification and fibrocartilage-like tissue were found in the degenerated disc (Rutges et al., 2010). It is speculated that these pathological tissues might have derived from IVD progenitor and resident stem cells. Secondly, the application of autologous disc cells was beneficial for IVD regeneration. A number of these cells maintained self-renewal potential and multipotent differentiation, activating these progenitor cells could be a valuable strategy for IVD regeneration (Oehme et al., 2015; Hu et al., 2018).

Recently, tissue-specific progenitor cells have been identified in the IVD, and these cells could be targeted to promote intrinsic repair, suggested that IVD progenitor cells might have multiple

TABLE 1 Surface markers of IVD progenitor cells.

Species	Cell type	Surface markers	Ref
Human	CESC	$CD14^-$, $CD19^-$, $CD34^-$, $CD45^-$, HLA-DR-, $CD44^+$, $CD73^+$, $CD90^+$, CD105+, CD133+, CD166+, Stro-1+	Liu et al. (2011)
Human	CESC	HLA-DR-, $CD14^-$, $CD19^-$, $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, CD105+	Yuan et al. (2018)
Human	AFSC, NPSC	$CD34^-$, CD49a+, $CD63^+$, $CD73^+$, $CD90^+$, CD105+, CD166+, p75 NTR+, CD133/1+	Risbud et al. (2007)
Human	IVDSC	$CD90^+$, CD105+, Stro-1+	Brisby et al. (2013)
Rhesus macaque	NPSC	$CD90^-$, $CD45^-$, $CD44^+$, $CD90^+$, CD146+, CD166+, HLA-DR+	Huang et al. (2013a)
Human	NPSC	$CD29^-$, $CD45^-$, $CD24^+$, $CD73^+$, $CD90^+$, CD105+	Qi et al. (2019)
Rat	NPSC	$CD34^-$, $CD45^-$, $CD44^+$, $CD90^+$, CD105+	Tao et al. (2015)
Human	NPSC	$CD29^-$, $CD44^-$, $CD73^-$, $CD90^-$, CD105-, $CD29^+$, $CD44^+$, $CD73^+$, $CD90^+$, CD105+	Shen et al. (2015)
Human	NPSC	$CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, CD105+	Lama et al. (2018)
Human	NPSC	HLA-DR-, $CD34^-$, $CD45^-$, $CD73^+$, $CD90^+$, CD105+	Liu et al. (2017)

TABLE 2 Clinical studies utilizing cell-based therapies to treat chronic low back pain.

Author, year	Clinical details	Cells transplanted	Results
Orozco et al. (2011)	10 patients with low back pain and evidence of DDD	Autologous MSCs	Clinical improvement in back pain, leg pain and disability. Disc height not recovered. Increased MRI T2 signal
Yoshikawa et al. (2010)	2 patients with back pain and sciatica, with radiological evidence of DDD and lumbar canal stenosis	Autologous marrow MSCs	Clinical improvement in both patients. Increased MRI T2 signal. Less instability
Henriksson et al. (2019)	4 patients undergo MSC injection and who later opted for spinal surgery	autologous MSCs	The labelled MSCs have differentiated into chondrocyte-like cells and were distributed at different parts of the IVD
Centeno et al. (2017)	33 patients with lower back pain and disc bulge	Autologous BM-MSCs	Mean 60% Clinical improvement in all patients. MRI showed a reduction in disc bulge. Patients with greater bulge reduction reported less pain
Kumar et al. (2017)	10 patients with low back pain due to DDD	Autologous AT-MSCs	No statistical difference between high and low dose arms. 1 Patient with improved Pfirmann grade. Greater than 50% (Oswestry Disability Index) ODI and (visual analog scale) VAS in 13 patients
Elabd et al. (2016)	5 patients with back pain and sciatica	Autologous hypoxic-cultured BM-MSCs	A positive association between clinical improvement and stem cell therapies. MRI showed reduced disc protrusion
Noriega et al. (2017)	24 patients with low back pain	Allogenic BM-MSCs	MRI showed improvement in MSC patients VS. disc degeneration in controls. Stem cell patients had significant ODI VAS and ODI VAS reductions at 3, 6, 12 months. MRI showed disc degeneration in controls VS. improvement in MSC patients
Meisel et al. (2007)	28 patients undergoing microdiscectomy with back pain	Autologous culture expanded disc derived chondrocytes	Patients receiving cell transplantation had reduced back pain at 2 years. Increased MRI T2 signal of treated and adjacent discs

origins. The tissue-specific progenitor cells are a form of three types of stem-like cells: annulus fibrosus-derived stem cells (AFSCs), nucleus pulposus-derived stem cells (NPSCs), and cartilage endplate-derived stem cells (CESCs) (Liang et al., 2017). Despite the different morphology in the structural organization of different anatomy of IVD, AFSCs, NPSCs and CESCs exhibited common multi-differentiation characteristics, proliferative and immunophenotype during cell culture (Blanco et al., 2010). Researchers have identified specific markers of IVD progenitor cells that are compatible with MSCs, such as CD105+, $CD90^{+}$, $CD73^{+}$, $CD79^{-}$, $CD34^{-}$, $CD19^{-}$, $CD14^{-}$, and HLA-DR (De Luca et al., 2020). Another study explored the multipotency of progenitor cells derived from NP cells, they found that NPSCs expressed stemness marker genes such as Nanog, CD133, OCT3/4, Sox2 and Nestin (Sakai et al., 2012; Huang S et al., 2013). Followed by: The current characterization and related study findings of IVD progenitor cell characteristics are described in Table 1. NPSCs are capable of differentiating into multiple neural cell types including neuron, oligodendrocyte and astroglial specific precursor cells *in vivo*, this provides a promising source of seeded cells for neural repair. Yao et al. identified that CESCs exist in the human degenerated CEP and are similar to bone-marrow mesenchymal stem cells (BMSCs) among the immunophenotype, proliferation rate, cell cycle, and stem cell gene expression (Yao et al., 2016). In our previous study, we separated and characterized a subpopulation of cells that possessed self-renewal, osteogenesis, and adipogenesis potential from rabbit AF tissue, and AFSCs exhibited several stem marker genes similar to that of MSCs (Liu et al., 2014). This suggests the AFSCs may be considered an ideal seeded cell for treating degenerative IVD. In general, IVD progenitor cells seem to meet the requirements for definition as MSCs, having multilineage potential expressing MSC markers, and being plastic adherent (Gan et al., 2021). However, further investigation of the *in vivo* characteristics is needed to fully address the cell localization, differentiation and functional role in the maintenance of IVD homeostasis (Kim et al., 2003).

3 The degenerated IVD microenvironment

To date, the pathophysiology of the degenerative disc disease is not completely clear, but it is accepted that aging, smoking, mechanical overload, obesity, and diabetes contribution to disc degeneration (Wang et al., 2017; Zhang Y. et al., 2021). IVD stem cells are involved in cell replenishment during wound healing and tissue regeneration. However, it is still ambiguous how IVD stem cells are exhausted during degeneration and aging (Gan et al., 2021). One possibility is that the increasingly harsh microenvironment and metabolic imbalance in the IVD might cause the progressive reduction of the number and the function of the stem cells (Figure 2). Another possibility is

that after multiple rounds of differentiation and proliferation, the self-renewal ability of IVD stem cells becomes exhausted in response to degeneration-related or age-related damage. The possible reason includes nutrition deprivation, hypoxia, less reactive oxygen species, inflammatory factors, increased extracellular matrix (ECM) stiffness, and cyclic tension (Li et al., 2014; Zhang et al., 2020b; Zehra et al., 2022).

The calcification of the endplate happens as the IVD degeneration, which seriously influences the nourishment and oxygen supply of the IVD, would cause loss of NP ECM, NP cell death, and the collapse of IVD, leading to decreased height, eventually accelerating the progress of the IVD degeneration (Xu et al., 2021). Hypoxia can not only induce apoptosis and inhibit proliferation but also promote the IVD progenitor cells differentiate to chondrogenic cells (Huang S. et al., 2013). Yao et al. reported that the CESCs derived from degenerated IVDs had impaired osteogenic potential after the induction of HIF-1α or in hypoxic conditions (Yao et al., 2017a). In addition, the inflammatory cytokines (TNF-α, IL-1β, IL-8 and IL-6) could remodel the ECM metabolism from anabolism to catabolism, leading to an imbalance ECM and aggravating the pain response of IVD. The NPSCs with neurogenic potential may also contribute to innervation under inflammatory conditions, thus leading to DDD (Navone et al., 2012).

Substrate elasticity and stiffness occur as a result of the biomechanical changes that regulate the differentiation of stem cells. Cells can sense the interactions with ECM via integrin mediation. When the matrix stiffness microenvironment of the IVD changes, stem cells make corresponding alterations (Zhang W. et al., 2020). For example, NPSCs exhibit reduced chondrogenic gene expression but promoted osteogenic gene expression cultured in a stiff synthetic hydrogel matrix (Navaro et al., 2015). In our previous study, we investigate the effects of fiber size and stiffness of scaffolds on the differentiation of AFSC. We found that the fiber size and the stiffness of scaffolds play critical roles in regulating the microenvironment for stem cells (Chu G. L. et al., 2019). In addition, mechanical loading could induce ECM synthesis, thus regulating the fate of stem cells. It is proved that applied forces, such as compression, hydrostatic, and tension, could significantly affect the lineage specification and maintenance of AF progenitor cells (Stemper et al., 2014; Rasoulian et al., 2021). Furthermore, continuous cyclic tensile strain can induce apoptosis and osteogenic gene expression in human CESCs (Zuo et al., 2019).

Acidic conditions could lead to reduced proliferation, ECM production and cell viability of stem cells in degenerated IVD. Owing to low nutrition and hypoxia in IVD, there is plenty of lactic acid generated and accumulated within IVD, this leads to the IVD microenvironment being slightly acidic (between 7.0 and 7.2). Consequently, the pH of the IVD microenvironment would be lower, and the situation for cells to survive would be rougher (Han et al., 2014). According to previous studies, the pH may decrease to 6.5 in the early stages of IVD degeneration. In more severe cases, it can drop to 5.6 (Liu et al., 2017).

After all, these results demonstrate that the components of the IVD microenvironment could influence the physiological activity of progenitor cells. Several studies have tried to inject MSCs into the degenerate IVD, but rapidly the cells could not be detectable, which shows the severe effects of the microenvironment of the degenerate IVD on cells (Borem et al., 2019; Wangler et al., 2019). IVD stem cells seem to be both negatively and positively influenced by mechanical loading and hypoxia, whereas their physiological activity may be compromised by ECM stiffness changes, proinflammatory signaling, and acidic conditions. If stem cells or progenitor cells are truly resident in the healthy or degenerated disc, they will offer a new application for the regeneration of IVD.

4 The role of microenvironment in the application of stem cells-based IVD regeneration

Although stem cell transplantation therapy offers hope for IVD regeneration, their clinical use is still hampered by the harsh microenvironment of hypoxia, nutrition deprivation, acidic conditions, excessive cyclic tension, high osmolarity, and inflammatory factors (Huang Y. C et al., 2013). Therefore, appropriate regulation of the microenvironment could be used to enhance the physiological function of IVD stem cells in the complicated microenvironment.

4.1 Hypoxia and low nutrition

The IVD is inherently avascular, and the transport of nutrients and excretion of metabolites in IVD tissues strictly depends on the diffusion from capillaries that originate from the CEP. The lack of blood circulation establishes a hypoxic microenvironment with the average physiological oxygen tension (6 ± 2%) in human and falls to 1% in the degenerated IVD consequently (Peck et al., 2021). The properties of stem cells within a normoxia (20% O_2) microenvironment is quite different from their performance in the IVD microenvironment that is hypoxia (1–5% O_2). According to a recent study, hypoxia is beneficial in maintaining better stemness compared with normoxia. BMSCs exhibited greater colony-forming units and proliferated faster through the downregulation of E2A-p21 by Hypoxia-inducible factor 1 (HIF-1) (Elabd et al., 2018). Besides, when adipose-derived stem cells (ADSCs) stay in hypoxic and low glucose conditions *ex vivo*, their cell viability and the synthesis of the ECM increase (Hwang et al., 2020). Yao et al. demonstrated that physiological hypoxia promotes the chondrogenic differentiation of the CESCs (Yao et al., 2017b).

Likewise, de Vries et al. observed BMSCs could differentiate to acquire phenotypes similar to that of NP cells after exposure in 2% O_2 and 10 ng/ml transforming growth factor β (TGF-β) (de Vries et al., 2016). Hypoxia is an essential regulator for BMSCs, however, when the oxygen tension is less than 1%, prolonged exposure with serum deprivation would lead to complete cell mortality (Wang et al., 2020). It is reported that HIF is a transcriptional factor that can initiate cellular signals that regulate enzymes dealing with hypoxia. HIF is stable regardless of the oxygen concentration and can regulate cell proliferation, extracellular matrix production, energy supply, cell autophagy, and apoptosis (Luo et al., 2021). The absence of HIF would promote the degeneration of the IVD, and small leucine-rich proteoglycans (SLRPs) can activate HIF then help IVD stem cells survive in the hypoxic microenvironment (Meng et al., 2018; Silagi et al., 2021). Agents that could promote the upregulation of SLRPs might help NPSCs survive in the hypoxic microenvironment (Merceron et al., 2014).

Besides oxygen tension, glucose is another source of energy that significantly affects the differentiation, proliferation and viability of IVD progenitor cells. The consequence of low glucose availability is a decreased viability of IVD cells (Huang et al., 2014; Mahmoud et al., 2020). Some researchers have demonstrated the effect of glucose concentration on regulating the chondrogenic and osteogenic differentiation potential of CESCs to affect the differentiation of CESCs, which may represent a target for CE degeneration therapy (Sun et al., 2019). Furthermore, to adapt to the changes in the microenvironment, IVD progenitors correspondingly change their metabolism by increasing matrix synthesis and anaerobic glycolysis in low glucose conditions (5 mM), which allows generating energy while producing less reactive oxygen species (ROS) and consuming less O_2 (Yin et al., 2020).

4.2 pH

The glucose concentration and oxygen tension are easy to be kept accurately to simulate the microenvironment of healthy IVD, the biggest challenge for IVD progenitor cells to survive in the microenvironment should be the ECM acidity, ranging from 7.0 to 7.2 in a healthy disc, whereas in degenerated IVD is between 6.8 and 6.2 (Cai et al., 2019). It has shown that under IVD-like acidic pH, the viability of BMSCs was impaired due to apoptosis and secondary necrosis, and the proliferation was also inhibited. In addition, this descending tendency was amplified within the lower pH between 7.4 and 6.5. Compared with ADSCs, NPSCs demonstrated less inhibition of viability and proliferation, which showed better performance to adapt to the acidic pH in IVD (Li et al., 2012). Another study also claimed that the acidic microenvironment of the degenerated IVD can induce BMSCs apoptosis by activating Ca^{2+}-permeable ASIC1a (Cai et al., 2019). Additional modifiers might be used to enhance the function of progenitor cells located in the stem cell niche. A study demonstrated that the diuretic amiloride could block acid-sensing ion channels of cells and might help NPSCs to survive in an acidic microenvironment (Han et al., 2014).

4.3 Mechanical stimulation and osmotic conditions

Mechanical stimulation is a natural constituent of IVD, numerous studies have demonstrated the critical roles of mechanical loadings in regulating the fate of progenitor cells, including compression, shear, torsion, flexion and hydrostatic pressure (Fernando et al., 2011; Tsai et al., 2014). The intradiscal pressure of the healthy IVDs between L4 and L5 is about 0.1–0.24 MPa in the supine position. When the back is flexed with a loading of 20 kg, the intradiscal pressure can increase to 2.0 MPa (Liang et al., 2018). Moreover, the complex mechanical loading makes microenvironmental osmotic condition change. In daily life activities, the IVD osmolarity varies from 430 mOsm/L to 496 mOsm/L. Their osmolarity changes by around 25% during each diurnal cycle. The main component to regulate the IVD osmotic pressure is negatively charged sulphated GAGs linked with aggrecan, which can intake the water into the IVD tissue. The IVD osmotic conditions is fluctuating all the time corresponding to many factors such as tissue hydration, mobile ions transportation and disc loading (Lyu et al., 2019). IVD osmolarity has different impacts on the stem cells owing to its variation. Zhang et al. demonstrated that hyperosmolarity inhibited ADSCs proliferation and viability, promoted the ADSCs differentiate to NP-like cells, activated Foxa1/2-Shh signaling and increased the expression of KDM4B and (Zhang et al., 2020c).

External mechanical stimuli also influence the migration, inflammatory response, proliferation and ECM synthesis of stem cells, which depend on the loading level and mode. Our study demonstrated that AFSCs presented different behaviors when subject to cyclic tensile strain with the magnitudes of 2, 5, and 12%. The catabolic and inflammatory gene increased at high magnitude of cyclic tensile strain with 12% by activating Caveolin-1 mediated NF-κB and integrin β1 signaling pathways. However, cell proliferation, migration and anabolism were found to increase after exposure to moderate cyclic tensile strain with 5% magnitude (Zhang W. et al., 2021). Hence, AFSCs seem to be affected positively and negatively by mechanical stimulation, and physiological mechanical loading might play a therapeutic role in AF regeneration (Figure 3).

4.4 Proteinase and cytokines

The ECM is biochemical and structural support to the IVD stem cells. There are collagen type I, collagen type II, hyaluronic acid (HA) and other components in the ECM. ECM can retain

water, arrange collagen tissue and provide viscoelastic properties (Penolazzi et al., 2020). The IVD degeneration is related to excessive matrix catabolism. Many cytokines and endogenous proteases increased during the degeneration progress, creating another complicated microenvironment for stem cell transplantation. The interaction between matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) is biologically important for the remodeling of IVD tissue, which could impact many aspects of the cells, such as viability, proliferation, differentiation and apoptosis (Hingert et al., 2020). Therefore, maintaining the ECM metabolism of natural tissues is very important. A mechanotransducer-targeted drug that mediates ECM stiffness to maintain the phenotype of IVD progenitor cells can prevent IVD degeneration. A study reported that the upregulation of TIMPs could restore the balance between catabolic and anabolic environments, suggesting TIMPs might be a possible therapeutic target for promoting the IVD regeneration (Le Maitre et al., 2004).

Apart from the effects of MMPs and TIMPs on stem cells, interleukins and other pro-inflammatory cytokines in IVD also regulated the behavior of stem cells. First, Interleukins (IL-1α, IL-1β) and TNF-α, prostaglandin E2 (PGE2), chemokines, and IFNγ played an important role in inducing the cell apoptosis, autophagy, and senescence to upregulate the synthesis of ADAMTS (-1, -4, -5, -9, and -15) and MMPs (-1, -3, -7, -9, and -13), thus leading to ECM breakdown and the development of discogenic low back pain (Jacobsen et al., 2021; Kim et al., 2021). Furthermore, TNF-α has been demonstrated to inhibit the differentiation of adipocytes, as well as the adipogenesis of stem cells. Besides, TNF-α plays a critical role in ECM remodeling by reducing the expression of YAP/TAZ and consequently impairing the chondrocytogenesis of IVD progenitor cells (Ekram et al., 2021).

Conversely, many cytokines also indicate a more favorable outcome to treatment. It was shown that many interleukins exhibit chemotactic features, which might play an important role in stem cell recruitment into degenerated IVD (Zhao et al., 2020). Besides, IVD progenitor cells have been demonstrated to secrete cytokines and proteinase that support NP cell functionality and viability under stress. For instance, insulin-like growth factor 1 (IGF-1) and TGF-β have been described to reduce ECM degradation and inflammation of IVD tissue, while bone morphogenetic protein 7 (BMP-7) and IGF-1 protected NP cells against apoptosis (Yang et al., 2015). Moreover, many interleukins significantly upregulated the immunosuppressive ability of stem cells. It was shown that IL-6 regulated the stemness of stem cells and maintained the undifferentiated state and the proliferation of MSCs through ERK signaling pathway (Marimuthu and Pushpa Rani, 2021). Because of the complicated molecular network in the degenerative disc disease, the synergistic and antagonistic effects of proteinase and cytokines on stem cells required further investigation.

5 Role of IVD graft substitutes in stem cell microenvironment

An ideal graft for IVD regeneration should provide prolonged and instant mechanical properties, biochemical cues and adequate space for seeded stem cells to differentiate, proliferation, and produce ECM (Wei et al., 2021; Luo et al., 2022). Illustrating the interactive mechanisms between the components such as seeded stem cells, environmental factors, ECM components and biological factors in the IVD microenvironment makes therapeutic strategies more rational. In addition, the hostile degenerated IVD microenvironment will be considered when developing IVD substitutes. The IVD graft substitutes should mimic the native IVD tissue with good mechanical strength to withstand the multidirectional and complex mechanical stimulations. Considering the pathological changes of degenerative disc disease, they should also mimic the anisotropic biochemical property, viscoelasticity and topography of native IVD tissue, meeting biological and mechanical compatibilities are crucial for the longevity and efficacy of the IVD repair and regeneration.

5.1 Elasticity

Substrate elasticity and stiffness have regulatory in stem cell adhesion, migration, proliferation and differentiation, thus become an important designing factor in IVD tissue engineering. NPSCs cultured in a stiff synthetic hydrogel matrix showed reduced chondrogenic gene expression and increased osteogenic gene expression (See et al., 2012). In addition, the elastic modulus of the scaffolds was found to affect stem cell fate and direct their lineage specification significantly. Recently, in order to mimic the gradient stiffness close to that of native AF tissue, we fabricated a series of electrospun poly (ether carbonate urethane) urea membranes and evaluated the behavior of AFSCs cultured on it. The expression of phenotypic marker genes of inner AF zone decreased with the substrate stiffness, while the expression of phenotypic marker genes of outer AF zone showed an opposite trend (Zhu et al., 2016). Similarly, Wan et al. fabricated a biphasic substrate to structurally and elastically mimic the AF, the outer phase of the scaffold is demineralized bone matrix gelation (BMG), while the inner part of the scaffold is poly (polycaprolactone triol malate) (PPCLM). The biphasic structure attempt to recapitulate the distribution and mechanical properties of native AF, thus offers enhanced tensile stress and compressive strength than uniphasic scaffold, making it a promising candidate for AF repair and regeneration (Wan et al., 2008).

NP replacement has been explored in various stages of clinical development for the treatment of degenerative disc disease. A variety of synthetic polymeric materials and natural materials such as collagen, chitosan, agarose, and alginate have been studied for creating a successful engineered NP tissue

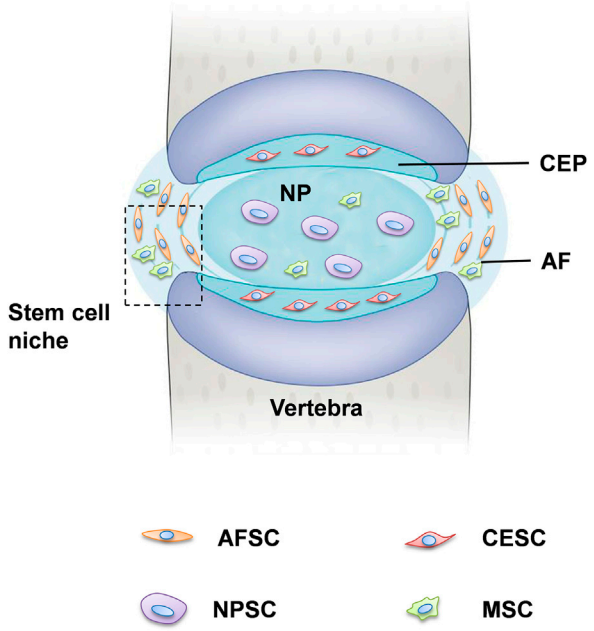

FIGURE 1
Tissue-specific progenitor cells and the location of stem cell niche. The IVD is the fibrocartilaginous part of a three three-component construct that consists of the nucleus pulposus (NP) in the center and annulus fibrosus (AF) in the surrounding, both of which are connected by cartilaginous endplates (CE). Progenitor cells have been found in all three constructs, respectively, referred to NPSCs, AFSCs, and CESCs. The perichondrium region adjacent to the epiphyseal plate and the AF border to the ligament zone has been suggested to be the IVD stem cell niche.

replacement, and the equilibrium Young modulus (E_y) of unconfined human NP was ~5 kPa, and a percent relaxation of ~65% (Yao et al., 2006). Lin et al. used photocross-linked alginate to encapsulate bovine NP cells, which promote the proliferation and maintain the viability of the seeded cells *in vitro* and *in vivo* (Lin et al., 2016).

After all, physical cues such as elasticity and stiffness, originated from cellular microenvironment, can direct stem cell differentiation and influence the physiological function of cultured stem cells. These findings will facilitate IVD bioengineering with mechanical functions approximate to the native IVD tissue for IVD regeneration.

5.2 Viscoelasticity

Besides the commonly known elasticity, a number of studies have demonstrated that viscoelasticity of matrix markedly affects cellular physiological functions, such as differentiation, proliferation and cell spreading (Lang et al., 2021; Jia et al., 2022). When cultured in hydrogel (17 kpa) with fast relaxation rate, MSCs preferentially differentiate toward osteoblast and produce rich mineralized collagen type I (Yue et al., 2019). In order to recapitulate the fibrillar architecture and viscoelasticity of the ECM, Lou et al. develop an interpenetrating network hydrogel system by using the crosslink of hyaluronic acid with collagen through dynamic covalent. They demonstrated that the faster relaxation of the interpenetrating network hydrogel system could improve the fiber remodeling, cell spreading, and hyaluronic acid formation of stem cells embedded in this system (Lou et al., 2018).

Degenerative disc disease is associated with alternations in the biochemical composition of the IVD tissue, such as increased collagen deposition, decreased proteoglycan concentration, and decreased water content, which would have great influence on mechanical function of IVD in shear and compression. Such changes may also affect the dynamic viscoelasticity of IVD tissue and thus alter the ability of IVD to dissipate energy under physiologic loading (Lee and Teo, 2004). In addition, IVD cells within tissues use their contractile machinery to probe and surveil the local microenvironment in both healthy and diseased states. By utilizing a puncture model in rabbit AF, Bonnevie et al. showed that the loss of residual strain in ruptured AF could lead to aberrant cellular changes to fibrotic phenotype and promote the short-term apoptosis of AF cells (Bonnevie et al., 2019). In addition, Eberhardsteiner et al. suggested that viscoelasticity of the AF ECM function is essential in resistance to bulk tissue failure by stress distribution between fibers (Eberhardsteiner et al., 2014). Therefore, viscoelasticity changes initiated the activation of aberrant mechanotransductive events with a loss of prestrain that targets cellular contractility and ultimately leads to disc degeneration.

5.3 Topography

Besides stiffness or viscoelasticity of the ECM, topographic factors including shape, size, and geometric arrangement are also considered critical biophysical cue that influences cell behaviors (Johnson et al., 2006; Peng et al., 2021). Bhattacharjee et al. fabricated scaffolds with fibers alignment that mimics the fibrous orientation of AF by using silk fibroin, the fibers offer the chondrogenic re-differentiation support and the cell/ECM alignment (Bhattacharjee and Ahearne, 2020). At both micro- and nano-scale, behaviors of stem cells can be significantly influenced by the size of topographical features. In a study using aligned nanofibrous scaffolds which resembled nonlinear dependence of modulus native AF. Research have demonstrated that the scaffolds guided the alignment of AF cells and formed ECM with considerable alignment (Nerurkar et al., 2011).

In order to engineer the structural anisotropy and key length scales of AF, aligned fibrous scaffolds with different fiber sizes are currently preferred for AF regeneration (Figure 4). Our group recently found that the fiber size of the grafts significantly

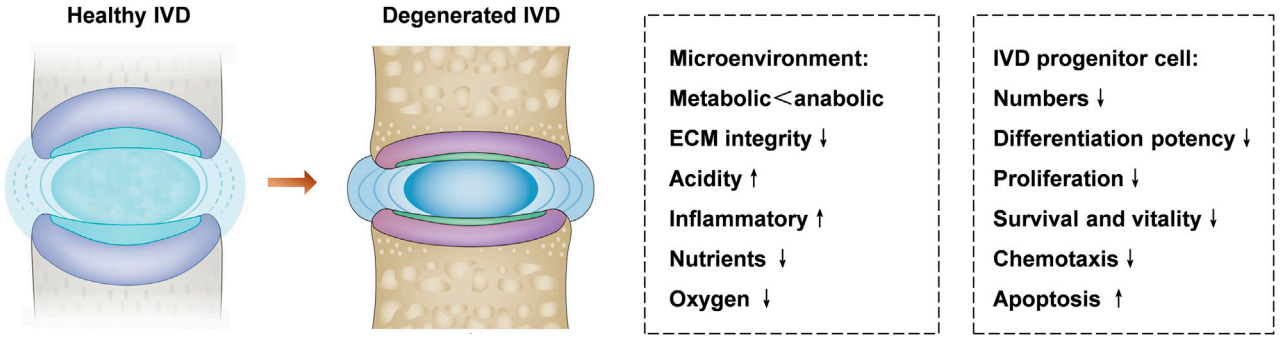

FIGURE 2
IVD microenvironment changes and IVD progenitor cells in healthy and degenerated IVD. The microenvironment of the degenerated IVD is characterized by metabolic disorders, acidic, inflammatory, poor nutrient supply, and low oxygen level, which offer a hostile microenvironment for IVD progenitor cells. Degenerative disc disease can lead to decreased differentiation potency, survival, chemotaxis, and proliferation of the IVD progenitor cells, the hostile microenvironment in the IVD might be unfavorable to the activity of progenitor cell.

affected the morphology and differentiation of stem cells cultured on it, AFSCs were able to differentiate into cell phenotypes similar to AF cells in various zones. Moreover, we found that microstructure features and mechanical property of firbous scaffolds exert a combine effect on AFSC differentiation, possibly through a Yes-associated protein (YAP) dependent mechanotransduction mechanism. (Chu G. et al., 2019; Chu et al., 2021). Above all, the topographical and geometric arrangement of graft could regulate physiological behavior of stem cells such as differentiation, matrix synthesis and apoptosis.

5.4 Chemical composition

In addition to providing topographic support and mechanical stimulus for cells, IVD grafts could also be loaded with bioactive agents (Guan et al., 2021; Chen and Jiang, 2022). In order to achieve antioxidant properties in NP, Cheng et al. fabricated an injectable thermosensitive hydrogel scaffold incorporated with ferulic acid as a controlled release system. After loading with the ferulic acid, the hydrogel scaffolds achieved excellent antioxidant properties (Cheng et al., 2013). Vadala et al. fabricated a bioactive electrospun PLLA scaffold with TGF-β1 for the repair and regeneration of damaged AF, the PLLA/TGF scaffold promoted cell proliferation as well as glycosaminoglycan and collagen production. The slow-releasing profile of TGF-β1 from the scaffold exhibited a sustained delivery that allows envisaging an application for AF repair and tissue engineering strategies (Vadala et al., 2012). Conventional technologies have constructed amounts of scaffolds with single bioactive factor, which have limitations in replicating the composition of native tissues or organs. Bao et al. reported an anti-bacterial PCL scaffolds incorporation of antimicrobial agents for AF tissue engineering, the combining scaffolds showed a sustained strong anti-bacterial and anti-fungal activity after 1 month (Bao et al., 2013). In a study using silk hydrogels recombined with human IGF and BMP-2 could induce the stem cells to differentiate into chondrogenic and osteogenic phenotypes. The silk hydrogels are finally used for the regeneration of EP cartilage-to-bone interface (Wang et al., 2009). Guillaume et al. developed a covalently cross-linked alginate hydrogel combined with continuous delivery of active compounds as FGF-2 and TGF-β3, the combined porous scaffolds exhibit successful ECM deposition to promote the regeneration of AF defects (Guillaume et al., 2014). Above all, substrates combined with bioactive factors attract increasing attention for IVD regeneration.

6 Clinical applications and future perspectives

There have been promising results in clinical trials for stem cell treatment of LBP. Therapies that have been verified helpful in preclinical trials are in different phases of being attempted in clinical trials to demonstrate their safety and efficacy (Urits et al., 2019; Binch et al., 2021). In a case of EuroDISC study, IVD cells were introduced to the discs after discectomy. No adverse events were found in clinical trials, and relief of pain was noted in the injection group compared to discectomy alone on a 2-years follow-up. Furthermore, patients who underwent discectomy and autologous disc chondrocyte transplantation were proved to have less adjacent segment disease than those who underwent discectomy alone (Yoshikawa et al., 2010). In another clinical trial, autologous MSCs were injected into the disc of 13 patients followed for 1 year as part of a pilot study. Results have shown the technique to be feasible and safe, while there was no disc height change in these patients, some benefits with pain relief occurred in most patients, and improved disc hydration was noted in 10/

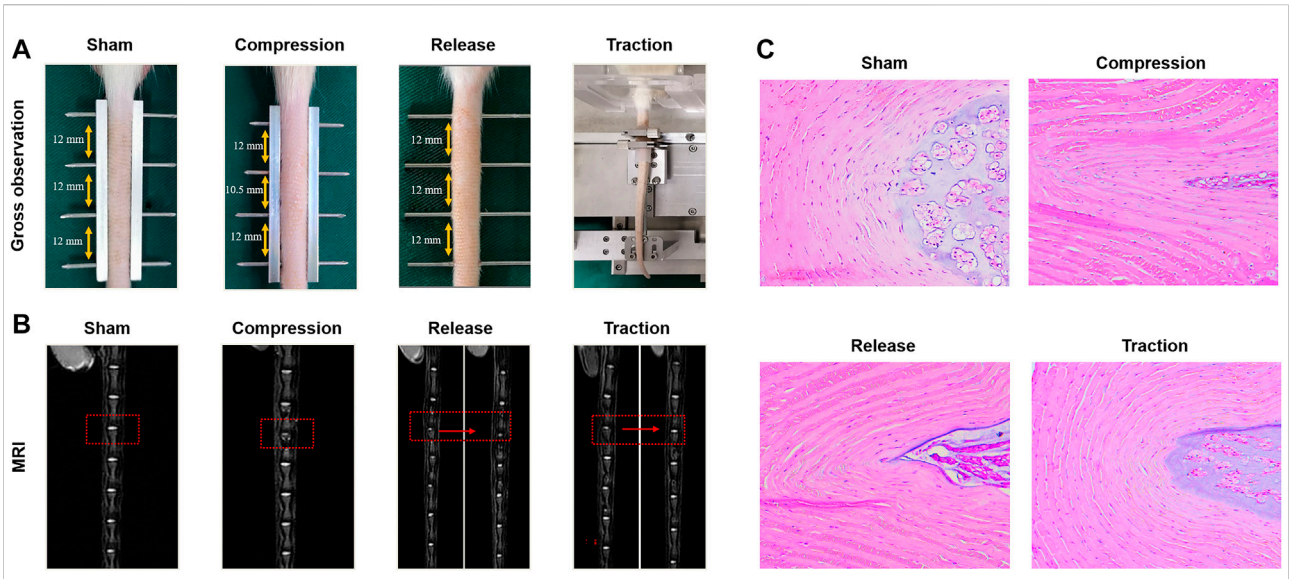

FIGURE 3
Effects of different modes of mechanical loading on the influence of IVD *in vivo*. **(A)** Gross observation of rat tail IVDs in the sham group, compression group, release group and traction group. **(B)** Magnetic resonance images (MRI) of IVDs from the groups as described above. **(C)** H&E staining of IVDs from the groups as described above. Reproduced with permission from Zhang et al. (Zhang W. et al., 2021).

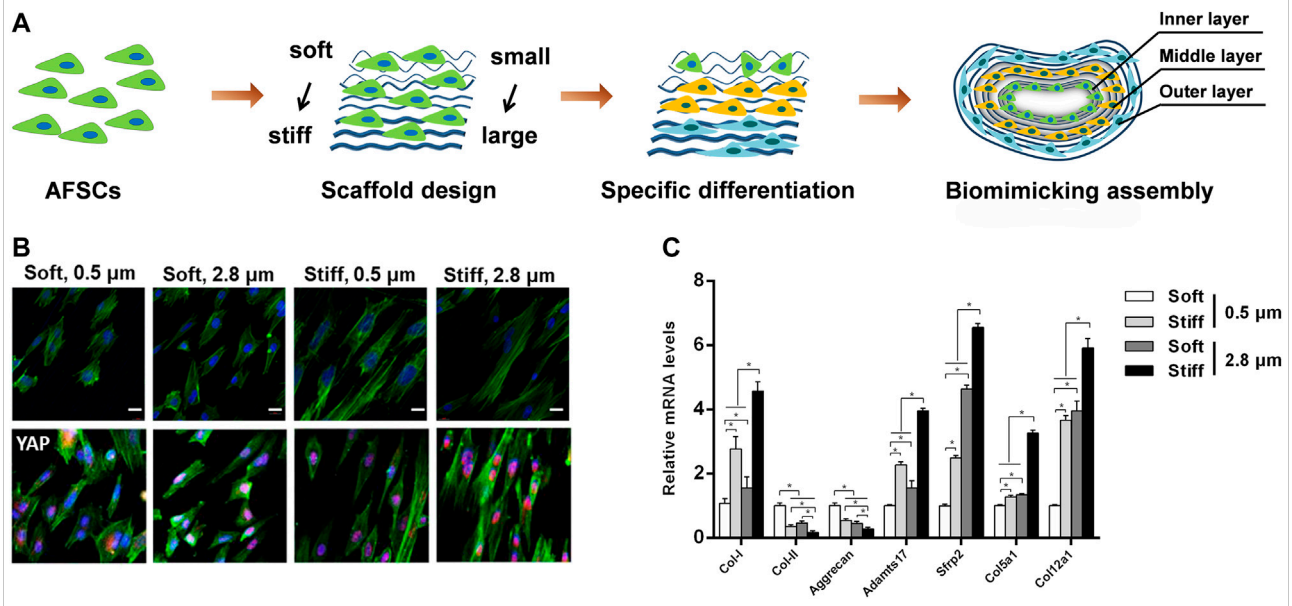

FIGURE 4
Combined effects of substrate topography and stiffness on the influence of AFSC differentiation. **(A)** Assembly of electrospun fibrous membranes with different fiber size and stiffness led to specifically differentiated AFSCs that mimic the hierarchical stratified structure of native AF tissue. Reproduced with permission from Zhou et al. (Zhou et al., 2021). **(B)** The morphology and YAP expression of AFSCs on scaffolds. **(C)** Expression of AF phenotypic marker genes cultured on electrospun fibrous membranes scaffolds for 7 days. Reproduced with permission from Chu et al. (Chu G. et al., 2019).

13 on MRI at 6 months (Orozco et al., 2011). Another study by Henriksson et al. traced the MSC injected into the discs of patients who subsequently undergoing spinal fusion surgery, the investigators were able to detect the labeled MSCs and found that the MSCs had the tendency of differentiating into chondrocyte-like cells (Henriksson et al., 2012). These measurements proved to compare favorably to disc replacement and spinal fusion while offering the benefit of being less invasive (Table 2). However, despite the encouraging clinical trials outcomes, there are many difficulties that remain prior to the cell-based therapies in clinical applications. The biggest obstacle was the harsh microenvironment such as hypoxia, nutrition deprivation, acidic conditions, excessive cyclic tension, high osmolarity, and inflammatory factors during the disc degeneration. The hostile microenvironment that may negatively impact cell-based and biological therapy in the progress of disc regeneration (Kong et al., 2020). According to the results of the clinical and experimental trials, stem cells could be effective to mildly degenerative disc, but not be sufficient to support the view that the stem cells will regenerate the advanced degenerative IVD (Du et al., 2021). In NP cells, HIF is stable in an oxygen-independent fashion, which enables NP cells to survive the harsh microenvironment. Future studies of cell-based therapy should focus on identifying the correct properties of microenvironment to adapt to the harsh environment that is present (Guerrero et al., 2021). Furthermore, the EP role as the nutrition transport regulator for disc regeneration and degeneration. The potential cross-talk between the cartilaginous EP and transplanted MSCs may open a new dimension to investigate the regenerative mechanisms.

7 Concluding remarks

Over the past decade, understanding the potential stem cell-based clinical application for IVD regeneration has dramatically improved. These advances have led to the development of new candidate therapies and, in some cases, clinical trials. So far, the use of stem cells for IVD repair and degeneration is still at the stage of pre-clinical and Phase 1 stage, mainly due to the low survival rate post-transplantation, the complicated microenvironment cells interact with and the detail functions for IVD regeneration are not fully understood. Among the harsh microenvironment posed to biological therapy for the degenerative disc disease are the chronic inflammation, reduced PH, excessive mechanical loading, and metabolic disorders. To overcome these challenges, it is necessary to figure out the influence of the microenvironment on the stem cell for IVD regeneration. Newly designed IVD graft substitutes may be able to simultaneously regulate cellular physiological function, and the controlled release of bioactive factors and therapeutic drugs while providing proper structural support, antibiosis, anti-inflammation. However, most strategies are still in the experimental stage, the relief of the symptoms do not mean that degeneration has stopped, as IVDs remain in a hostile microenvironment. Every therapeutic strategy has a deficiency. Future studies should pay more attention to making full use of biomaterials-mediated delivery of biomechanics or mechanical stimulation to stimulate stem cell differentiation and survival effectively. Therefore, an in-depth understanding of the microenvironment during degeneration and regeneration of IVD will help find a new way to restore the homeostatic microenvironment of IVD and ultimately achieve effective treatment for degenerative disc disease.

Author contributions

GC and BL contributed to the conception and design of this review article. GC, WZ and FH drafted the manuscript. YL and FH performed searches, analyses, and interpretations. KL, CL, QW and HW searched for some manuscripts and figures and contributed to the final version of the manuscript. YL, FH and BL substantially revised the paper. BL gave final approval of the version to be submitted.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Targeting regulation of stem cell exosomes: Exploring novel strategies for aseptic loosening of joint prosthesis

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Periprosthetic osteolysis is a major long-term complication of total joint replacement. A series of biological reactions caused by the interaction of wear particles at the prosthesis bone interface and surrounding bone tissue cells after artificial joint replacement are vital reasons for aseptic loosening. Disorder of bone metabolism and aseptic inflammation induced by wear particles are involved in the occurrence and development of aseptic loosening of the prosthesis. Promoting osteogenesis and angiogenesis and mediating osteoclasts and inflammation may be beneficial in preventing the aseptic loosening of the prosthesis. Current research about the prevention and treatment of aseptic loosening of the prosthesis focuses on drug, gene, and stem cell therapy and has not yet achieved satisfactory clinical efficacy or has not been used in clinical practice. Exosomes are a kind of typical extracellular vehicle. In recent years, stem cell exosomes (Exos) have been widely used to regulate bone metabolism, block inflammation, and have broad application prospects in tissue repair and cell therapy.

KEYWORDS

stem cell exosomes, joint replacement, aseptic loosening, bone metabolism, anti-inflammatory

Introduction

Arthroplasty is currently the treatment of choice for terminal osteoarticular diseases and is the mainstay of treating joint diseases, relieving joint pain, and reconstructing joint function (Rachner et al., 2011; Beckmann et al., 2021; Szczesiul and Bielecki, 2021). Among them, revision surgery accounts for a significant proportion of joint replacements, and one of the significant reasons lies in aseptic loosening caused by particulate wear debris around the prosthesis (Rachner et al., 2011; Hampton et al., 2020; Hodges et al., 2021). Searching for exosome and aseptic loosening in PubMed, only three results could be obtained. Therefore, bone metabolism, angiogenesis, and aseptic loosening were searched, and 481 and nine results were obtained. The articles and reviewers with

high credibility in recent years were selected from 481 results, and the contents were sorted out to obtain this article. Aseptic loosening mainly involves macrophages, osteoblasts, and osteoclasts. Macrophages release a series of pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-23. After recognizing and phagocytosing wear particles, dysregulation of the receptor activator of nuclear factor κB-receptor activator of nuclear factor κB (NF-κB) ligand-osteoclastogenesis inhibitory factor (RANK-RANKL-OPG) axis is caused. For example, TNF-α and IL-1 stimulate osteoblasts to express RANKL; IL-23 promotes the differentiation of $CD4^+$ T cells into the T helper cell 17 (Th17) phenotype, and IL-17 secreted by Th17 cells is a potent inducer of RANKL expression (Lubberts et al., 2003; Lin et al., 2017b). The RANK–RANKL–OPG signaling pathway is a vital pathway regulating osteoclast formation, activation, and survival (Boyce and Xing, 2007). RANK is expressed by osteoclast precursor cells and mature osteoclasts. Activation of RANK promotes the RANK-mediated NF-κB signaling pathway, which in turn encourages osteoclastogenesis and activates osteoclasts (Rachner et al., 2011; Nagy and Penninger, 2015). RANKL is a ligand for RANK that activates RANK on the surface of osteoclast precursor cells, causing a series of activations such as tumor necrosis factor receptor–associated factor 6 (TRAF6), mitogen-activated protein kinases (MAPKs), and transcription factors NF-κB and activator protein-1 (AP-1), which in turn promote the differentiation, activation, and survival of osteoclasts (Boyce and Xing, 2007; Altaf and Revell, 2013; Park et al., 2017). OPG, a decoy receptor that binds to RANKL and inhibits RANK–RANKL interaction, is expressed by vascular endothelial cells and fibroblasts in periprosthetic tissues and inhibits osteoclast activation (Crotti et al., 2004; Koreny et al., 2006). In aseptic loosening of the prosthesis, the expression of OPG is downregulated, and the RANKL/OPG ratio is activated, indicating enhanced osteolysis at the time of OPG decompensation (Hartmann et al., 2017). In summary, regulating bone metabolism and inhibiting inflammation after macrophages phagocytose wear particles are significant entry points for the prevention and treatment of aseptic loosening of prostheses.

Exosomes are small endogenous vesicles with a diameter of about 40–160 nm secreted by cells and contain proteins, lipids, metabolites, and nucleic acids (mRNA, non-coding RNA, and DNA). Exosomes have been reported to play a critical role in removing excess or unnecessary intracellular components and regulating intercellular communication (Kalluri and LeBleu, 2020). In recent years, stem cell exosomes have played a vital role in the treatment of osteoarticular diseases, including bone marrow–derived mesenchymal stem cells (BMSCs), adipose-derived stem cells (ADSCs), umbilical cord blood–derived mesenchymal stem cells (UCB-MSCs), and urine-derived stem cells (USCs). BMSCs are the earliest primary source of pluripotent stem cells, and their culture time is relatively short (Berebichez-Fridman et al., 2017; Berebichez-Fridman and Montero-Olvera, 2018). However, their cell yield, lifespan, and differentiation potential decrease with donor age (Kern et al., 2006; Cagliani et al., 2017; Berebichez-Fridman and Montero-Olvera, 2018). ADSCs are stem cells derived from adipose tissue. Subcutaneous adipose tissue is found throughout the body, and 98–100 percent of cells derived from adipose tissue are viable (Liras, 2010; Choudhery et al., 2014). Studies have indicated that age influences the expansion and differentiation of ADSCs, especially in osteogenic and cartilaginous lineages (Choudhery et al., 2014). UCB-MSCs are derived from the umbilical cord and are considered the most primitive cells among MSCs of various tissue origins, with easily accessible and non-invasive properties (Yang et al., 2020a). UCB-MSCs secrete more wound healing factors (such as the extracellular matrix–degrading enzymes, matrix metalloproteinase-2 and urokinase-type plasminogen activator) than other MSCs (Doi et al., 2016; Kim et al., 2017) and promote fibroblast migration, proliferation, and collagen synthesis (Luo et al., 2010). USCs are derived from fresh human urine and have the advantages of non-invasiveness, easy access, sustainable production, and the relative absence of ethical issues (Li et al., 2020). USCs also have the ability for solid proliferation, lipogenesis, endothelial differentiation, and vascularization compared with BMSCs (Wu et al., 2018).

Recent studies have demonstrated that stem cell exosomes play a crucial and essential role in the process of bone metabolism and anti-inflammation (Qin et al., 2016; Li et al., 2018; Shi et al., 2019; Yang et al., 2020a). Different stem cell exosomes can play substantial roles in enhancing osteogenesis, suppressing osteoclast activity, augmenting angiogenesis, and resisting inflammation (Tofiño-Vian et al., 2017; Shi et al., 2019). Therefore, the application of stem cell exosomes is theoretically promising as an effective intervention to prevent and treat the aseptic loosening of prostheses.

Enhance osteogenesis and suppress osteoclast activity

Bone formation and bone resorption are central components of bone metabolism in the ternary regulation theory of bone metabolism (Marie and Kassem, 2011). Bone formation is the primary process of bone development. When bone formation is more significant than bone absorption, bone develops. When bone mass reaches its peak, bone formation and absorption are in a dynamic equilibrium stage (Buettmann et al., 2019; Zhang et al., 2020). Osteoclasts are more active than osteoblasts in a pathological state, and bone resorption exceeds bone formation. Unbalanced bone resorption and bone formation eventually result in bone loss (Wu et al., 2010). While in other pathological states, with excessive osteoblasts, unbalanced bone formation and resorption also lead to

excessive bone formation rather than bone loss (Gao et al., 2018). Osteoblasts are equipped with the potential to differentiate into osteocytes (Marie and Kassem, 2011; Pajarinen et al., 2017). Osteoclasts gather around apoptotic bone cells to further recruit osteoclasts. Interestingly, communication between osteoblasts and osteoclasts occurs through EVs (Yuan et al., 2018). Wear debris triggers bone resorption by activating macrophages and osteoclasts and directly impairs bone formation by attenuating osteoblast function (Pajarinen et al., 2017). Studies have focused on periprosthetic osteolysis on osteoclasts, macrophages, and fibroblasts. Once worn debris is exposed *in vitro*, these cells release pro-inflammatory cytokines that may activate osteoclasts through multiple pathways, leading to bone loss (Kusano et al., 1998; Lader and Flanagan, 1998). In addition, osteoblasts secrete cytokines to recruit inflammatory cells into the periprosthetic space and stimulate bone resorption by osteoclasts (Vermes et al., 2001). Therefore, silencing osteoclast-mediated osteolysis around the prosthesis is of great significance for preventing aseptic loosening of the prosthesis.

Various stem cell exosomes block osteoclast activation or directly differentiate into osteoblasts to regulate bone remodeling. BMSC-Exos activates osteogenesis and downregulates osteoclastogenesis through multiple pathways. BMSC-Exos transplantation plays a key role in the treatment of osteoporosis by promoting osteogenesis, which is attributed to the activation of bone morphogenetic protein-2-drosophila mothers against decapentaplegic protein1runt-related transcription factor-2 (BMP-2/Smad1/RUNX2) and hypoxia-inducible factor-1-vascular endothelial growth factor (HIF-1α/VEGF) signaling pathways (Zhang et al., 2020). BMSC-Exos contribute to bone healing during fracture healing by carrying miR-126 and alleviate radiation-induced bone loss by activating the Wnt/β-catenin pathway (Lu et al., 2020). In aged BMSCs, the expression level of miR-31a-5p was higher, which leads to adipogenesis and cell senescence and attenuates cell osteogenesis (Xu et al., 2018). In addition, exosomes secreted by pre-differentiated human mesenchymal stem cells (hMSCs) for a certain period induce osteogenic differentiation, including upregulating osteogenic miRNA (Hsa-miR-146a-5p, Hsa-miR-503-5p, Hsa-miR-483-3p, and Hsa-miR-129-5p) and downregulating anti-osteogenic miRNA (Hsa-miR-32-5p, Hsa-miR-133a-3p, and Hsa-miR-204-5p) to activate phosphoinositol-3-kinase-protein kinase B (PI3K/Akt) and MAPK signaling pathways. hMSC exosomes are used as inducers to induce osteogenic differentiation of hMSCs *in vitro* (Zhai et al., 2020). Pathologically, BMSC-Exos extracted from patients with osteoporosis attenuate osteogenesis by downregulating SMAD7 *via* miR-21 (Jiang, Tian, Zhang). In summary, BMSC-Exos have obvious bone-promoting and bone-suppressing effects under physiological conditions. The treatment centered on BMSC-Exos is expected to become a strategy for clinical prevention of aseptic loosening of the prosthesis.

Adipose-derived, stem-cell-derived exosomes (ADSC-Exos) have a decent osteogenic effect. It was found that the overexpression of miR-130a-3p, the exosome of ADSCs, could enhance osteogenic differentiation of ADSCs and reduce the protein and mRNA levels of silent information regulator 7 (SIRT7), the target of miR-130a-3p. Overexpression of miR-130a-3p resulted in downregulation of SIRT7 and upregulation of Wnt signaling pathway–related proteins, suggesting that exosome miR-130a-3p upregulates osteogenic differentiation of ADSCs by partially mediating the SIRT7/Wnt/β-catenin axis (Yang et al., 2020b). ADSC-Exos decreased RANKL expression at mRNA and protein levels and decreased RANKL/OPG ratio at the gene level. ADSC-Exos antagonized hypoxia and serum deprivation–induced osteocyte apoptosis and osteoclastogenesis (Ren et al., 2019). Transplantation of UCB-MSCs derived from human umbilical cord blood or its conditioned medium prevents bone loss in ovariectomized nude mice, drastically enhances the survival rate of bone-like MLO-Y4 cells, and mediates osteoclast differentiation. *In vitro*, the conditional medium (CM) of UCB-MSCs activates alkaline phosphatase (ALP) in human BMSCs and the mRNA expression of type 1 collagen, RUNX2, osterix, and ALP in C3H10T1/2 cells, indicating that it had apparent osteogenic activity (An et al., 2013), which was mediated by a paracrine mechanism.

USCs also have more vigorous proliferation and stronger abiogenesis, endothelial differentiation, and vascularization than BMSCs (Wu et al., 2018). Autologous USC-Exos are promising osteoporosis therapeutic agents that enrich osteogenesis and block osteoclastogenesis by transporting silent information regulator 7 (CTHRC1) and OPG (Chen et al., 2019). In addition, our previous research results show that USC-Exos effectively cause osteogenic differentiation and generation and attenuate osteoclast differentiation.

In summary, wear debris downregulates bone formation by activating macrophages and osteoclasts to trigger bone resorption or silencing osteoblast function. At the same time, a variety of stem cell exosomes play an essential regulatory role in promoting osteogenesis and blocking osteoclasts. Therefore, targeted regulation of stem cell exosomes is expected to play a preventive and therapeutic role in the process of aseptic loosening of the prosthesis (Figure 1) (Table 1).

Augment angiogenesis

Angiogenesis is the process of generating new blood vessels from the original blood vessels (Olsen et al., 2017). In 2014, nature reported a new capillary subtype in the murine skeletal system with distinct morphological, molecular, and functional properties (Kusumbe et al., 2014). These vessels are found in specific locations, mediate growth of the bone vasculature, generate distinct metabolic and molecular microenvironments, maintain perivascular osteoprogenitors, and couple angiogenesis

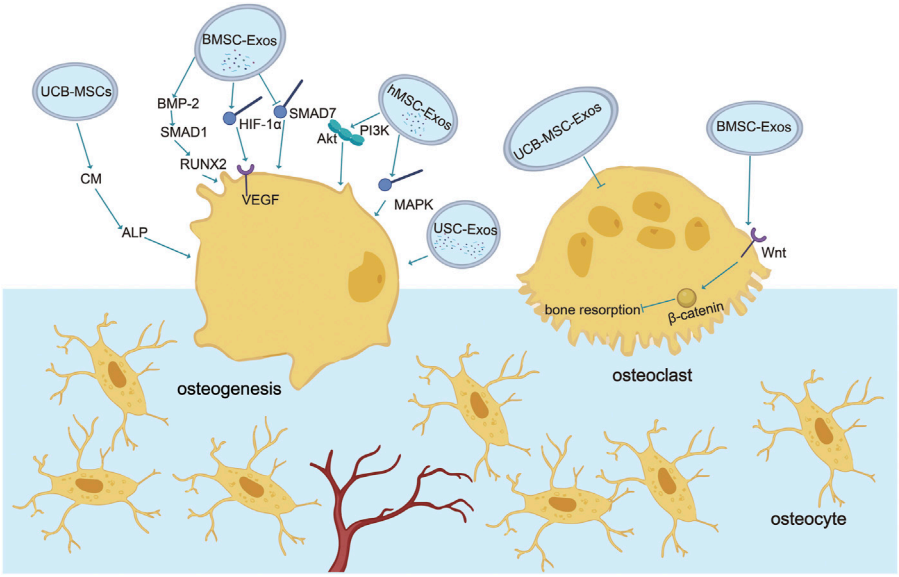

FIGURE 1
Role of exosomes in osteogenesis and osteoclasts. Various stem cell exosomes block osteoclast activation or directly differentiate into osteoblasts to regulate bone remodeling. Communication between osteoblasts and osteoclasts occurs through EVs. BMSC-Exos activate osteogenesis through BMP-2-SMAD1-RUNX2, HIF-1α-VEGF, and SMAD7 pathways and block osteoclastogenesis through the classic Wnt-β-catenin pathway. UCB-Exos activate ALP in human BMSC and the mRNA expression of type 1 collagen, RUNX2, osterix, and ALP in C3H10T1/2 cells. USC-Exos enrich osteogenesis and block osteoclastogenesis by transporting CTHRC1 and OPG. hMSC-Exos promote osteogenesis by PI3K/Akt and MAPK.

TABLE 1 Main exosomes in bone metabolism.

Exosome species	Key target	Reference
BMSC-Exos	BMP-2/Smad1/RUNX	Zhang et al. (2020)
	HIF-1α/VEGF	
	miR-126-Wnt/β-catenin	Lu et al. (2020)
	miR-31a-5p	Xu et al. (2018)
	miR-2148	(Jiang, Tian, Zhang)
	ALP, type 1 collagen, RUNX2, and osterix	An et al. (2013)
hMSC-Exos	Hsa-miR-146a-5p, Hsa-miR-503-5p, Hsa-miR-483-3p, and Hsa-miR-129-5p	Zhai et al. (2020)
	Hsa-miR-32-5p, Hsa-miR-133a-3p, and Hsa-miR-204-5p	
	PI3K/Akt	
	MAPK	
ADSC-Exos	miR-130a-3p	Yang et al. (2020b)
	SIRT7/Wnt/β-catenin	
	RANKL	Ren et al. (2019)
USC-Exos	CTHRC1 and OPG	Chen et al. (2019)

to osteogenesis (Kusumbe et al., 2014). Vessels not only mediate the circulation of cells, oxygen, nutrients, and waste, especially the wear particles of the prosthesis, but also provide vascular secretion signals that control organ growth and homeostasis (Red-Horse et al., 2007; Butler et al., 2010; Tashiro et al., 2012). Local blood supply or angiogenesis plays a vital role in bone metabolism (Wang et al., 2017) and forms a network with surrounding bone tissue to further regulate bone metabolism. During bone development, homeostasis, and repair, dense vascular systems provide oxygen and nutrients to highly

anabolic bone cells (Wang et al., 2007; Hu and Olsen, 2016). New blood vessels provide sources of circulating factors, such as parathyroid hormone and vitamin D, which are essential for the stability of the bone environment (Hankenson et al., 2011).

After joint replacement, no wear debris around the prosthesis attenuates osteoblast function, impairs bone formation, and blocks angiogenesis (Pajarinen et al., 2017). MAO-650 is a coating of microporous TiO_2 decorated with hydroxyapatite (HA) nanoparticles. MAO-650 supports the proliferation and differentiation of osteoblasts and endothelial cells, mediates macrophage inflammatory response, and triggers favorable bone immune regulation to function as a positive regulator of bone/vascular formation and prevent aseptic loosening of prosthesis (Bai et al., 2018). Therefore, reducing the inhibitory effect of wear debris around prosthesis on angiogenesis may be an effective means for clinical prevention of aseptic loosening.

MicroRNAs in stem cell exosomes play a crucial role in angiogenesis spinal cord injury (SCI) mouse MSC loading miR-126 into exosomes. Exosomes derived from miR-126-modified MSCs contribute to human umbilical vein endothelial cell (HUVEC)–related angiogenesis and neurogenesis and attenuate apoptosis by mediating the expression of Sprouty-related EVH1 domain protein 1 (SPRED1) and phosphoinositide 3 (Huang et al., 2020). The level of miR-29a in BMSC-Exos derived from bone marrow mesenchymal stem cells is high, which is transported to HUVECs to restore angiogenesis sensitivity. Angio-inhibitory protein 1 (VASH1) was identified as a direct target of miR-29a, mediating miR-29a in BMSC-Exos to activate angiogenesis (Lu et al., 2020). Human ADSCs contribute to angiogenesis by activating the PKA signaling pathway and promoting VEGF expression. This result is used to find safe and effective treatments for traumatic diseases (Xue et al., 2018). UCB-MSC-derived exosomes reduce cisplatin-induced renal oxidative stress and apoptosis *in vivo*, increase the proliferation of cultured renal epithelial cells, promote angiogenesis, and regenerate damaged kidneys (Dorronsoro and Robbins, 2013; Xue et al., 2018). In addition, exosomes derived from UCB-MSCs contribute to injury repair. UCB-MSC-derived exosomes are encapsulated in new nanogels and injected into the sheath of the spinal cord model. The number, volume fraction, and connectivity of blood vessels in the spinal cord are dramatically raised, which regulates diabetic wounds (Zhang, Zhang, Gao, Chang, Chen, Mei, et al.). Over-metastasis of malignant brain tumor 1 (DMBT1) protein in USC-Exos causes angiogenesis, providing a new prospect for diabetic soft tissue wound healing (Chen et al., 2018). Umbilical cord mesenchymal stem cell–derived exosomes combined with Pluronic F127 hydrogel enhance granulation tissue regeneration and upregulate VEGF and transform growth factor-β1 (TGF-β1) to trigger wound healing and complete skin regeneration in chronic diabetes mellitus (Yang et al., 2020c).

In summary, stem cell exosomes play various vital roles in the aseptic loosening of the prosthesis, including hematopoietic stem cells supporting perivascular niches and repairing and regenerating damaged bone, cartilage, and vascular tissue (Pajarinen et al., 2017; Saribas et al., 2020). Promoting angiogenesis is beneficial for providing more nutrients, metabolizing, transporting worn particles, and maintaining bone metabolism homeostasis (Red-Horse et al., 2007; Butler et al., 2010; Tashiro et al., 2012). Therefore, the use of stem cell exosomes to enhance angiogenesis is conducive to preventing the aseptic loosening of the prosthesis (Figure 2).

Regulate immune cells and cytokines

Wear particle–induced aseptic inflammation is the leading cause of aseptic loosening of prostheses after joint replacement, so inhibition of inflammation may be a viable clinical alternative for preventing aseptic loosening of prostheses (Rachner et al., 2011; Hodges et al., 2021). Several studies have revealed that stem cell exosomes have the ability to resist inflammation mainly by regulating immune cells (macrophages, T cells, and B cells) and the cytokines they secrete.

For macrophages, stem cell exosomes play a role in anti-inflammation by inducing M2 macrophage polarization. During wound healing, after macrophages took up BMSC-Exos, exosomes induced M2 macrophage polarization through miR-223, resulting in higher IL-10 levels and decreased TNF-α levels, as shown by accelerated wound healing (He et al., 2019). In the bronchopulmonary dysplasia (BPD) model, after uptake of BMSC-Exos by alveolar macrophages, the expression levels of pro-inflammatory factors secreted by M1 macrophages such as TNF-α, IL-6, and CCL5 were blocked, and the expression levels of anti-inflammatory factors secreted by M2 macrophages such as arginase-1 (Arg-1) were increased, that is, macrophages transformed from M1 to M2, and the process occurred in a dose-dependent manner (Willis et al., 2018). In a cutaneous wound model in streptozotocin-induced diabetic rats, the induction of LPS-preconditioned umbilical cord stem-cell-derived exosomes (LPS pre-UCMSC-Exos) of M1 macrophages was sharply reduced. In contrast, the density and distribution of M2 macrophages were significantly increased. THP-1 cells produced more anti-inflammation cytokines (IL-10 and TGF-β) and M2 macrophage surface marker CD163 and fewer pro-inflammatory cytokines (IL-1, IL-6, and TNF-α). Taken together, LPS pre-UCMSC-Exos facilitated the differentiation of macrophages to M2, but not M1[70]. When peripheral blood mononuclear cells (PBMCs) were cocultured with ADSC-Exos, the mRNA expression levels of M2 macrophage markers (CD163 and Arg1) in PBMCs and the percentage of CD206 (a specific M2 macrophage marker)-positive cells were significantly increased. Moreover, M2 macrophage-specific transcription factors signal

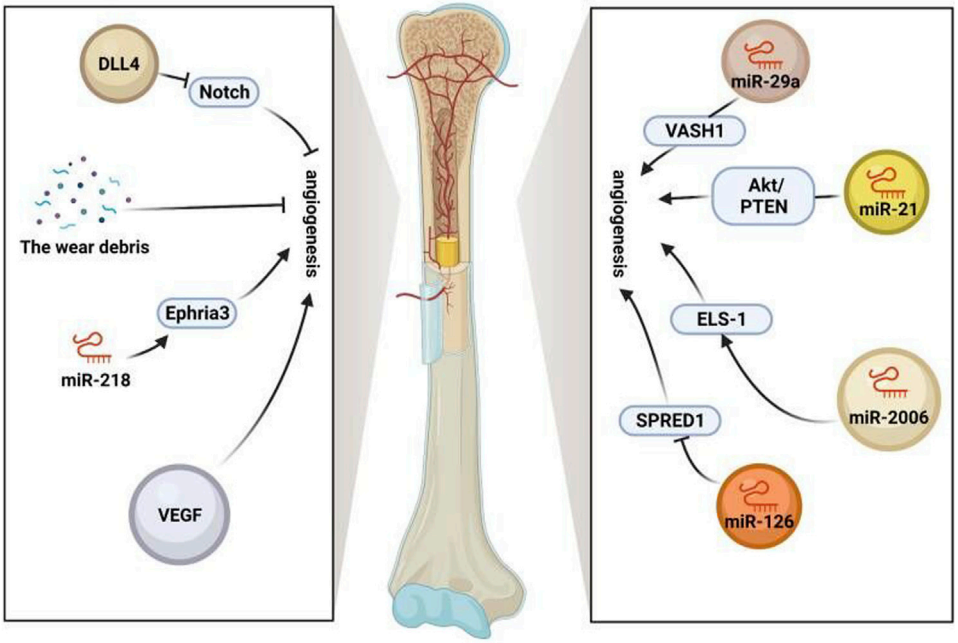

FIGURE 2
Role of exosomes in vessels. Stem cell exosomes play various vital roles in the aseptic loosening of prosthesis, including hematopoietic stem cells supporting perivascular niches and repairing and regenerating damaged bone, cartilage, and vascular tissue. Angiogenesis is promoted by VEGF, miR-218-Ephria3, miR-29a-VASH1, miR-21-PTEN/Akt, and miR-2006-ELS-1 while inhibited by the wear debris, miR-126-SPRED1, and notch (https://app.biorender.com/).

transducer and activator of transcription 6 (Stat6) and MAF BZIP transcription factor B (MafB) were activated considerably, indicating that ADSC-Exos induce the M2 phenotype of PBMCs and play a vital role in anti-inflammation (Heo et al., 2019).

For T cells, stem cell exosomes play a role in anti-inflammation by upregulating the expression of pro-inflammatory or anti-inflammation cytokines, regulating the differentiation of T cells, and inhibiting the proliferation of PBMCs. After treatment of PBMCs with BMSC-Exos, the expression levels of pro-inflammatory cytokines TNF-α and IL-1β decreased, and the expression levels of anti-inflammation cytokines TGF-β increased. The ability of exosome-induced Th1 cells to transform into Th2 cells reduced the differentiation of T cells into Th17 and reduced the production of IL-17. The expression of CTLA-4 in Treg cells emerged, which could inhibit the immune response by competing with CD28 for ligands CD80 and CD86 and then played a role in anti-inflammation (Chen, Huang, Han, Yu, Li, Lu, et al.). Treatment of PBMCs with UCB-MSC-Exos obtained by treatment with TGF-β or IFN-γ or a combination of both (MSCs-T/I) inhibited the proliferation of PBMCs, which became more pronounced with increasing dose. After treatment with MSCs-T/I exosomes, the proportion of PBMCs that transformed into Treg cells increased, and the expression of IL-10, IDO, and other anti-inflammation factors also increased, so the immunosuppressive effect and the anti-inflammation effect were enhanced (Zhang et al., 2018). In T1DM mice treated with ADSC-Exos, the number of Treg cells was significantly increased, and the levels of IL-4, IL-10, TGF-β, and other anti-inflammation factors were improved considerably. In contrast, the levels of IFN-γ, IL-17, and other pro-inflammatory factors were significantly decreased, showing a significant anti-inflammation effect (Nojehdehi et al., 2018).

For B cells, after treatment of PBMCs with BMSC-Exos, the expression levels of CXCL8 (IL-8) and marginal zone B- and B1-cell-specific protein (MZB1) were higher. The increased CXCL8 could inhibit T-cell activation and proliferation through myeloid-derived suppressor cells (MDSCs), and the increased MZB1 could cause significant inhibition of B-cell proliferation by regulating Ca^{2+}. In conclusion, exosomes play a role in anti-inflammation by reducing the number and function of immune cells (Khare et al., 2018).

To sum up, the effect of stem cell exosomes is mainly achieved by regulation of immune cells and cytokines, and the effect of exosomes may be used as an essential means to inhibit particle-induced aseptic inflammation. Therefore, applying stem cell exosomes is a new idea to prevent the aseptic loosening of prostheses in the future (Figure 3) (Table 2).

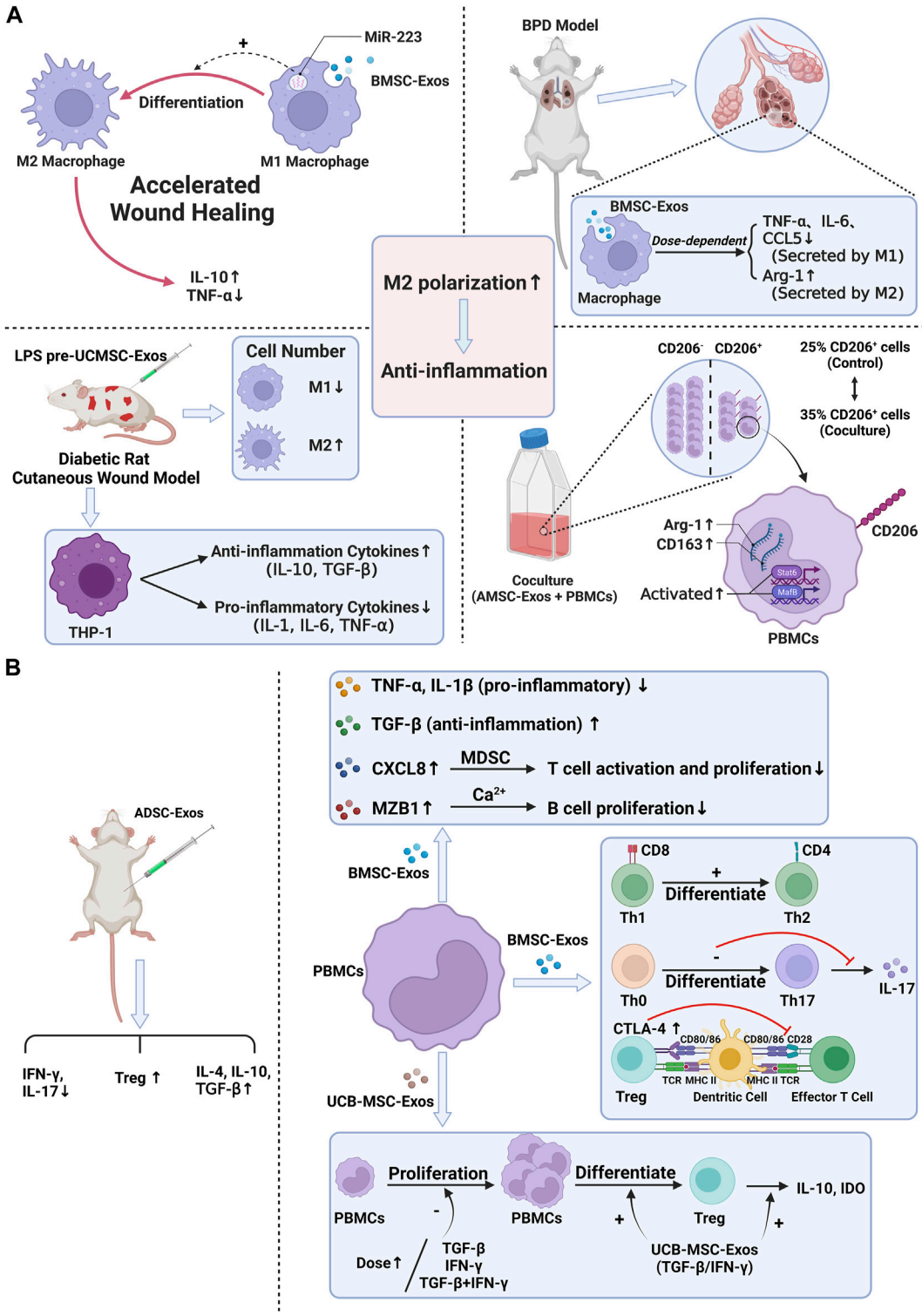

FIGURE 3
Anti-inflammation ability of stem cell exosomes. Panel **(A)**: Stem cell exosomes play anti-inflammation roles by inducing M2 macrophage polarization. BMSC-Exos induced M2 macrophage polarization through miR-223, resulting in higher IL-10 levels and lower TNF-α levels. After uptake of BMSC-Exos, the pro-inflammatory factors decreased, and the anti-inflammation factors increased. Under the induction of LPS pre-UCMSC-Exos, the M1 macrophages were decreased, while the M2 macrophages were increased. THP-1 produced more anti-inflammation cytokines and fewer pro-inflammatory cytokines. When PBMCs were cocultured with AdMSC-Exos, CD163, Arg1, and CD206-positive cells were increased. M2 macrophage–specific transcription factors were activated. Panel **(B)**: Stem cell exosomes play anti-inflammation roles by regulating the
(*Continued*)

FIGURE 3
expression of cytokines, inhibiting the proliferation of PBMC, and reducing the number and function of immune cells. For T cells, after BMSC-Exos treatment, the pro-inflammatory cytokines decreased, and the anti-inflammation cytokines increased. Moreover, exosomes induced the differentiation of T cells and the higher expression of CTLA-4. UCB-MSC-Exos inhibited PBMC proliferation. More PBMCs transformed into Treg cells, and IL-10 and IDO were increased. After ADSC-Exos treatment, the number of Treg cells and the levels of IL-4, IL-10, and TGF-β were increased, while IFN-γ and IL-17 decreased. For B cells, BMSC-Exos treatment induced higher levels of CXCL8 and MZB1, which, respectively, inhibited T-cell activation and proliferation and inhibited B-cell proliferation.

TABLE 2 Key variants in the anti-inflammatory pathway.

Type of immune cell	Type of exosome	Upregulated factor	Downregulated factor	Reference
Macrophages	BMSC-Exos	IL-10 and Arg-1	TNF-α	Willis et al. (2018) and He et al. (2019)
	LPS pre-UCMSC-Exos	IL-10, TGF-β, and CD163	IL-1, IL-6, and TNF-α	Ti et al. (2015)
	PBMCs cocultured with AdMSC-Exos	CD163, Arg1, CD206, Stat6, and MafB		Heo et al. (2019)
T cells	PBMCs with BMSC-Exos	CTLA-4	TNF-α, IL-1β, and IL-17	(Chen, Huang, Han, Yu, Li, Lu, et al.)
	PBMCs with UCB-MSC-Exos		PBMC	Nojehdehi et al. (2018)
	PBMC with MSC-Exos	IL-10 and IDO		Nojehdehi et al. (2018)
	ADMSC-Exos	Number of Treg cells, IL-4, IL-10, and TGF-β	IFN-γ and IL-17	Nojehdehi et al. (2018)
B cells	PBMCs with BMSC-Exos	IL-8 and MZB1		Khare et al. (2018)

Comparison of treatments for aseptic loosening

In the face of aseptic loosening of prostheses, most of the current clinical treatments for aseptic loosening are in the experimental stage, and most of the current treatment strategies focus on the use of new materials or the adjustment of prosthesis components. Revision arthroplasty is often the ultimate measure of severe loosening. At present, the methods that have been put into research on aseptic loosening include drug therapy, gene therapy, and cell therapy.

Some drugs have been shown to prevent osteolysis, such as drugs that suppress osteoclast activity (e.g., bisphosphonates); drugs that promote osteogenesis, such as BMP; and drugs that act on inflammatory signaling pathways or cytokines, such as TNF-α antagonists. However, these drugs cannot be put into clinical use. On the one hand, these drugs are still in preclinical trials, and on the other hand, some drugs (drugs acting on cytokines) may have unknown adverse effects on other systems of the body (Smith and Schwarz, 2014). The use of drugs for the treatment of aseptic loosening still needs further clinical trials and studies.

Gene therapy is a treatment that has emerged in recent years. Ulrich-Vinther et al. (2002) investigated the use of a recombinant adeno-associated viral (RAAV) vector expressing OPG for gene therapy to construct a RAAV vector co-expressing OPG (RAAV-OPG-IRES-EGFP) and then found that OPG can effectively inhibit wear particle–induced osteoclastogenesis and osteolysis. However, this method is also in the experimental stage, and gene regulation, vector selection, and other aspects also need to be further improved.

Local therapeutic cell delivery can directly or indirectly affect osteolysis. Autologous bone grafting is a form of local cell therapy in which osteoblasts and other cells in the bone graft complex can be implanted into the bone graft to regulate the inflammatory cascade and provide autocrine and paracrine factors to support bone healing. Some researchers have used methods of local delivery of MSCs to modulate the inflammatory response and promote osteogenic differentiation and bone healing, which may be used as a potential treatment in the future (Lin et al., 2017a; Lin et al., 2019).

In contrast to the previously mentioned methods, the use of exosomes for the treatment of aseptic loosening has many advantages. Exosomes are smaller, which makes them quickly circulate *in vivo* and reach the injured site (Mendt et al., 2018). Derived from cells, exosomes are safer and lower in immunogenicity. Good membrane-bound characteristics make the contents have good biocompatibility and stability, and it is easier to cross the blood–brain barrier (Akbari and Rezaie, 2020). As a non-cellular product, exosome transplantation does not undergo rejection and harmful differentiation and malignant

transformation that may occur when MSCs are transplanted (Harrell et al., 2019). Unique materials combined with stem cell exosomes have broad prospects for the treatment of aseptic loosening of the prosthesis. Korda et al. (2008) confirmed that the combination of autologous mesenchymal stem cells and allogenic bone enhanced the integration of femoral prosthesis in a sheep hemiarthroplasty model. Compared with the allograft alone treatment group, the graft healing rate in the MSC treatment group was increased, the graft absorption decreased, and the failure rate decreased (Hernigou et al., 2014). Vulcano et al. (2013) reported similar results for the reconstruction of bone defects around the acetabulum after aseptic loosening in five unrelated patients. A case report by Jäger et al. (2006) described the treatment of periacetabular osteolysis with BMP2/MSC composites. Progressive healing was reported with satisfactory results (Jäger et al., 2006). In summary, the use of exosomes for the treatment of aseptic loosening is a promising approach.

Conclusion and foresight

In recent years, total joint replacement (TJR) has been the most cost-effective and successful surgical intervention for end-stage osteoarticular disease. However, the operation of total joint replacement has been dramatically increased and modified by international organizations, the main reason behind which is the periprosthetic osteolysis and aseptic loosening caused by TJR (Kurtz et al., 2005; Kurtz et al., 2007). Implant wear and subsequent biomaterial wear particles released into the surrounding tissue are the leading causes of periprosthetic osteolysis (Schmalzried et al., 1992). These wear particles disperse through the articular fluid along the bone–implant interface (Revell, 2008). In the tissue surrounding the prosthesis, wear debris is consumed by macrophages, activating inflammatory phenotype, secreting cytokines and chemokines (Nich et al., 2013; Pajarinen et al., 2014), and recruiting more macrophages (Lin et al., 2017b). Exosomes have tremendous therapeutic potential in related bone diseases, such as aseptic loosening of prostheses. Exogenous stem cell exosomes enhance bone binding and alleviate peri-implant osteolysis through paracrine regulation. Osteoblasts promote osteogenic mRNAs in exosomes, mediate anti-osteogenic miRNAs, and upregulate bone growth through Wnt/MAPK/PI3K-Akt pathways (Yang et al., 2020b; Zhai et al., 2020). Bone resorption is activated by RANK, tartrate-resistant acid phosphatase (TRAP), and OPG in exosomes (Inder et al., 2014; Raimondi et al., 2015). Vascular growth is closely related to bone regeneration. Although the wear debris of prosthesis blocks vascular growth, a large number of MSC exosomes contain the inclusions that stimulate vascular regeneration except for human umbilical vein endothelial cells (Zhao et al., 2020). Over-transfer of DMBT1 protein functions as a positive regulator of angiogenesis and wound healing of diabetic soft tissue. Thus, its operation in aseptic loosening of prosthesis needs further study (Chen et al., 2018). Enhancing osteogenesis and angiogenesis and suppressing osteoclast is a new idea to solve the aseptic loosening of prostheses. In summary, different stem cell exosomes play an important role in promoting osteogenesis, angiogenesis, and silencing osteoclasts and macrophage-mediated inflammation, providing a new idea for the clinical prevention and treatment of aseptic loosening of the prosthesis.

In the face of aseptic loosening of prostheses, clinical attention should be paid to prevention. Most of the current treatment strategies focus on the use of new materials or the adjustment of prosthesis components. Unique materials combined with stem cell exosomes have broad prospects for the treatment of aseptic loosening of prostheses. Korda et al. (2008) confirmed that the combination of autologous mesenchymal stem cells and allogeneic bone enhanced the integration of femoral prosthesis in the sheep hemiarthroplasty model. Compared with the allograft alone treatment group, the graft healing rate in the MSC treatment group was increased, the graft absorption decreased, and the failure rate decreased (Hernigou et al., 2014). Vulcano et al. (2013) reported similar results for the reconstruction of bone defects around the acetabulum after aseptic loosening in five unrelated patients. A case report by Jäger et al. (2006) described the treatment of periacetabular osteolysis with BMP2/MSC composites. Progressive healing was reported with satisfactory results. Although it has been less effective in alleviating the problems caused by TJR with materials, the effects of prosthesis on osteoclastogenesis and angiogenesis in patients are completely avoided. In recent years, stem cell exosomes have been widely used to regulate bone metabolism and inhibit inflammation by promoting osteogenesis and angiogenesis, having broad application prospects in tissue repair and injury prevention. Focusing on the regulation of stem cell exosomes combined with targeted drug therapy will provide more possibilities for patients to adapt to prostheses. Stem cell exosomes offer an effective treatment for bone metabolic diseases such as osteoporosis (Pajarinen et al., 2017). Exosomes from MSCs may be a promising alternative therapy based on cells (Saribas et al., 2020). Exosomes are replicated, so there is no risk of tumor formation. In addition, exosomes are much smaller than stem cells, which quickly circulate *in vivo* and reach the injured site (Mendt et al., 2018). At the same time, considering that most studies on exosomes derived from MSCs are currently in the preclinical stage, the traditional methods of exosome isolation and characterization are not effective for clinical application. The exact mechanism of MSC-derived exosomes in osteogenesis, osteoclast differentiation, angiogenesis, and inflammation remains unclear and needs further study.

Most of the current clinical treatments for aseptic loosening are in the experimental stage, and most of the current treatment strategies focus on the use of new materials or the adjustment of prosthesis components (Jäger et al., 2006; Korda et al., 2008; Vulcano et al., 2013). At present, the methods that have been put into research on aseptic loosening include drug therapy, gene therapy, and cell therapy (Hernigou et al., 2014). Some drugs have been shown to prevent osteolysis, but these drugs cannot be put into clinical use because they have not passed the clinical trial and will have other adverse effects (Jäger et al., 2006). Gene therapy has emerged in recent years. However, this method is also in the experimental stage, and gene regulation, vector selection, and other aspects also need to be further improved. Cell therapy like local delivery of MSC may serve as a future treatment for aseptic loosening (Mendt et al., 2018; Saribas et al., 2020). In contrast to the previous methods, exosomes have the merits of smaller size, greater safety, lower immunogenicity, better membrane-bound characteristics, and so on. In addition, unique materials combined with stem cell exosomes have broad prospects for the treatment of aseptic loosening of the prosthesis, which makes them a better method for treating aseptic loosening. In summary, the use of exosomes for the treatment of aseptic loosening is a promising approach.

Author contributions

T-LM and J-XC wrote the original draft. T-LM, J-XC, Z-RK, and PZ wrote and edited the review. J-XC and Z-RK prepared the figures. Y-HH and JX edited the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Glossary

AdMSC-Exos Adipose-derived exosomes

ADSC-Exos Adipose-derived stem cell-derived exosomes

ALP Alkaline phosphatase

Akt Protein kinase b

AP-1 Activator protein-1

ADSC Adipose-derived stem cells

BMP Bone morphogenetic protein

BMSC Bone marrow-derived mesenchymal stem cell

BPD Bronchopulmonary dysplasia

Arg-1 Arginase-1

CM Conditional medium

CTHRC1 Silent information regulator 7

DMBT1 Malignant brain tumor 1

Exos Exosomes

HA Hydroxyapatite

HUVEC Human umbilical vein endothelial cell

hAD-MSC Human adipose-derived MSC

HIF-1α Hypoxia-inducible factor-1

UCB-MSC Human umbilical cord mesenchymal stem cellUmbilical cord blood-derived mesenchymal stem cell

IL-1 Interleukin-LPS pre-UCMSC-Exos

MSC Human mesenchymal stem cell

MafB MAF BZIP transcription factor B

MAPKs Mitogen-activated protein kinases

MDSC Myeloid-derived suppressor cell

MZB1 Marginal zone B- and B1-cell-specific protein

OPG Osteoclastogenesis inhibitory factor

PI3K Activate phosphoinositol-3

RAAV Recombinant adeno-associated viral

RANK Receptor activator of nuclear factor κB

RANKL Receptor activator of nuclear factor κB ligand

PBMC Peripheral blood mononuclear cell

RUNX2 Runt-related transcription factor-2

SCI Spinal cord injury

SIRT7 Silent information regulator 7

Smad1 Drosophila mothers against decapentaplegic protein 1

Stat6 Signal transducer and activator of transcription 6

SPRED1 Sprouty-related EVH1 domain protein 1

TNF-α Tumor necrosis factor-α

TGF-β-1 Transform growth factor-β-1

Th17 T helper cell 17

TJR Total joint replacement

TRAF6 Tumor necrosis factor receptor-associated factor 6

TRAP Tartrate-resistant acid phosphatase

UCB-MSC Human umbilical cord mesenchymal stem cellUmbilical cord blood-derived mesenchymal stem cell

USC Urine-derived stem cell

VEGF Vascular endothelial growth factor.

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Effects of *HSD11B1* knockout and overexpression on local cortisol production and differentiation of mesenchymal stem cells

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Exogenous glucocorticoids increase the risk for osteoporosis, but the role of endogenous glucocorticoids remains elusive. Here, we describe the generation and validation of a loss- and a gain-of-function model of the cortisol producing enzyme 11β-HSD1 (*HSD11B1*) to modulate the endogenous glucocorticoid conversion in SCP-1 cells — a model for human mesenchymal stem cells capable of adipogenic and osteogenic differentiation. CRISPR-Cas9 was successfully used to generate a cell line carrying a single base duplication and a 5 bp deletion in exon 5, leading to missense amino acid sequences after codon 146. These inactivating genomic alterations were validated by deep sequencing and by cloning with subsequent capillary sequencing. 11β-HSD1 protein levels were reduced by 70% in the knockout cells and cortisol production was not detectable. Targeted chromosomal integration was used to stably overexpress *HSD11B1*. Compared to wildtype cells, *HSD11B1* overexpression resulted in a 7.9-fold increase in *HSD11B1* mRNA expression, a 5-fold increase in 11β-HSD1 protein expression and 3.3-fold increase in extracellular cortisol levels under adipogenic differentiation. The generated cells were used to address the effects of 11β-HSD1 expression on adipogenic and osteogenic differentiation. Compared to the wildtype, *HSD11B1* overexpression led to a 3.7-fold increase in mRNA expression of lipoprotein lipase *(LPL)* and 2.5-fold increase in lipid production under adipogenic differentiation. Under osteogenic differentiation, *HSD11B1* knockout led to enhanced alkaline phosphatase (ALP) activity and mRNA expression, and *HSD11B1* overexpression resulted in a 4.6-fold and 11.7-fold increase in mRNA expression of Dickkopf-related protein 1 *(DKK1)* and *LPL*, respectively. Here we describe a *HSD11B1* loss- and gain-of-function model in SCP-1 cells at genetic, molecular and functional levels. We used these models to study the effects of endogenous cortisol production on mesenchymal stem cell

differentiation and demonstrate an 11β-HSD1 dependent switch from osteogenic to adipogenic differentiation. These results might help to better understand the role of endogenous cortisol production in osteoporosis on a molecular and cellular level.

KEYWORDS

11β-HSD1, osteoporosis, glucocorticoids, CRISPR-Cas9, targeted chromosomal integration

Introduction

Osteoporosis is characterized by reduced bone mineral density leading to an increased risk of fractures, which results in an elevated morbidity and mortality (Abrahamsen et al., 2015; Ensrud et al., 2019). Every year, osteoporosis accounts for 8.9 million fractures worldwide. In Europe, 32 million people suffer from osteoporosis with women being more affected than men (Kanis et al., 2021).

Therapy with exogenous glucocorticoids is a major risk factor for osteoporosis (van Staa et al., 2002). In contrast, endogenous glucocorticoids play an essential role in bone homeostasis. Whereas systemic levels of cortisol are regulated centrally by the hypothalamic–pituitary–adrenal (HPA) axis, local cortisol levels are regulated by two isoenzymes: 11β-hydroxysteroid dehydrogenase 1 (11β-HSD1) and 2 (11β-HSD2) (Tannin et al., 1991; Agarwal et al., 1994; Albiston et al., 1994; Krozowski et al., 1995; Roland und Funder 1996; Bujalska et al., 1997; Cooper et al., 2000; Whorwood et al., 2002). 11β-HSD2 oxidizes the biologically active cortisol to inactive cortisone. Locally, cortisone can be re-activated to cortisol by 11β-HSD1 (gene name: *HSD11B1*) which is a crucial mechanism for mediation of the anti-inflammatory therapeutic effects of glucocorticoids (Fenton et al., 2021). 11β-HSD1 is most strongly expressed in liver (Tannin et al., 1991). Other relevant tissues include the adipose tissues, skeletal muscle and bone (Bujalska et al., 1997; Cooper et al., 2000; Whorwood et al., 2002). In bone, 11β-HSD1 is the predominant isozyme (Bland et al., 1999; Cooper et al., 2000) and plays an important role in osteoblast differentiation by providing the necessary glucocorticoid stimulus (Eijken et al., 2005). Notably, *HSD11B1* expression in osteoblasts and suppressed cortisol levels in patients evaluated for osteoporosis increase with age (Cooper et al., 2002; Siggelkow et al., 2014).

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiation into osteoblasts, adipocytes or chondrocytes. Osteoblasts are crucial for vital bone remodeling due to their ability to mineralize bone and to regulate osteoclast activity through paracrine signaling (Han et al., 2018). With age, apoptosis of human MSCs increases (Zhou et al., 2008) and differentiation into osteoblasts decreases in favor of differentiation into adipocytes (Moerman et al., 2004; Zhou et al., 2008).

SCP-1 cells are immortalized human MSCs that can be differentiated towards the osteogenic, adipogenic or chondrogenic lineage (Böcker et al., 2008). Previously we showed that transient *HSD11B1* overexpression and pharmacological inhibition of 11β-HSD1 affect osteogenic differentiation in SCP-1 cells by affecting cortisol production (Blaschke et al., 2021).

However, transient transfections and inhibition experiments limit the insight into the coherences of the overexpressed *HSD11B1* and changes in differentiation. Pharmacological inhibition may elicit off-target effects which potentially affect differentiation. With genetic inactivation or constant overexpression of *HSD11B1*, long-term effects of the absence or presence of 11β-HSD1 on differentiation can be monitored. Therefore, stable genetic modifications of MSCs resulting in complete inactivation or constant overexpression of *HSD11B1* would deliver a well-defined cell model leading to reliable and well interpretable results on differentiation capacity.

While the effects of exogenous glucocorticoids on bone homeostasis are well known [for a recent review see (Gado et al., 2022)], the role of endogenous cortisol and especially its local production by 11β-HSD1 need further investigations. Therefore, we here propose an *in vitro* approach to address the critical role of endogenous glucocorticoids on osteogenic and adipogenic differentiation of MSCs. We aimed 1) to use the CRISPR-Cas9 technology and targeted chromosomal integration in SCP-1 cells to generate a *HSD11B1* loss-of-function and gain-of-function model and 2) to use these models for analyzing the role of local cortisol levels on adipogenic and osteogenic differentiation of MSCs. With these models we aim to improve the understanding of the onset of age-related osteoporosis and thereby to identify new potential therapeutic strategies.

Materials and methods

Culturing and differentiation of SCP-1 cells

SCP-1 cells were cultured at 37°C in a humidified atmosphere (95%) with 5% CO_2 in DMEM containing 4.5 g/l glucose, 0.58 g/l L-glutamine, 3.7 g/l NaHCO3 (without pyruvate) and supplemented with 10% fetal bovine serum, 100 U/ml

penicillin and 100 µg/ml streptomycin. Medium and medium supplements were obtained from Thermo Fisher Scientific (Waltham, MA, United States).

For differentiation, 4×10^4 SCP-1 cells per well were plated in 6-well plates. Adipogenic differentiation was induced by supplementing the culture media with 500 µM IBMX, 0.1 mg/ml insulin (Insuman® Rapid), 200 µM indomethacin and 1 µM dexamethasone. For the first four days of adipogenic differentiation, only insulin was added. Osteogenic differentiation was induced by supplementing with 173 µM L-ascorbic acid 2-phosphate, 10 mM β-glycerol phosphate and 50 nM 1α,25-dihydroxy-vitamin D3. All stimulants were obtained from Sigma-Aldrich (St. Louis, MO, United States), except for insulin which was obtained from Sanofi (Paris, France). The differentiation medium was replaced every 3–4 days. For analyses of cortisol production over time and the influence of 11β-HSD1 on adipogenic and osteogenic differentiation, differentiation was performed in 24-well plates with 8×10^3 SCP-1 cells per well. In the course of these experiments, dexamethasone was substituted by 5 µM cortisone (adipogenic differentiation) or was additionally added (osteogenic differentiation).

Generation of *HSD11B1* knockout SCP-1 cells

The Alt-R™ CRISPR-Cas9 System (Integrated DNA technologies, Carolville, IA, United States) with the predesigned crRNAs Hs. Cas9. HSD11B1.1. AB (5′-CTACTA CTATTCTGCAAACG-3′) targeting exon 2 and Hs. Cas9. HSD11B1.1. AA (5′-AGTCAACTTCCTCAGTTACG-3′) targeting exon 5 was chosen to knockout *HSD11B1*. SCP-1 cells were reverse transfected with RNP-complexes in 24-well plates as described before (Schwefel et al., 2018) with the following modifications: the medium was changed to normal culture medium after 24 h. As the cells reached confluence, they were detached and one half of the cell suspension was transferred into a 6-well plate to 2 ml culture medium. The other half was used for DNA isolation. Upon reaching confluence in the 6-well plate, the cells were transferred into T25 flasks. Subsequently, the cells were diluted and transferred into 96-well plates applying the low-density dilution method. As our preliminary analyses showed poor clonal growth, the cells were diluted in the following densities: 0.8 cells/well, 2.5 cells/well and 5.0 cells/well. Both single cell clones and clones arisen from 2–3 cells were further expanded in 12-well plates and T25 flasks and further analyzed.

For DNA isolation, the pelleted cells were resuspended in 50 µl QuickExtract™ DNA Extraction Solution (Epicentre, Middleton, WI, United States) and were heated at 65°C for 10 min, followed by heating at 98°C for 5 min. The isolate was diluted 1:5 with sterile water.

Potential off-target sites were chosen based on their ranking in the IDT and CCTop off-target prediction. For CCTop analyses (CCTop, RRID:SCR_016890), the default settings were used (PAM type: NGG, target site length: 20 bp, core length: 12 bp, max. Total mismatches: 4, max. Core mismatches: 2). Sequences of predicted off-target sites were determined by capillary Sanger sequencing.

Generation of *HSD11B1* overexpressing SCP-1 cells

HSD11B1 was stably overexpressed in SCP-1 cells applying the Flp-In™ System (Invitrogen, Carlsbad, CA, United States). The coding sequence of *HSD11B1* was amplified from the plasmid pcDNA3.1-HSD11B1 and was subsequently cloned into the pcDNA5/FRT Expression Vector (Invitrogen) using EcoRV and HindIII restriction sites. The Rapid DNA Ligation Kit (Thermo Fisher Scientific) was used for ligation.

To generate the Flp-In™ host cell line, 2×10^5 SCP-1 cells were plated and cultured for 24 h. The cells were transfected with 4 µg of the linearized pFRT/*lacZ*eo plasmid using 12 µl FuGENE 6 (Promega, Madison, WI, United States), in a 6-well plate. After 24 h, the culture medium was changed to complete medium. Upon reaching 100% confluence, the cells were transferred into cell culture dishes (100 mm) and after 24 h the medium was replaced by selection medium (complete medium supplemented with 400 µg/ml Zeocin™). When resistant clones became visible, they were transferred into 12-well plates. The medium was changed every 3–4 days. When the cells reached confluence, they were sequentially transferred into a 6-well plate, a T25 flask and finally into a T75 flask. Cells were cultured in selection medium until cryopreservation. Success of transfection was determined by β-galactosidase activity with the β-Gal Assay Kit (Invitrogen, Carlsbad, CA, United States).

Stable transfection of SCP-1/FRT cells with the *HSD11B1* encoding Expression Vector (pcDNA5/FRT:*HSD11B1*) was performed in a 6-well plate as described above with the following modifications: the cells were transfected with 400 ng pcDNA5/FRT:*HSD11B1* and 3.6 µg pOG44 using 12 µl FuGENE 6 (Promega). One well was transfected with 400 ng pcDNA5/FRT:*HSD11B1* and 3.6 µg GFP-tpz and served as a transfection control. As selection antibiotic hygromycin was used with a final concentration of 200 µg/ml. After transfer into 12-well plates, a reduced hygromycin concentration of 100 µg/ml was applied. A reduced hygromycin concentration of 50 µg/ml was used in the 6-well plate, T25 flask and T75 flask.

Validation PCRs

PCRs for the genetic validation of overexpressing cells were performed as described before (Saadatmand et al., 2012).

Additionally, a third PCR was performed to check for multiple integration (Supplementary Table S1). Amplification was performed using the QIAGEN Multiplex PCR Kit (Qiagen, Hilden, Germany) and the following reaction conditions: 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min 30 s, 72°C for 2 min, and finally 72°C for 10 min.

T7 endonuclease I assay

Efficiency of CRISPR-Cas9 mediated gene editing was determined by T7EI (New England Biolabs, Ipswich, MA, United States) digest of annealed PCR amplicons of the regions of interest (Supplementary Table S1). For *CCM3*, the primers for the T7EI assay were kindly provided by S. Spiegler and U. Felbor (Department of Human Genetics, University Medicine Greifswald) (Schwefel et al., 2018). Following gel electrophoresis, the fragment pattern was analyzed and the DNA amount of each fragment was determined by measuring the integrated intensity applying Fiji (Schindelin et al., 2012). The efficiency of CRISPR-Cas9 treatment was validated by estimating the indel occurrence as described before (Ran et al., 2013).

TOPO® TA cloning

Cloning was performed with the amplification products of the T7EI PCR and TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's protocol. The generated TOPO clones were sequenced using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific).

Deep sequencing

For deep sequencing, a Next Generation Sequencing (NGS) approach was used. The region of interest was amplified by PCR (Supplementary Table S1). Purification and sequencing was performed as described before (Römer et al., 2021) with the primers listed in Supplementary Table S1 and the MiSeq Reagent Nano Kit v2 (500-cycles) (Illumina, Inc. San Diego, United States) on the MiSeq System (Illumina) with paired-end reads.

To perform the following three steps we used the software tool VSEARCH version 2.14.2 (Rognes et al., 2016). First, we merged the paired-end reads using default parameters. We then kept only reads with maximum number of expected errors 1 and length of at least 200 bp. Finally, the reads with an identity of 99% in each clone were clustered together. The centroid sequence from each cluster that contained more than 50 reads was aligned against the reference sequence (NG_012081.1) to analyze sequence modifications.

Gene expression analysis

For validation experiments, RNA was isolated with the RNeasy *Plus* Mini Kit (Qiagen). Approximately 1×10^6 cells were pelleted and 350 µl RLT *Plus* Buffer supplemented with 1% β-mercaptoethanol were added. The suspension was either stored at −20°C or directly submitted to the QIAcube robot.

RNA of differentiated SCP-1 cells was isolated with a modified protocol of the single-step method (Chomczynski und Sacchi 1987) using TRIzol™ Reagent (Thermo Fisher Scientific) and chloroform. Per well of a 24-well plate 500 µl TRIzol™ were added and the lysates of two wells were pooled and stored at −20°C. Lysates were thawed on ice and 200 µl chloroform were added and mixed with the lysates by shaking. After a 10-min incubation on ice, the samples were centrifuged at 12,000 *g* and 4°C for 10 min. The aqueous phase was carefully transferred into a fresh 1.5 ml reaction tube. The RNA was precipitated by addition of 500 µl ice-cold isopropanol followed by incubation at −20°C for 10–30 min, depending on the expected yield. By centrifugation at 12,000 *g* for 20 min, the RNA was pelleted. The supernatant was removed and the RNA was washed with 500–800 µl ice-cold 70% ethanol, depending on the pellet size. The centrifugation was repeated and the ethanol was completely removed with a pipette. The pellet was allowed to dry at room temperature until it became transparent. To dissolve the RNA, 25–30 µl sterile water were added followed by incubation at 60°C for 5 min.

Isolated mRNA was reverse transcribed into cDNA applying the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Gene expression was analyzed by RT-qPCR using predesigned TaqMan® Gene Expression Assays (Thermo Fisher Scientific): Hs01060665_g1 (*ACTB*), Hs01029144_m1 (*ALPL*), Hs00183740_m1 (*DKK1*), Hs00194153_m1 (*HSD11B1*), Hs00173425_m1 (*LPL*), Hs99999910_m1 (*TBP*). For graphical representation, the Ct values were converted into relative expression by applying the ΔCt method. The expression was calculated as gene of interest transcripts per 1,000 housekeeping gene transcripts. Gene expression of *HSD11B1* in differentiated SCP-1 cells was normalized to *ACTB* expression, as *ACTB* is the most stably expressed housekeeping gene in differentiated SCP-1 cells.

Protein quantification by Western Blot

SCP-1 cells were either differentiated adipogenically for 14 days or cultured without stimulants. The cells were detached and pelleted by centrifuging at 300 *g* for 3 min. The cell pellets were washed with PBS and subsequently resuspended in 30 µl 5 mM Tris-HCl (pH 7.4) supplemented with 100 µM PMSF, 1 µM leupeptin and 3 µg/ml aprotinin. After five freeze-thaw cycles, the lysate was transferred into a 1.5 ml tube and centrifuged at 100,000 *g* and 4°C for 30 min. The supernatant was

discarded and the pelleted membranes were resuspended in 20 µl 5 mM Tris-HCl supplemented with protease inhibitors (see above). The pelleted membranes were further disrupted by passing through a 27 G needle. The protein amount was quantified using a BCA assay (Thermo Fisher Scientific) and 50 µg protein were mixed with 4x Laemmli Buffer (20% glycerol, 100 mM Tris-HCl pH 6.8, 0.02% Orange G, 6% SDS, 2% DTT) supplemented with 2-mercaptoethanol (1:10). The proteins were gently denatured at 37°C for 30 min. SDS-PAGE was performed with a 12.5% separation gel and a 4% stacking gel. The proteins were transferred to a nitrocellulose membrane (GE Healthcare, Chicago, IL, United States) applying the tank blot procedure on ice using pre-cooled Towbin buffer (Towbin et al., 1979) with increased methanol content (30%) at a constant current of 370 mA. The membranes were blocked in TBST (25 mM Tris, 136 mM NaCl, 3 mM KCl, 0.04% Tween 20) with 10% (v/v) FCS and as both primary antibodies used originate from rabbit, the membrane was cut horizontally to circumvent cross-detection with the secondary antibody. Incubation with anti-HSD11B1 (1:1,000; Abcam Cat# ab157223, RRID:AB_2630342) and anti-Na^+/K^+-ATPase (1:2000; Abcam Cat# ab76020, RRID:AB_1310695) followed at 4°C in a tube rotator for 48 h and overnight, respectively. The secondary antibody was incubated at room temperature on a shaking platform for 1 h (1:20,000; LI-COR Biosciences Cat# 925-68071, RRID:AB_2721181; LI-COR Biosciences Cat# 925-32211, RRID:AB_2651127). All antibodies were diluted in TBST supplemented with 0.05% sodium azide. Anti-HSD11B1 binds at the C-terminus of the protein. The blot was developed with the Odyssey® CLx (LI-COR) by detection of fluorescence at 700 nm (HSD11B1) or 800 nm (Na^+/K^+-ATPase). Signals were quantified with the Image Studio™ software (LI-COR).

Protein quantification by targeted proteomics

Targeted proteomics were performed as described before (Meyer et al., 2020). Here, wildtype, *HSD11B1* knockout and overexpressing SCP-1 cells were differentiated adipogenically for 14 days. For the LC-MS/MS measurement, an injection volume of 20 µl was used. Solvent A (0.1% formic acid in acetonitrile) and Solvent B (0.1% formic acid in water) were mixed applying a gradient over time (Supplementary Table S2). The peptide QEEVYYDSSLWTTLLIR (JPT Peptide Technologies GmbH, Berlin, Germany) was chosen for detection of 11β-HSD1, targeting the C-terminus at 253–269 aa (UniProtKB P28845). For normalization, Na^+/K^+-ATPase protein level was detected with the peptide LSLDELHR (Thermo Fisher Scientific). The MS detection parameters applied are listed in Supplementary Table S3. The measurement was performed in three replicates. The peak areas were automatically determined by the Analyst 1.6.3 software (Sciex, Darmstadt, Germany). Peak areas of 11β-HSD1 were determined for wildtype, knockout and overexpressing cells and normalized to the respective peak area of Na^+/K^+-ATPase, which allows a relative quantitation of 11β-HSD1. For 11β-HSD1 four and for Na^+/K^+-ATPase three mass transitions were analyzed. The relative 11β-HSD1 protein level was calculated as ratio of the mean of the four 11β-HSD1 measurements and the mean of the three Na^+/K^+-ATPase measurements.

Measurement of 11β-HSD1 activity

To functionally validate the knockout and overexpressing SCP-1 cell lines, wildtype, *HSD11B1* knockout and overexpressing cells were adipogenically differentiated for 14 days. On day five and twelve of differentiation, cells were stimulated with 5 µM cortisone. To this end, FCS was substituted by BSA which was diluted in the medium to a final concentration of 0.1%. As an additional control, cell-free medium was incubated. After 48 h, the medium was removed and analyzed.

To quantify extracellular cortisol levels, the medium was prepared by heavily mixing 200 µl with 800 µl 100% acetonitrile supplemented with 50 ng/ml cortisol-d4 and centrifuged at 16,000 *g* for 15 min to pellet the debris. Subsequently, 350 µl of the supernatant were evaporated to dryness under nitrogen flow at 40°C. The dried pellet was resuspended in 200 µl 0.1% formic acid (50% 0.2% formic acid, 50% acetonitrile + methanol (6 + 1)) and centrifuged at 16,000 *g* for 5 min. Fifteen µl were injected into the LC-MS/MS system which consisted of an API 4000 QTRAP® tandem mass spectrometer (AB Sciex Ontario, Canada) with ESI interface coupled to a Shimadzu Nexera X2 UHPLC system with LC 30AD pumps and SIL 30AC autosampler (Shimadzu, Kyoto, Japan) (Supplementary Table S4). Samples were separated using a Brownlee SPP RP-Amide (4.6 × 100 nm, 2.7 µm particle size) column (Perkin Elmer, Waltham, MA, United States) and a flow rate of 500 µl/min. Solvent A (0.1% formic acid in 90% acetonitrile + methanol (6 + 1)) was mixed in equal volumes with solvent B (0.1% formic acid in water).

Staining and quantification of lipid droplets

Cells were fixed with 4% paraformaldehyde. Fixed cells were stored in PBS at 4°C. Lipid droplets were stained with Nile Red, nuclei were stained with DAPI as described before (Rakow et al., 2016; Andrzejewska et al., 2019). The cells were incubated with 400 µl staining solution (1 µg/ml Nile Red and 1 µg/ml DAPI in PBS; both from Sigma-Aldrich, St. Louis, MO, United States) in the dark at room temperature for 15 min. Thereafter, the cells were washed and overlaid with PBS. Fluorescence and background for Nile Red and DAPI were detected at 538 nm

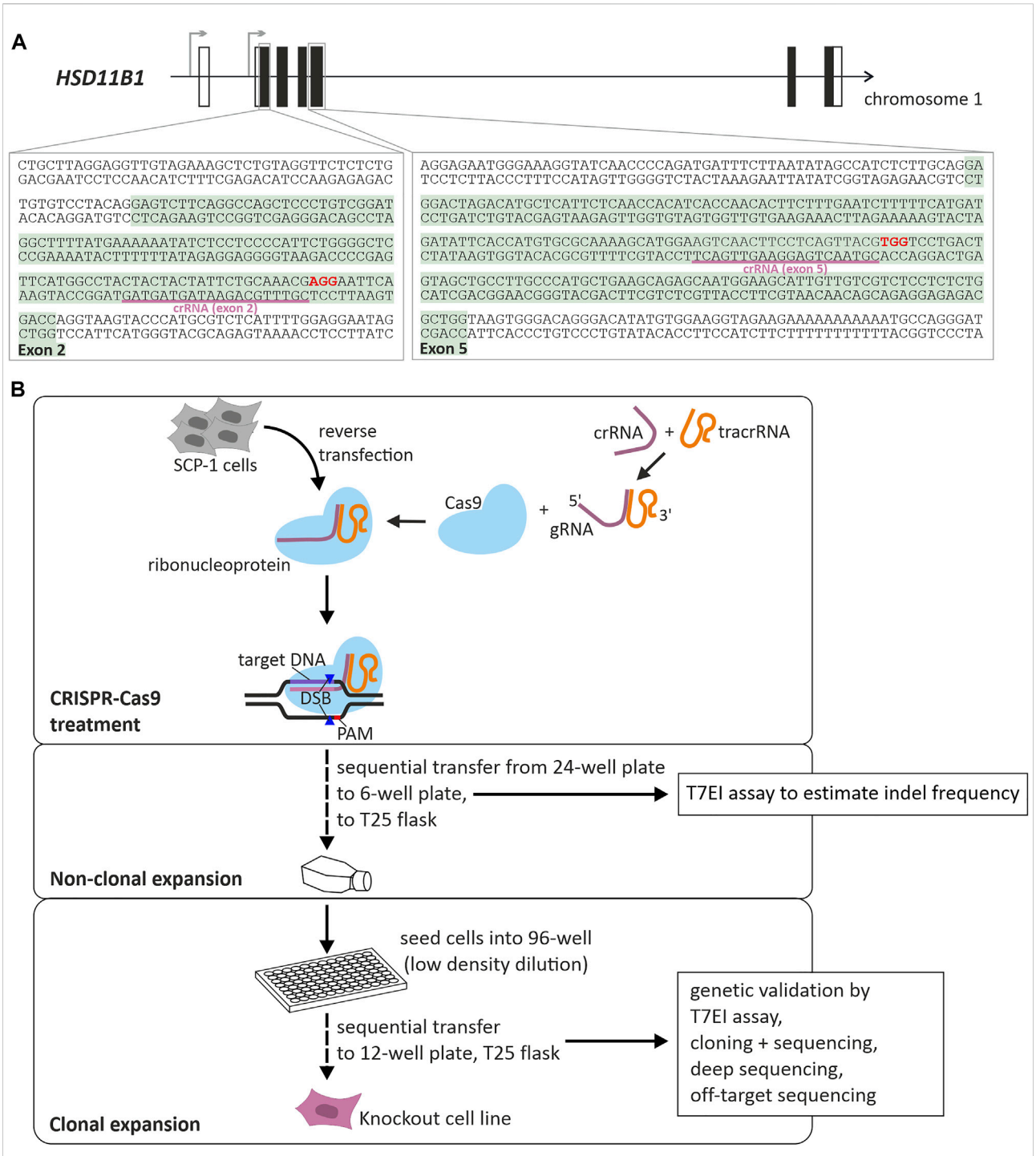
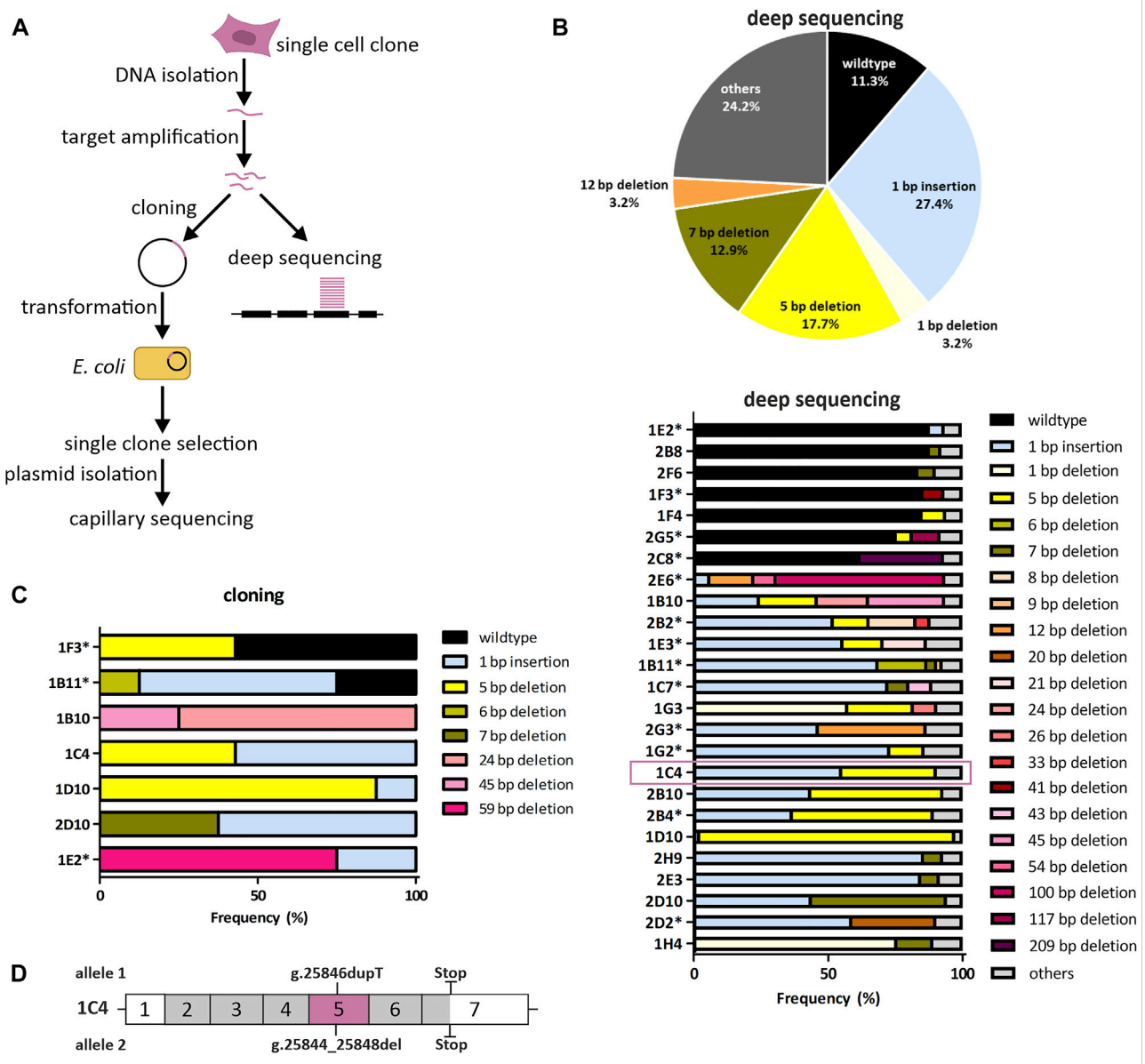


FIGURE 1
Generation of *HSD11B1* knockout SCP-1 cells using CRISPR-Cas9 mediated gene editing. **(A)** Schematic representation of the localization of the targeted regions within *HSD11B1*. Two crRNAs were applied, one targeting exon 2, the other targeting exon 5. The binding sites of the crRNAs is underlined, the PAM sites are shown in bold print. **(B)** Workflow and components of CRISPR-Cas9 mediated gene editing applying the ribonucleoprotein approach of IDT. A crRNA is designed so as to contain a sequence complementary to the target site. It is mixed with a tracrRNA to form the gRNA. This gRNA forms a ribonucleoprotein with the Cas9 endonuclease. After transfection of target cells, Cas9 will be directed to the target site and will create a double strand break (DSB) upstream of the PAM site.



E

Target sequence	PAM	Gene	Position	Locus
AGTCAACTTCCTCAGTTACG	TGG	*HSD11B1*	Exonic	chr1:+209707033
ATTCCAC-TCCTCAGTTACG	TAG	*C1orf35*	Intronic	chr1:+228101603
AGTCAAATTTCTCAGTTACA	GGG	*ATRNL1*	Intronic	chr10:-115635604
ATTAAACATCCTCAGTTACG	TGG	*ZRANB1*	Intronic	chr10:+124980616
ATTCAA-TTCCTCAGTTACA	CAG	*LTBP1*	Intronic	chr2:-33247742
TGACAACTTCCTGAGTTCCG	GGG	*FMNL3*	Intronic	chr12:+49671673

FIGURE 2
Genetic validation of *HSD11B1* knockout SCP-1 cells generated using the crRNA targeting exon 5. **(A)** Sequencing strategy to identify sequence modifications in the CRISPR-Cas9 treated cells. **(B)** Summarized allele frequencies of sequence modifications detected by NGS. Others represents additional modifications that had a frequency of less than 2% (top). Read frequencies of sequence modifications across all clones determined by NGS. Others represents the clusters that were not analyzed in detail due to a read number <50 (bottom). Asterisks indicate clones with potential origin from more than one cell. **(C)** Read frequencies of sequence modifications determined by cloning and subsequent capillary sequencing. **(D)** Schematic representation of sequence modifications and consequences in clone 1C4. Positions given are based on the reference sequence NG_012081.1. **(E)** Potential off-target sites of the applied *HSD11B1* exon 5 crRNA predicted by IDT and CCTop. Mismatches in the target sequence are marked red, hyphens indicate gaps. Sequences of the respective sites were controlled by capillary sequencing.

after excitation at 485 nm and at 454 nm after excitation at 364 nm, respectively, in a Tecan infinite M200 microplate reader (Tecan Group Ltd. Männedorf, Switzerland). For both dyes, the background values were subtracted from the fluorescence values and Nile Red measurements were normalized on DAPI. Microphotographs were taken using a LSM780 with a 10x EC PlnN 10X/0.3 DICI M27 objective.

Alkaline phosphatase assay

The ALP assay was performed as described before (Ode et al., 2013). After washing with 400 µl PBS and 500 µl AP Buffer (100 mM NaCl, 100 mM Tris, 1 mM $MgCl_2$, pH 9.0), 250 µl AP Buffer and 250 µl *p*-nitrophenyl phosphate (*p*NPP) solution (1 mg/ml in 1 M diethanolamine, pH 9.8; Sigma-Aldrich) were added. After a 10-min incubation at 37°C in a CO_2-incubator, the reaction was stopped by addition of 500 µl 1 N NaOH. The absorbance at 405 nm was determined in a microplate reader and the concentration of *p*-nitrophenol (*p*NP) (i.e., consumed *p*NPP) was determined using the Beer-Lambert equation.

Statistical methods

Descriptive statistics and data plotting were performed with GraphPad Prism v.5.01. One-way ANOVA with post-hoc Tukey's Test for multiple comparisons was performed with IBM SPSS Statistics v.26. Results were called significant when $p \leq 0.05$.

Results

Generation and genetic validation of *HSD11B1* knockout cells

We applied CRISPR-Cas9 mediated gene editing to knockout *HSD11B1* in SCP-1 cells. Two independent targets were used, one in exon 2 and one in exon 5 (Figure 1A). The efficiency of CRISPR-Cas9 mediated gene editing was controlled using T7EI assays and the cells were diluted to propagate single cell clones (Figure 1B; Supplementary Figure S1). Following the clonal selection, a T7EI assay was applied to select 38 clones for further analyses: 13 clones where exon 2 was targeted and 25 clones where exon 5 was targeted. The knockout in the chosen clones was validated by cloning with subsequent capillary sequencing and by deep sequencing using a Next Generation Sequencing technology (Figure 2).

Deep sequencing was performed for all clones with an average depth of 2,431 reads (range 555–8,016). Following clustering to account for sequencing errors, an average of 2.3 clusters per clone were analyzed (range 1–6).

The knockout efficiency was substantially better when exon 5 than when exon 2 was targeted. Among the exon 5 clones analyzed, 88.7% did not contain the wildtype sequence. The most predominant modifications detected with deep sequencing in exon 5 were a 1 bp insertion, 5 bp deletion and 7 bp deletion (Figure 2B). In parallel, we analyzed seven exon 5 clones in a "classical" low-throughput analyses by cloning the targeted region in bacteria and resequencing single bacterial clones (mean number of bacterial clones sequenced: 7.3 per cell clone, range 4–8). In five out of seven clones analyzed no wildtype sequences were detected (Figure 2C). Three clones showed similar distribution of the genetic changes both in the capillary and in the deep sequencing: 1C4, 1D10 and 2D10. The clone 1C4 was chosen for further analyses.

The validation of exon 2 clones revealed that eight out of thirteen clones analyzed (62%) contained either completely or to more than 40% wildtype reads. Further three clones showed changes that did not lead to a frameshift (Supplementary Figure S2). Only two clones (1B7, 1F4) indicated a frameshift. However, single wildtype reads were found also in these clones that might would have become predominant after several passages. Hence, no exon 2 clones were further used.

We chose the clone 1C4 for subsequent experiments. This clone had a 1 bp insertion originating from a duplication of T at position 25846 (NG_012081.1) and a 5 bp deletion occurring at positions 25844–25848 (NG_012081.1). Both nucleotide changes caused frameshifts leading to missense amino acid sequences after codons 146 and 145 (Supplementary Figure S3), which resulted in premature stop codons at amino acids 257 and 259, respectively (Figure 2D).

To exclude off-target effects, we sequenced five gene loci in the human genome that were predicted due to sequence homology to be the most probable off-targets of the used crRNA (Figure 2E). None of them showed alterations compared to the wildtype sequence in the clone 1C4 suggesting no off-target artefacts of the CRISPR-Cas9 mediated gene editing.

Generation and genetic validation of *HSD11B1* overexpressing cells

We used targeted chromosomal integration to generate SCP-1 cells stably overexpressing *HSD11B1*. To this end, first, the target FRT site was integrated into SCP-1 cells to create the host cell line (Figure 3). The resulting SCP-1/FRT cells were used to integrate the complete ORF of the human *HSD11B1*. Flp-In™ T-REx 293 (HEK293) were also transfected with *HSD11B1* as a control. Correct chromosomal integration was validated by PCR (Supplementary Figure S4) and the *HSD11B1* expression levels were determined by RT-qPCR (Figure 3B). Compared to the untransfected cells, *HSD11B1* expression increased 243-fold in the stably transfected SCP-1 cells and 13,974-fold in the

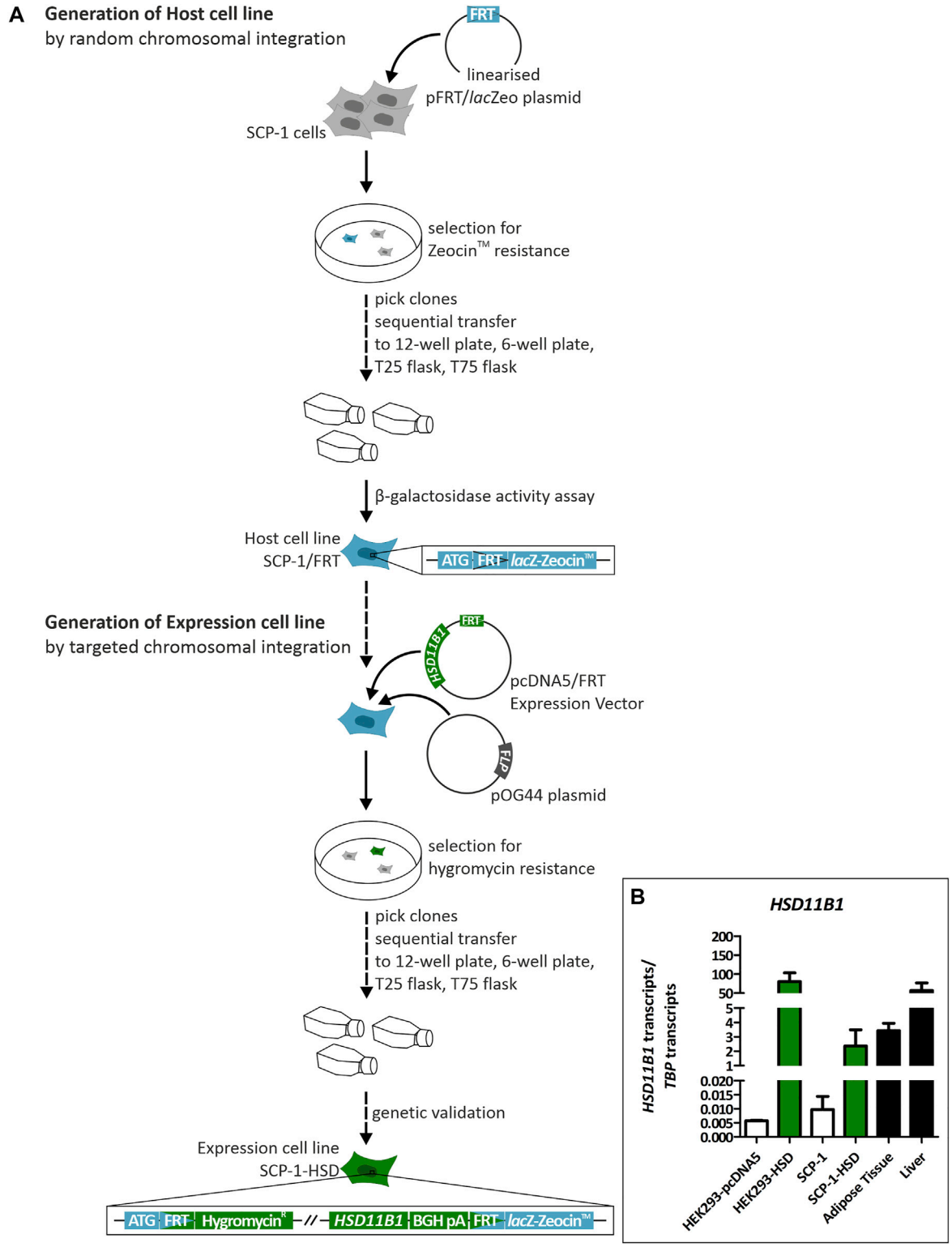


FIGURE 3
Generation and genetic validation of stably *HSD11B1* overexpressing SCP-1 cells using the Flp-In™ system. **(A)** The host cell line is created by transfection with the pFRT/*lac*Zeo plasmid. The FRT site is randomly integrated. The expression cell line is generated by transfection with the pcDNA5/FRT expression vector carrying *HSD11B1* and the pOG44 plasmid expressing *Flp* recombinase. **(B)** *HSD11B1* mRNA expression in *HSD11B1* overexpressing HEK293 and SCP-1 cells compared to expression in human adipose tissue and liver. As controls untransfected SCP-1 cells and HEK293 cells transfected with the empty pcDNA5 vector were used. Shown are the HEK293-HSD clone I/6 and the SCP-1 clone I/1. Shown are means ± SEM of 2–5 biological replicates.

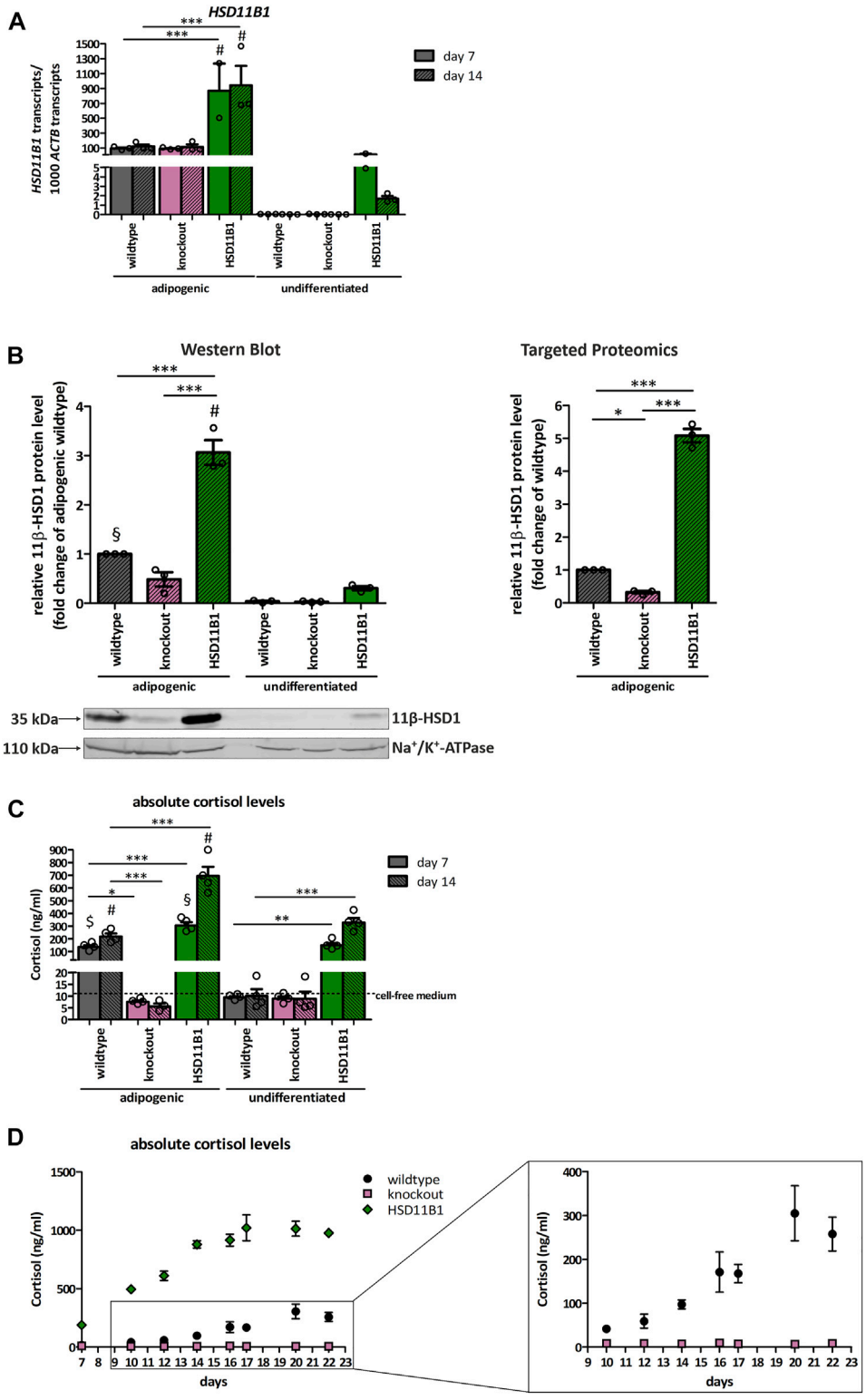


FIGURE 4
Functional validation of the *HSD11B1* knockout and overexpression in SCP-1 cells after adipogenic differentiation. Functional validation has been performed by analyzing **(A)** mRNA expression, **(B)** protein expression, 11β-HSD1 activity measuring **(C)** extracellular cortisol levels and **(D)** extracellular cortisol levels over time. **(A)** *HSD11B1* mRNA expression was determined by RT-qPCR. Shown are means ± SEM of 2–3 biological replicates. **(B)** Protein expression after 14 days of adipogenic differentiation as determined by Western Blot analysis (left) and Targeted

(*Continued*)

FIGURE 4
Proteomics (right). A representative Western Blot is given. In both approaches, 11β-HSD1 protein levels were normalized to Na^+/K^+-ATPase. Cells were differentiated for 14 days. Shown are means ± SEM of 3 Western Blot quantifications from two independent differentiation experiments and 3 Targeted Proteomics measurements from a single differentiation experiment. **(C)** 11β-HSD1 activity was determined by cortisol measurements after a 48-h stimulation with cortisone. Shown are means ± SEM of 3–4 biological replicates. **(D)** 11β-HSD1 activity was determined by cortisol measurements in samples taken with every medium replacement. Absolute cortisol levels in days are depicted. Shown are means ± SEM of 3–4 biological replicates. Cells were differentiated with IBMX, indomethacin, insulin, and dexamethasone **(A–C)** or cortisone **(D)**. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; $ $p \leq 0.05$, § $p \leq 0.01$, # $p \leq 0.001$ compared to respective undifferentiated control, determined by one-way ANOVA with post-hoc Tukey's Test.

HEK293 cells (Figure 3B). The resulting *HSD11B1* expression in the stably transfected SCP-1 cells (2.4 transcripts per *TBP* transcript) were comparable with the *HSD11B1* expression levels in human adipose tissue. *HSD11B1* expression in the overexpressing HEK293 clone (80.3 transcripts per *TBP* transcript) was comparable with the levels in the liver (Figure 3B).

Functional validation of *HSD11B1* knockout and overexpression in SCP-1 cells

To validate the *HSD11B1* knockout and overexpression at functional level, we analyzed mRNA and protein expression, and cortisol production during adipogenic differentiation of SCP-1 cells. Adipogenic differentiation was chosen as this resulted in the highest increase in endogenous *HSD11B1* expression in SCP-1 cells. *HSD11B1* expression increased 3,000-fold in wildtype SCP-1 cells already on day 7 and remained stably on the same level on day 14 of differentiation (Figure 4A). Compared to the wildtype cells, *HSD11B1* expression in overexpressing cells was more than 100-fold higher in the undifferentiated, 9.4-fold higher at day 7 ($p < 0.001$) and 7.9-fold higher at day 14 of differentiation ($p < 0.001$). *HSD11B1* expression was not diminished in the *HSD11B1* knockout cells.

11β-HSD1 protein expression was analyzed by Western blot and targeted proteomics at day 14 of adipogenic differentiation (Figure 4B). With both approaches, the 11β-HSD1 protein level in overexpressing cells significantly increased (3-fold in Western blot and 5-fold in targeted proteomics) compared to wildtype cells ($p < 0.001$ and $p < 0.001$). In knockout cells, 11β-HSD1 protein levels were reduced by 50% (Western Blot) and by 70% (targeted proteomics, $p < 0.05$) when compared to wildtype cells. In undifferentiated cells, 11β-HSD1 protein was only detectable in overexpressing cells.

More importantly, the functional effects of *HSD11B1* overexpression and knockout were clearly observed when we analyzed cortisol production in medium supplemented with cortisone. The concentration of extracellular cortisol of wildtype cells increased 14-fold, upon seven days of differentiation (Figure 4C). The extracellular cortisol level increased constantly from day 10 and reached its maximum of 305 ng/ml on day 20 (Figure 4D). In *HSD11B1* overexpressing cells, the cortisol production exceeded the production in the wildtype cells by at least 3.3-fold at any time point of the measurements. A constant increase of the cortisol level was detected, with the highest cortisol concentration of 1,020 ng/ml reached at day 17 of the differentiation. Importantly, despite lack of changes in *HSD11B1* mRNA levels and the detection of trace amounts of 11β-HSD1 protein, no cortisol production was detectable at any time point of differentiation of the *HSD11B1* knockout cells (Figures 4C,D).

Analyses on the effects of endogenous cortisol on adipogenic and osteogenic differentiation

The here generated cell models were used to study the influence of endogenously produced cortisol on adipogenic and osteogenic differentiation. To evaluate the effects of *HSD11B1* mediated cortisol production on adipogenic differentiation, lipid droplets were visualized and quantified using Nile Red staining. At day 16 of adipogenic differentiation, a significant increase in lipid droplets was detected in all three cell lines: wildtype, *HSD11B1* knockout and overexpressing (Figures 5A,B), demonstrating a successful differentiation of SCP-1 cells towards the adipogenic lineage. The overexpressing cells showed the highest lipid accumulation. After 16 days of adipogenic differentiation, in a protocol where dexamethasone was substituted with cortisone, Nile Red fluorescence increased 3.1-fold in the wildtype and 7.5-fold in the overexpressing cells ($p < 0.001$, Figure 5B).

Additionally, the success and the extent of adipogenic differentiation in the three cell lines were analyzed by gene expression analyses of the adipogenic marker gene Lipoprotein Lipase (*LPL*). The increase in *LPL* expression on day 16 of differentiation was significantly stronger in the overexpressing cells (915-fold) compared to the increase in the wildtype cells (132-fold, $p < 0.05$; Figure 5C).

The *HSD11B1* knockout did not lead to a decrease in adipogenic differentiation, neither in terms of *LPL* expression nor in accumulation of lipid droplets (Figures 5B,C). The higher adipogenic differentiation efficiency was observed in the

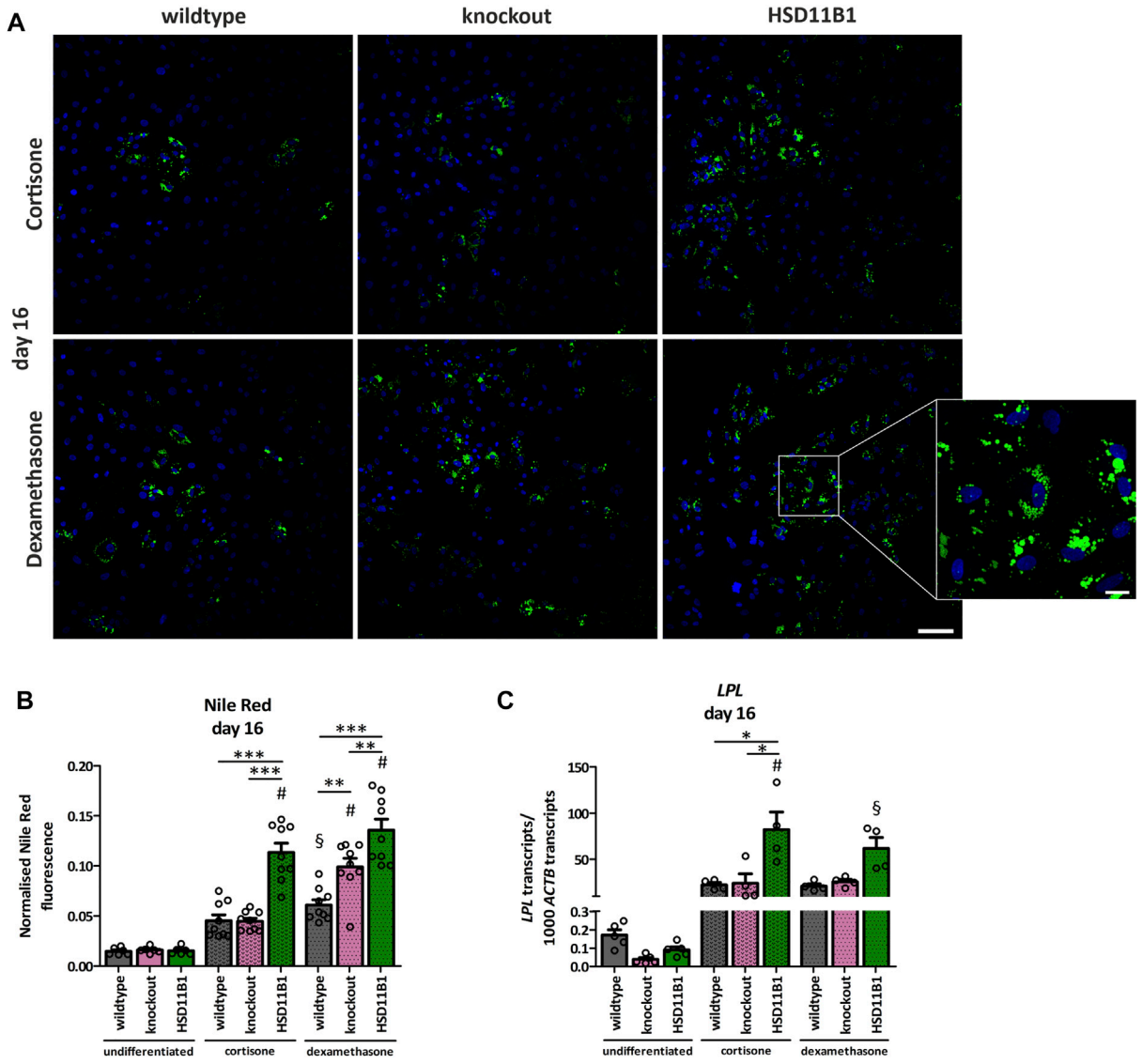


FIGURE 5
Analyses of the effects of *HSD11B1* knockout and overexpression on adipogenic differentiation. **(A)** Lipid droplets were visualized by Nile Red staining, nuclei were stained with DAPI. Representative images for each cell line are given. Scale bar: 100 µm. Zoom into overexpressing cells differentiated with dexamethasone (zoom factor: 5.1x, scale bar: 20 µm). **(B)** Nile Red was quantified directly after staining by fluorescence measurement and normalized on DAPI fluorescence. Shown are means ± SEM of 6 wells of undifferentiated and 9 wells of differentiated cells from 3 independent differentiation experiments. **(C)** mRNA expression of the adipogenic marker *LPL*. Shown are means ± SEM of 5 (undifferentiated) and 4 (differentiated) independent differentiation experiments. Cells were differentiated with IBMX, indomethacin, insulin, and cortisone or dexamethasone. *$p \leq 0.05$, **$p \leq 0.01$; §$p \leq 0.01$, #$p \leq 0.001$ compared to the respective undifferentiated cells, determined by one-way ANOVA with post-hoc Tukey's Test.

HSD11B1 overexpressing cells also under a control stimulation with dexamethasone (Figures 5A–C). These experiments suggest that *HSD11B1* overexpression increases adipogenic differentiation of SCP-1 cells independent of cortisone stimulation.

A dexamethasone-free protocol that included 1α,25-dihydroxyvitamin D_3 was used to analyze the effects of *HSD11B1* knockout and overexpression on osteogenic differentiation. In presence of cortisone, the expression of the Wnt signaling marker Dickkopf-related protein 1 (*DKK1*) significantly increased in the overexpressing cells compared to both the undifferentiated (7.7-fold, $p < 0.001$) and the differentiated wildtype cells (4.6-fold, $p < 0.01$, Figure 6A). This increase was less pronounced when cortisone was not present in the medium, suggesting cortisone dependent effects of *HSD11B1* in osteogenic differentiation.

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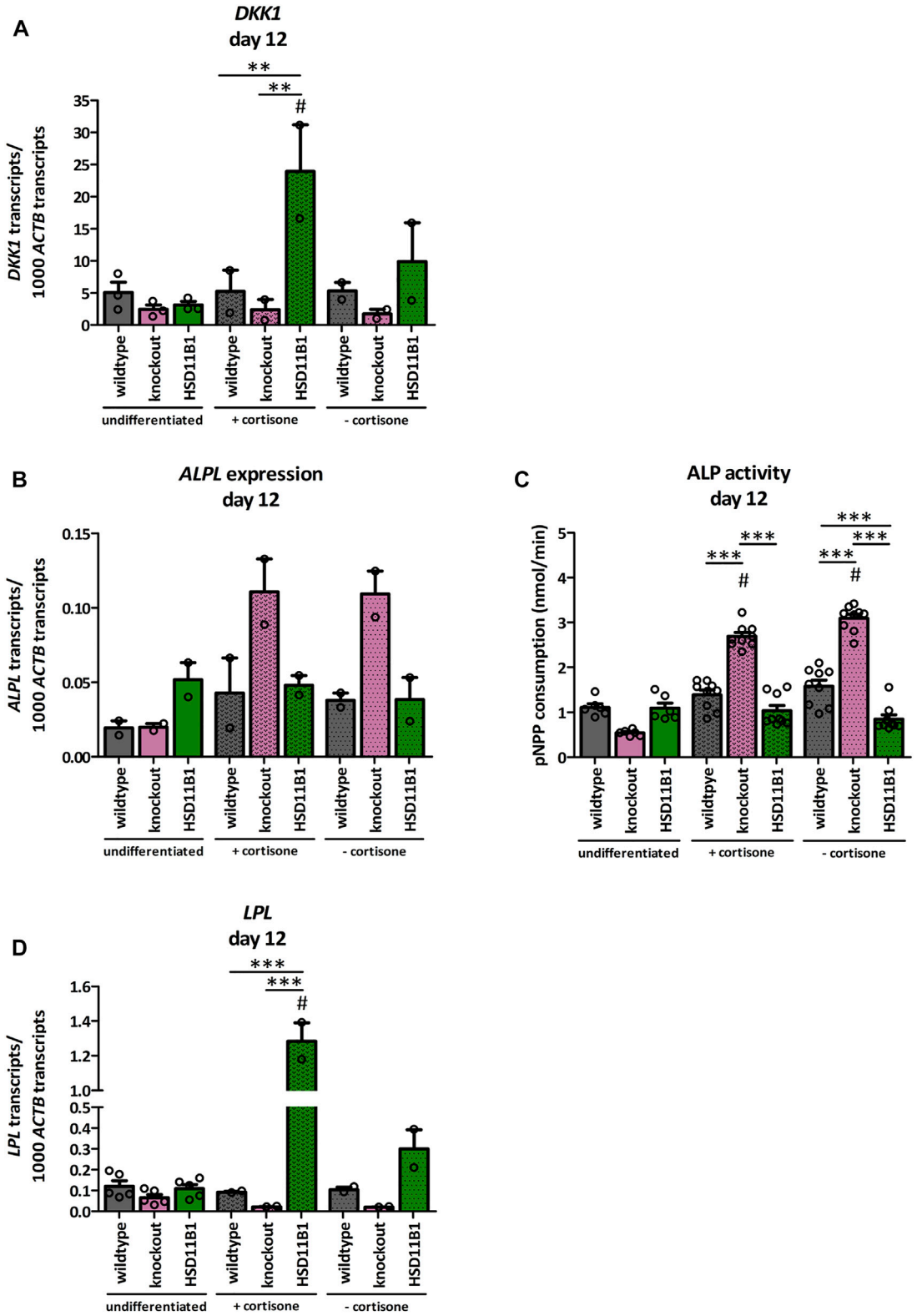

FIGURE 6
Analyses of the effects of *HSD11B1* knockout and overexpression on osteogenic differentiation. mRNA expression of **(A)** the Wnt signaling marker *DKK1*, **(B)** the osteogenic marker *ALPL*, and **(D)** the adipogenic marker *LPL*. Shown are means ± SEM of 2 independent differentiation experiments (*DKK1*, *ALPL*, *LPL*) and 2 to 5 independent biological replicates of the undifferentiated cells (*DKK1* 3 replicates, *ALPL* 2 replicates, *LPL*
(*Continued*)

FIGURE 6
5 replicates). **(C)** ALP activity. Shown are means ± SEM of 6 wells of undifferentiated and 9 wells of differentiated cells from two independent differentiation experiments. Cells were differentiated with ascorbate, β-glycerol phosphate, 1α,25-dihydroxyvitamin D3 with or without cortisone. Mean. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$; \$ $p \leq 0.05$, §$p \leq 0.01$, #$p \leq 0.001$ compared to the respective undifferentiated cells, determined by one-way ANOVA with post-hoc Tukey's Test.

More importantly, the expression of the osteogenic marker alkaline phosphatase (*ALPL*) was increased only 2.2-fold in the wildtype but 5.6-fold in the knockout cells when compared to undifferentiated control cells (Figure 6B). This represents a 2.6-fold higher increase in the *HSD11B1* knockout cells. The effects were observed both in the presence and in the absence of cortisone. In accordance with this, also ALP activity was increased only in the *HSD11B1* knockout cells when compared to undifferentiated cells (4.8-fold in the presence of cortisone, $p < 0.001$, Figure 6C). The activity was also independent of the presence of excess cortisone in the medium.

Based on the hypotheses that cortisol production by 11β-HSD1 is sufficient to induce adipogenesis in bone cells, also mRNA expression of the adipogenic marker *LPL* was analyzed under osteogenic differentiation (Figure 6D). In *HSD11B1* overexpressing cells and in presence of cortisone, *LPL* expression increased 11.7-fold compared to the undifferentiated controls ($p < 0.001$). This effect was also cortisone dependent (4.3-fold higher *LPL* expression when cortisone was present in the differentiation medium). In contrast, there was no detectable increase in the wildtype cells. In the *HSD11B1* knockout cells, even a decrease in the expression of *LPL* was observed under these conditions. Taken together, these results suggest that *HSD11B1* overexpression may promote adipogenic whereas *HSD11B1* knockout may promote osteogenic differentiation.

Discussion

Local cortisol metabolism plays a critical role in bone tissue homeostasis (Hardy et al., 2008; Lavery et al., 2012; Morgan et al., 2014; Hardy et al., 2018; Fenton et al., 2021). Here, we generated cell models with stable knockout and overexpression of *HSD11B1* to analyze the impact of local cortisol production by 11β-HSD1 on differentiation of hMSCs.

With the generated cell lines, it is possible to overcome the limitations of transient overexpression and pharmacological inhibition. Properly validated and characterized immortalized cells deliver consistent results and are suitable for initial analyses of molecular mechanisms compared to primary MSCs whose isolation, growth and characterization is time and cost intensive. Moreover, the functionality, i.e., stem cell characteristic of primary MSCs, highly depends on *in vitro* age (Bonab et al., 2006) which makes it particularly difficult to obtain reliable functional outcomes following long-term cultures such as those required for the genetic modifications shown here. We demonstrated the ability of the cells to differentiate into the adipogenic and osteogenic lineage proving the preservation of multipotency of the SCP-1 cells. Furthermore, we generated a SCP-1 cell line carrying a target sequence for flippase-based targeted chromosomal integration and successfully applied these cells. When addressing further scientific questions, these cells could be easily used for stable transfection and overexpression of other genes to generate gain-of-function models. Recently, we modified the Flp-In system to enable targeted integration of multiple genes (Jensen et al., 2020).

In none of the sequenced CRISPR-Cas9 off-target sites, sequence modifications were detected, indicating a high specificity of the used crRNA and thus a specific knockout of *HSD11B1*. Knockout in exon 5 was more efficient than in exon 2. This may result from a lower estimated efficiency of the crRNA targeting exon 2 when compared to the efficiency of the crRNA targeting exon 5. To our knowledge, nothing is known about differences in general target efficiency between exons of the same gene. We could not detect nonsense mediated mRNA decay (NMD) as the premature stop codon in the clone 1C4 is located downstream of the 3′-most exon-exon junction (Nagy und Maquat 1998; Popp und Maquat 2016).

We aimed to generate a knockout cell line from a clonally derived cell clone as mixed clones increase the variability and thereby the probability of inconsistent results. Eight exon 5 clones exhibited more than two types of modifications (Figure 2B) that could be explained by one of the three following points: 1) polyploidy of the analyzed cell clones, 2) a methodological artefact, or 3) the presence of a mixture of cells rather than a single cell clone. Upon generation, SCP-1 cells were shown to be diploid (Böcker et al., 2008) which makes us exclude this possibility. To our knowledge, no other studies on chromosome number have been performed since then. Alternatively, additional sequence modifications may have been detected due to sequencing errors. However, as only sequence clusters with more than 50 reads were analyzed, we would exclude this possibility, too. In our opinion, the most probable scenario is the propagation of cell mixtures rather than single cell clones. In our hands, SCP-1 cells did not grow clonally very well, so that the dilution factor was chosen to be higher than the recommended 0.5 cells/well. After seeding, the cells were strictly observed to identify clones originating from more than one cell. However, despite careful observation of the cells, undocumented mixed clones may have arisen. This proves the necessity of proper genetic validation and the indispensability of deep sequencing.

We applied the here generated *HSD11B1* knockout and overexpression SCP-1 cells to analyze the role of local cortisol in adipogenic and osteogenic differentiation. The lack of a glucocorticoid stimulus in the knockout cells did not abolish lipid droplet formation. This makes the remaining substances of the adipogenic differentiation cocktail (insulin, IBMX and indomethacin) sufficient to stimulate adipogenic differentiation which was also shown in mouse preadipocytes (Park und Ge 2017). *HSD11B1* overexpressing cells formed significantly more lipid droplets and expression of the adipogenic marker *LPL* increased when compared to wildtype or knockout cells, indicating a promoting role of 11β-HSD1 in adipogenic differentiation. Indeed, production of cortisol by 11β-HSD1 was shown to promote adipogenesis in committed omental preadipocytes (Bujalska et al., 2002) and subcutaneous preadipocytes even in the presence of indomethacin, IBMX and insulin (Bujalska et al., 2008). In contrast to our expectations, also in the presence of dexamethasone, the overexpressing cells showed increased lipid droplet formation and *LPL* expression. As dexamethasone is not a substrate of 11β-HSD1 but of 11β-HSD2 (Best et al., 1997; Hult et al., 1998), analyses on the expression and activity of 11β-HSD2 should be considered in further investigations. 11β-HSD2 converts dexamethasone to 11-dehydrodexamethasone which is a substrate for both isozymes *in vitro* (Best et al., 1997) and can therefore be reactivated to dexamethasone by 11β-HSD1 (Best et al., 1997; Rebuffat et al., 2004). Moreover, dexamethasone might induce the expression of *HSD11B2* in SCP-1 cells as it was shown for lung and placental cells (Suzuki et al., 2003; van Beek et al., 2004).

Following osteogenic differentiation, *ALPL* expression and ALP activity was increased in the knockout cells, but cortisone independently. The origin of these cortisone independent effects needs to be investigated in further analyses. *DKK1* expression significantly increased in *HSD11B1* overexpressing cells in the presence of cortisone. Wnt signaling is important for osteoblastogenesis and as *DKK1* is an inhibitor of Wnt signaling (Fedi et al., 1999; Krupnik et al., 1999; Semënov et al., 2005), its increase indicates a reduced osteogenic differentiation. Additionally, expression of the adipogenic marker *LPL* increased in overexpressing cells when cortisone was added to the differentiation medium. It was previously reported that cortisol induces adipogenic differentiation of stromal cells (Pereira et al., 2002). Our data show that high amounts of cortisol generated by enzyme activity of the overexpressed *HSD11B1* promote the switch from osteogenic to adipogenic differentiation. This is in line with our previous observations (Blaschke et al., 2021).

In this study, a limited number of adipogenic and osteogenic markers were analyzed to obtain first results on the effect of 11β-HSD1 on MSC differentiation. Further analyses are needed to investigate the effects of *HSD11B1* knockout and overexpression on adipogenic and especially on osteogenic differentiation in more detail, e.g., its impact on specific matrix generation in microphysiological bone models (Schoon et al., 2020).

We previously showed that suppressed cortisol levels are associated with bone mineral density in patients evaluated for osteoporosis (Siggelkow et al., 2014) indicating a role for 11β-HSD1 in the development of age-related osteoporosis. *HSD11B1* expression increases with age (Cooper et al., 2002; Tiganescu et al., 2011). Therefore, accelerated local cortisol regeneration by increased 11β-HSD1 levels would result in increased adipogenesis in bone and could contribute to the development of age-related osteoporosis. In osteoblasts from subjects aged between 50 and 59 years, expression of adipogenic markers was detected (Clabaut et al., 2021). Specific inhibition of 11β-HSD1 was shown to decrease adipogenesis *in vitro* (Blaschke et al., 2021), to promote osteogenic differentiation and to improve bone microstructure and density *in vivo* (Park et al., 2014; Li et al., 2020). It was further shown that co-culturing of osteoblasts derived from MSCs with bone marrow adipocytes promoted transdifferentiation and increased the expression of adipogenic markers, including *HSD11B1*, in the osteoblasts (Clabaut et al., 2010; Clabaut et al., 2021). This raises the question of whether endogenous cortisol produced by 11β-HSD1 in the adipocytes is involved in this process. Therefore, future analyses should comprise *ex vivo* analyses of 11β-HSD1 expression in human bone and bone marrow.

In conclusion, local cortisol production by 11β-HSD1 had an only limited influence on adipogenic differentiation of MSCs *in vitro*, whereas it clearly impacted osteogenic differentiation. While knockout of *HSD11B1* promoted the osteogenic differentiation, local cortisol production by 11β-HSD1 promoted a shift from osteogenic to adipogenic differentiation. This indicates a role for 11β-HSD1 in the development of osteoporosis by increasing adipogenesis in bone. Taken together, the here generated *HSD11B1* loss-of-function and gain-of-function cell models provide a novel and powerful tool for analyzing the role of 11β-HSD1 in the onset of age-related osteoporosis and could help to identify new therapeutic strategies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

AK, JS, and CW performed experiments. AK, MT, JS, AT, and CW analyzed data. WB contributed essential material. AK, JS, and MT wrote the manuscript. All authors read and edited the manuscript. MT, HS, AK, JS, and MB participated in research design.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2022.953034/full#supplementary-material

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A new frontier in temporomandibular joint osteoarthritis treatment: Exosome-based therapeutic strategy

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Temporomandibular joint osteoarthritis (TMJOA) is a debilitating degenerative disease with high incidence, deteriorating quality of patient life. Currently, due to ambiguous etiology, the traditional clinical strategies of TMJOA emphasize on symptomatic treatments such as pain relief and inflammation alleviation, which are unable to halt or reverse the destruction of cartilage or subchondral bone. A number of studies have suggested the potential application prospect of mesenchymal stem cells (MSCs)-based therapy in TMJOA and other cartilage injury. Worthy of note, exosomes are increasingly being considered the principal efficacious agent of MSC secretions for TMJOA management. The extensive study of exosomes (derived from MSCs, synoviocytes, chondrocytes or adipose tissue et al.) on arthritis recently, has indicated exosomes and their specific miRNA components to be potential therapeutic agents for TMJOA. In this review, we aim to systematically summarize therapeutic properties and underlying mechanisms of MSCs and exosomes from different sources in TMJOA, also analyze and discuss the approaches to optimization, challenges, and prospects of exosome-based therapeutic strategy.

KEYWORDS

temporomandibular joint osteoarthritis, Exosome, mesenchymal stem cell, therapeutic strategy, optimization in bioengineering

Introduction

Temporomandibular joint osteoarthritis (TMJOA) is a degenerative temporomandibular arthropathy characterized by progressive cartilage degeneration, abnormal subchondral bone remodeling and obvious synovitis (Scrivani et al., 2008; Toller, 1973). Due to the severe concomitant symptoms such as difficulties in chewing,

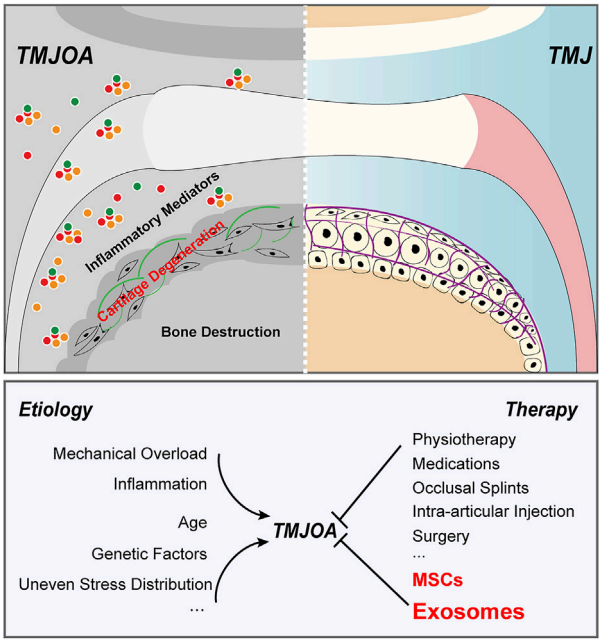

FIGURE 1
Pathogenesis and treatment strategies of TMJOA. TMJOA is a degenerative temporomandibular arthropathy characterized by progressive cartilage degeneration, abnormal subchondral bone remodeling and obvious synovitis. It is a pathological state affected by multiple factors. Traditional clinical strategies of TMJOA emphasize on symptomatic treatments and are unable to halt or reverse the destruction of cartilage or subchondral bone. MSCs and exosomes are highly promising for TMJOA alleviation.

acute or chronic pain, and even maxillofacial deformities, it severely deteriorates the quality of patient life and leads to the large resultant socioeconomic burden. Joint cartilage is composed of chondrocytes and extracellular matrix like collagen fibers, proteoglycans, and hyaluronic acid. Feature of avascular structure of cartilage is detrimental to the exchange of available signaling molecules, migration of progenitor cells, and adequate supply of nutrients and oxygen, resulting in the inability of damaged cartilage tissue to regenerate effectively (Chen et al., 2020). Because of the limited self-healing ability of cartilage, it has become one of the most difficult joint diseases to treat. Compared with other joints in the body, temporomandibular joint has its own characteristics (Macedo et al., 2017; David and Roberts, 2018). Besides, the layer of hyaline cartilage covering generalized joints mainly contained type II collagen, but the cartilage of TMJ is fibrocartilage, which is a kind of cartilage composed of both type I collagen and type II collagen. Because of the structural differences, there will be some differences in treatment strategies when the disease occurs. The cartilage of TMJ has better multidirectional bearing capacity and more dense fibers, which are not easy to degrade and are less affected by aging (Schwartz et al., 2015; Chandrasekaran et al., 2021). However, when it is damaged, the difficulty of restoring normal structure (Kuo et al., 2011). Numerous studies have confirmed that it is a pathological state affected by multiple factors (Figure 1). Excessive mechanical stress is a major factor leading to cartilage rupture in TMJ (Su et al., 2014; Huang et al., 2021; Ootake et al., 2021). Uneven stress distribution in TMJ caused by occlusal disorder was reported to induce the hyperactivity of osteoclasts in subchondral bone. Researchers have demonstrated that inflammation is one of the risk factors of TMJOA (Li et al., 2019a; Li et al., 2019b; Luo et al., 2019; Lei et al., 2022). Liu detected synovial fluid from TMJOA patients and found that the level of inflammatory cytokines was significantly increased.16 Moreover, genetic factors and age-related reduction of host-adaptive capacity are also vital in TMJOA (Xu et al., 2003; Yamaguchi et al., 2014). It is because of the specificality of TMJ structure and the ambiguity of etiological mechanism that the treatment of TMJOA has been set up a huge obstacle.

To date, treatment strategies for TMJAO are symptomatic and limited (Figure 1), only to reduce inflammation and relieve pain (Thie et al., 2001). Traditional clinical treatments can stop the progression of the disease to some extent, but they cannot actively restore degraded cartilage or damaged subchondral bone (Derwich et al., 2021; Liu Q. et al., 2022; Matheus et al., 2022). Novel radical therapies for osteoarthritis are urgently required. In recent years, cell-based disease treatment strategies have raised considerable concerns, especially mesenchymal stem cells (MSCs) -based therapies (Matheus et al., 2022). Abundant native MSCs are present in multiple niches in the joint, including subchondral bone, synovial fluid, and adipose tissue. In the last decade, increasing evidence has suggested that MSCs have great potential in the treatment of osteoarthritis. BMSCs have suggested promising therapeutic efficacy for TMJ cartilage repair (Ciocca et al., 2013). Although the role of MSCs in the field of disease treatment cannot be ignored, we still need to comprehensively understand its non-negligible bottlenecks as cell therapy strategies. The host exhibited immunological tolerance toward implanted MSCs and had a potential risk for malignancies, which might also pose a risk to immunological cells for controlling an inflammatory milieu (Lalu et al., 2012). Therefore, it is inevitable to find an alternative approach to solve the dilemma faced by MSC-based therapy. Numerous studies have summarized the bio-effect of MSCs is increasingly attributed to paracrine signaling to transfer its cargo to the body, among which exosomes are a vital carrier for message in many biological and pathological processes. Exosomes provide new perspectives for the development of cell-free and ready-to-use therapy for treatment of cartilage lesions and TMJOA.

Herein, the present review was aimed at discussing the therapeutic potential and corresponding mechanism of MSCs, the biological properties of exosomes derived from diversified cell sources, and advances in our knowledge of their emerging roles in managing TMJOA. We also discussed the detailed exosome-based tissue engineering strategies of TMJOA therapy in the hope

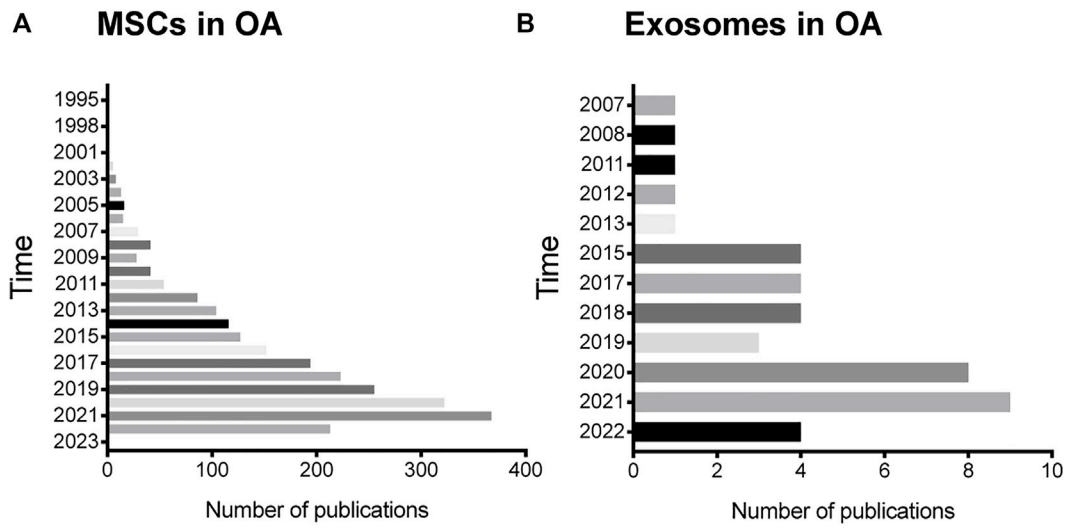

FIGURE 2
Status of MSCs and exosomes research in OA. **(A)** The annual number of publications related to MSCs research in OA in the past 27 years. **(B)** The annual number of publications related to exosomes research in OA in the past 15 years.

of providing inspiration for future investigations. Particularly, we proposed novel perspectives for the development and implementation of exosomes as a cell-free regenerative medicine therapeutic strategy for cartilage repair in TMJOA and discussed future opportunities and challenges in this exciting field.

Mechanisms of MSCs in the treatment of TMJOA

Since MSCs first discovery by Friedenstein (Friedenstein et al., 1982), they are commonly used in the treatment of various diseases, including TMJOA. We conducted a literature review and found that the application of MSCs in osteoarthritis was first reported in 1995, and more and more related research is being carried on, with over 89% of the published in the recent 10 years (Figure 2).

When TMJOA occurs, the dynamic balance between chondrocyte matrix anabolism and catabolism is disrupted, accelerating the progression of the disease (Weng et al., 2017). Zhang et al. (2017) reported BMSCs reversed the loss of cartilage matrix associated with osteoarthritis and enhanced scavenging activity of the degraded matrix in deep zone chondrocytes. Lu et al. (2015) also found that the implanted GFP-BMSCs differentiated into COL2-positive cells and relieved matrix degradation in TMJOA. It was indicated that human umbilical cord matrix-mesenchymal stem cells (hUCMSC) showed prominent cartilage protective effect and effective cartilage regeneration potential (Kim et al., 2019). Moreover, Maria revealed scaffolds loaded with dental pulp mesenchymal stem cells (DPSCs) effectively supported abundant fibrocartilaginous tissue formation. Besides, other MSCs, such as adipose-derived mesenchymal stem cells (ADMSCs) (Ahtiainen et al., 2013) and synovial fluid derived mesenchymal stem cells (SFDMSCs) (Koyama et al., 2011), have also been indicated to alleviate TMJOA by participating in cartilage matrix metabolism.

Uncoupled remodeling of subchondral bone is another pathological feature contributed to TMJOA (Jiao et al., 2011; Yang et al., 2014; Zheng et al., 2018; Ibrahim et al., 2019). Human exfoliated deciduous teeth stem cells (SHED) markedly improved surface smoothness and bone integrity of the destroyed condylar in TMJOA mice (Chen et al., 2013). Chen K investigated MSC-treated groups demonstrated pronounced micro-architectural changes of the subchondral bone (Tanaka et al., 2008). In addition, it was reported that the migration of BMSCs restored subchondral bone loss in mice with TMJOA (Lu et al., 2015).

Evidence has suggested that sustained inflammation is involved in the onset and progression of TMJOA (Liu W. et al., 2017). When stimulated, immune cells in inflammatory microenvironment release inflammatory factors to affect the matrix metabolism of chondrocytes to deteriorate TMJOA (Tanaka et al., 2008) and are also in close correlation with sensory neuron hyperexcitability to induce the pain of TMJOA (Magnano et al., 2007; Ou et al., 2021). Buul et al. found the decreased expression of IL-1β, MMP-1 and MMP-13 in synovial explants when cultured with MSCs conditioned medium (Van Buul et al., 2012). It was recently shown that BMSCs injection into the bilateral TMJ region significantly

TABLE 1 Comparison of MSC-based and Exosome-based TMJOA therapies.

Treatment strategy	Acquisition	Transportation	Storage	Mass production	Delivery	Immunogenicity	Tumorigenicity	Treatment effect
MSC-based	Easy	Difficult	Difficult, cryopreservation affects cell viability	Time-consuming, cell senescence, change in biological characteristics	May happen cellular embolism	Certain degree	Certain degree	Good, clear
Exosome-based	Relatively easy, but the isolation is complicated	Easy	Easy, cryopreservation hardly affects affect exosome activity	Low yield, poor consistency	Not happen	Temporarily not found	Temporarily not found	Good, lack of clinical studies

MSC, mesenchymal stem cell; TMJOA, temporomandibular joint osteoarthritis.

reversed high levels of TNF-α and IL-1β in TMJOA (Lu et al., 2015). This is further supported by Hyunjeong Kim's study (Kim et al., 2019). The self-assembled peptide hydrogels accelerated tissue regeneration by anti-inflammatory modulation (Kim et al., 2016).

Inflammation and immunity go hand in hand (Bartholomew et al., 2002; Koliaraki et al., 2020; Lim et al., 2021; Pham et al., 2021). In the pathogenesis of inflammatory diseases, dysregulation of the host immuno-inflammatory response is one of the important predisposing factors (Theill et al., 2002; Hernández et al., 2011). Similarly, Monasterio proposed cytokines, CCLs and CCRs of the Th1/Th17/Th22 axis were involved in TMJOA pathogenesis (Monasterio et al., 2018). A large number of studies have shown that MSCs regulate innate and acquired immunity in the treatment of OA (Yu et al., 2016). Tang et al. (2021a) reported that hUCMSCs protected cartilage from injury by regulating the macrophages polarization and affecting the joint immune microenvironment, but notably, there was a stronger regulation ability of immune effector process in hUCMSCs-exosomes treatment group.

In recent years, although the efficacy of MSCs in treating TMJOA has been widely studied in animal studies and human clinical trials, in fact, the problems encountered in clinical application have been deeply troubling researchers (Table 1). Donor's age affects the intrinsic activity and functionality of obtained cells (Kim et al., 2020). The lack of standardization for large-scale cell production results in inconsistent cell quality after expansion. Additionally, the senescence and dedifferentiation of cells during the expansion *in vitro* will also affect potential and increase the risk in the application (Siddappa et al., 2007). More cautiously, there is a potential of tumorigenicity (Le et al., 2012; Waterman et al., 2010). Moreover, the issue of cell storage is also a bottleneck of MSC-based strategy. Whether the biological activity of MSCs will be affected after repeated cryopreservation is a great question to be considered in future. It is noteworthy that there is a paradigm shift that, rather than direct differentiation to cells of the target tissue, the therapeutic efficacy of MSCs in tissue repair and regeneration is predominantly attributed to paracrine signaling, particularly exosomes (Mayourian et al., 2018; Li et al., 2019; Mori et al., 2019; Zhang et al., 2019; Zhou Q.-F. et al., 2020). Therefore, exosome-based therapeutic strategy of TMJOA may be a promising substitute for MSC-based therapy.

Characteristics of exosomes

It was not until 2006 that Ratajczak proposed for the first time that mRNA could be delivered by membrane-derived vesicles (MV) released from the surface of activated eucaryotic cells and exert positive effects on surrounding cells (Ratajczak et al., 2006). Exosome-mediated transfer of RNAs was suggested as a novel mechanism of genetic exchange between cells

(Waterman et al., 2010), occurring within the microenvironment or at a distance by traffic of exosomes. Exosomes are the smallest in size ranging from 40 to 160 nm in diameter among the three main subcategories of extracellular vesicles (EVs). Exosomes of different cell origin carry their own various bioactive molecules, containing different types of proteins, DNAs, mRNAs, microRNAs, lipids, metabolites and so on. It is the diversity of contents that illustrates the diversity of exosome functions (Valadi et al., 2007). They are ubiquitously involved in the basic processes of innate and adaptive immunity and immune-mediated disease processes (Garikipati et al., 2018). It was shown that miR-21-5, as a lead cardioactive MSC-exosomal-microRNA, mediated effects on increasing engineered cardiac tissues contractility and was suggested as a specific molecular target for optimizing cardio-therapies (Mayourian et al., 2018). In recent years, increasing studies have been conducted on the application of exosomes in the treatment of neurological diseases (Budden et al., 2021; Xu et al., 2017; Rufino-Ramos et al., 2017). More attention should also be paid to bottlenecks in exosome treatment, including the limitation of increasing exosome production, the difficulty of analyzing the effective components of exosomes and the better improvement of the functions of the active component. Encouragingly, the problems of exosomes faced in the diseases therapy have been gradually handled *via* various biotechnology modifies. The composition and secretion of exosomes are affected by the environment and signals of donor cells, including hypoxia, heat, and pharmacological intervention (Pegtel et al., 2010; Fan et al., 2020). Therefore, changing the culture conditions of donor cells can meet the clinical needs of exosomes in treating diseases. Because exosomes are excellent carriers, the direct insertion of miRNA mimics or siRNAs into exosomes through electroporation (Ma et al., 2018) and electric pulses (Yang et al., 2020) has attracted the attention of many researchers. By modifying exosomes membrane through genetic manipulation strategy by biotechnology, exosomes can reach the target cells and tissues according to the predetermined route and play a more specific role (Kanki et al., 2011; Wang et al., 2018). The intersection between different cells exosomes and chondrocytes offered a new insight into the pathogenesis and treatment of degenerative joint diseases. Many studies have proposed that exosomes play an irreplaceable role in the treatment of TMJOA. In the following section, we summarized the current studies on the therapeutic effects of exosomes from various cells in TMJOA.

Functional mechanisms and potential therapeutics of exosomes in TMJOA

The different responses of recipient cells to exosomes are mainly due to the heterogeneity of exosomes, including their inconsistent expression of cell surface receptors and different contents. It means that exosomes from different cells have different effects on the same type of cell and the same exosome may also have inconsistent or even contradictory effects on different target cell types or target tissues66 (Table 2). More and more researchers attempt to obtain diversified exosomes and apply them in OA treatment to have a deeper understanding of the occurrence and development of OA and hope to find more novel targets in molecular mechanisms of TMJOA treatment (Figure 3). To date, an increasing amount of literature has indicated that exosomes from different sources (Figure 2), such as MSCs, chondrocytes, and synovial fluid in TMJ cavity, are reportedly important in the treatment of TMJOA. In recent years, researchers have focused on identifying effective constituents in exosomes, such as miRNA, for the treatment of TMJOA, with a view to obtain a more direct, effective, and targeted therapeutic strategy.

MSCs-derived exosomes

BMSCs have been used the earliest to treat various diseases because of their outstanding biological characteristics (Li et al., 2012; Li et al., 2020; Dubus et al., 2022). Certainly, BMSCs-derived exosomes have also been demonstrated to be ideal agents for the treatment of osteoarthritis. He Lei investigated BMSCs-exosome stimulation obviously reversed the inhibition effect of IL-1β on the proliferation and migration of chondrocytes, significantly upregulated the expression of COL2A and downregulated MMP13 *in vitro* and vivo (Armiñán et al., 2010). Although the previous studies were less homogenous due to problems with dose, injection frequency and management timing, one of the therapeutic functionalities of MSC-derived exosome is anti-inflammatory efficacy in promoting functional recovery of matrix metabolism homeostasis. After evaluation of the influences on injections of embryonic stem cell-derived-exosomes in TMJ-OA induced by monosodium iodoacetate (MIA), the underlying molecular mechanisms of exosome-mediated matrix homeostasis in TMJ injury repair and cartilage regeneration were clearly elucidated (Zhang et al., 2019). The obstacle of S-GAG synthesis induced by IL-1β was broken by the exosome treatment. Also, consistent with previous experiments, MSCs exosomes reduced inflammation by suppressing NO and MMP13. Together, MSCs derivative exosomes inhibit cartilage degeneration and TMJOA-induced pain by alleviating inflammation in the early stage, and then promote matrix proliferation and expression as well as the recovery of subchondral bone structure, and finally achieve the repair and regeneration of the overall TMJ.

In addition to alleviating inflammatory events in TMJOA, MSC-exosomes could also inhibit apoptosis of chondrocytes and activation of the immunity (Cosenza et al., 2017; He et al., 2020; Wang Y. et al., 2021). A study by Zhu revealed the exosomes from

TABLE 2 Summary of Roles of Exosomes on Different Target cells in Osteoarthritis.

Target cell type	Sources of exosomes	Separation and extraction	Dose and delivery	Biological effects	Underlying mechanisms	Reference
Chondrocyte	BMSCs	Ultracentrifugation	20 μg, 40 μg *in vitro*; 40 μg/100 μl in vivo-IA	Proliferation; Migration; Matrix metabolism	Attenuate IL-1β-induced inhibition on proliferation and migration, downregulation of anabolic markers, and upregulation of catabolic markers	He et al. (2020)
	BMSCs	Ultracentrifugation	12.5 ng, 125 ng, 1.25 μg *in vitro*; 250 ng/5 μl in vivo-IA	Matrix metabolism; Apoptosis	Restore anabolic/catabolic equilibrium; Anti-apoptotic effect	Cosenza et al. (2017)
	SMMSCs	Ultracentrifugation	5 μg (10×10^{11} particles/ml) *in vitro*; 30 μ (10^{11} particles/ml) in vivo-IA	Catabolic metabolism	Promote proliferation and migration; Inhibited apoptosis	Wang et al. (2020)
	iPMSCs	Ultrafiltration	108particles/ml *in vitro*; 8 μl (1.0×10^{10}particles/ml) in vivo-IA	Migration; Proliferation	Enhance the motility; Stimulate proliferation	Zhu et al. (2017)
	iPFPMSCs	ExoQuick-TC kit; Ultrafiltration	1, 5, or 10×10^8 particles/ml *in vitro*; 10 μl (10^{10} particles/ml) in vivo-IA	Apoptosis; Migration; Metabolism; Autophagy	Inhibit apoptosis and promote anabolism; Enhance the level of autophagy *via* inhibition of mTOR pathway	Wu et al. (2019)
	Chondrocytes	Ultrafiltration	10 μg/ml, 20 μg/ml *in vitro*	Proliferation; Migration	Enhance proliferation and migration	Nikhil et al. (2022)
	Chondrocytes	Ultrafiltration	200 μg/ml *in vitro*; 200 μg in vivo-IA	Metabolism; Mitochondrial function	Restore chondrocyte metabolism; Eliminate mitochondrial dysfunction	Zheng et al. (2019)
	Chondrogenic progenitor cells	Ultracentrifugation	108 particles/ml *in vitro*; 8 μl (1.0×10^{10} particles/ml) in vivo-IA	Proliferation; Migration	Stimulate chondrocyte migration and proliferation *via* MiR-221-3p	Wang et al. (2020)
	Fibroblast-like synoviocytes	ExoQuick-TC Kit	Not reported	Proliferation; Migration; Matrix metabolism	Exosomal lncRNA H19 promotes cell viability and migration, and protects against ECM degradation by regulating miR-106b-5p and TIMP2 expression	Tan et al. (2020)
	Platelet-rich plasma	Ultrafiltration	200 μg/100 μl *in vitro*; 4 μg/2 μl in vivo-IA	Migration; Proliferation; Apoptosis; Degeneration	Promote proliferation, migration, and IL-1β-induced apoptosis and degeneration	Zhang et al. (2022b)
	Platelet-rich plasma; Hyperacute serum	Ultracentrifugation	$1.42 \times 10^9 \pm 2.12 \times 10^6$ particles *in vitro*	Inflammation	Elicit chondroprotective gene expression; Inhibit inflammation by reducing IL-6 secretion	Otahal et al. (2020)
	IL-1β-treated chondrocytes	Ultracentrifugation	10 μg *in vitro*	Catabolic metabolism	Stimulate catabolic events	Liu et al. (2020)
	OA sclerotic subchondral bone osteoblast	Ultracentrifugation	10, 20, 50 μg/ml *in vitro*	Matrix metabolism; Cellular bioenergetics; Chondrocyte activity	Trigger the catabolic gene expression; Suppress the oxygen consumption rate *via* miR-210-5p	Wu et al. (2021)
	M2 phenotype macrophages	CM	Not reported	Formation; Differentiation	Downregulate chondrogenic-specific genes; Upregulate differentiation-related genes *via* LncRNA MM2P-induced, exosome-mediated transfer of Sox9	Bai et al. (2020)
	miR-126-3p-overexpressing synovial fibroblasts	Ultracentrifugation	2×10^9 particles/ml *in vitro*; 40 μl (500 μg/ml) in vivo-IA	Proliferation; Colony formation; Inflammation	Suppress chondrocyte inflammation and apoptosis	Zhou et al. (2021)
	ATF4-modified serum	Ultrafiltration	10 μg/ml *in vitro*; 200 μg in vivo-IA	Proliferation; Apoptosis; Autophagy; Inflammation	Promote Proliferation and autophagy; Inhibit apoptosis; Decrease MMP13 and inflammatory cytokines	Cosenza et al. (2017)

(Continued on following page)

TABLE 2 (*Continued*) Summary of Roles of Exosomes on Different Target cells in Osteoarthritis.

Target cell type	Sources of exosomes	Separation and extraction	Dose and delivery	Biological effects	Underlying mechanisms	Reference
Macrophage	Chondrocytes	Ultrafiltration	200 μg/ml *in vitro*; 200 μg in vivo-IA	Immune reactivity	Increase M2 macrophage infiltration with a concomitant decrease in M1 macrophages	Zheng et al. (2019)
	Osteoarthritic chondrocytes	Ultrafiltration	200 μl, 10^8 particles/l *in vitro*; 10^9 paritcals in 5 μl in vivo-IA	Inflammation; Autophagy	Stimulate inflammasome activation; Increase the production of mature IL-1β *via* miR-449a-5p/ATG4B-mediated autophagy inhibition	Ni et al. (2019)
	Inflamed synovial fluid	ExoQuick-TC Kit	7.5×10^9 particles/ml *in vitro*	Inflammation; Matrix metabolism; Immune regulatory properties	Produce IL-1β and IL-16; Stimulate the production of CCL20, CCL15, and CXCL1 chemokines; Release MMP12 and MMP7	Domenis et al. (2017)
Synovial fibroblast	Apoptotic and activated T cells and monocytes	Differential Centrifugation	5×10^3 microparticles, 5×10^4 microparticles, 5×10^5 microparticles *in vitro*	Inflammation; Matrix metalloproteinases	Increase the synthesis of inflammatory mediators and MMPs consistent with activation of NF-κB	Distler et al. (2005)
	BMSCs	ExoQuick-TC Kit	2 μg *in vitro*; 250 ng/ 5 μl in vivo-IA	Proliferation; Apoptosis; Inflammation	Weaken proliferation; Enhance apoptosis of synovial fibroblasts treated with IL-1β	Jin et al. (2020)
	TGF-β1-modified MSCs	Extraction kit	100 μl (1×10^{11} particles/ml) in vivo-IA	Polarization	Promote M2 polarization *via* carrying miR-135b targeting MAPK6	Wang et al. (2021)
Mesenchymal stem cell	Chondrocytes	Ultracentrifugation	10 μg *in vitro*	Differentiation	Promote chondrogenic differentiation	Liu et al. (2020)
	BMSCs	Ultrafiltration	200 μg/500 μl in hydroge *in vitro*; 100 μg in vivo-IA	Migration; Proliferation; Differentiation; Recruitment	Promote proliferation, migration, and chondrogenic differentiation; Stimulate BMSC recruitment *via* the chemokine pathways	Zhang et al. (2017)
	Platelet-rich plasma	Ultrafiltration	200 μg/100 μl *in vitro*; 4 μg/2 μl in vivo-IA	Migration; Proliferation; Differentiation	Promote proliferation, migration, and chondrogenic differentiation	Zhang et al. (2022a)
	Tenocyte	Ultracentrifugation	0.016, 0.08, 0.2, 0.4 μg *in vitro*	Proliferation; Differentiation	Induce the tenogenic differentiation through TGF-β; Promote proliferation	Xu et al. (2019)
	IL-1β-treated chondrocytes	Ultracentrifugation	10 μg *in vitro*	Differentiation	Inhibit chondrogenic differentiation	Liu et al. (20117)
Endothelial cell	IL-1β-stimulated synovial fibroblasts	Ultracentrifugation; ExoQuick-TC Kit	15 ml of conditioned medium	Migration; Tube formation activity	Promote migration and tube formation activity	Kato et al

BMSCs, bone marrow mesenchymal stem cells; IA, intra articular; SMMSCs, synovial membrane mesenchymal stem cells; iPMSCs, induced pluripotent mesenchymal stem cells; iPFPMSCs, infrapatellar fat pad mesenchymal stem cells; OA, osteoarthritis; CM, condition media.

induced pluripotent stem cells (iPMSCs) or synovial membrane derived MSCs (SMMSCs) accelerated proliferation and migration of chondrocytes (Tang et al., 2021b). Notably, there was a proliferation promotion of chondrocytes in the co-culture studies of chondrocytes and MSCs (Zhu et al., 2017). Zhang found $CD163^+$ cells of the cartilage overlying synovium in exosome-treated defect increased but $CD86^+$ cells decreased, indicating higher M2 macrophages infiltration with a decline in M1 macrophages (Wu et al., 2011). Inflammatory cytokines, such as IL-1β, displayed a concomitant decrease like M1 macrophages. The senescence of chondrocytes appears generally during the progression of TMJOA (Clérigues et al., 2012; Zhang et al., 2018; Varela-Eirín et al., 2022). A wide range of evidence has shown that exosomes from ADMSCs declined the presence of phosphorylated histone H2AX, relieved DNA damage, restored the mitochondrial membrane changes and oxidative stress, and inhibited OA osteoblast senescence (Duarte, 2015). Additionally, numerous studies have indicated that there is a close relationship between autophagy and cartilage biology and the pathology of TMJOA (Almonte-Becerril et al., 2010; Lotz et al., 2011; Jeon and Im, 2017; Tofiño-Vian et al., 2017). It was demonstrated that infrapatellar fat pad (IPFP)

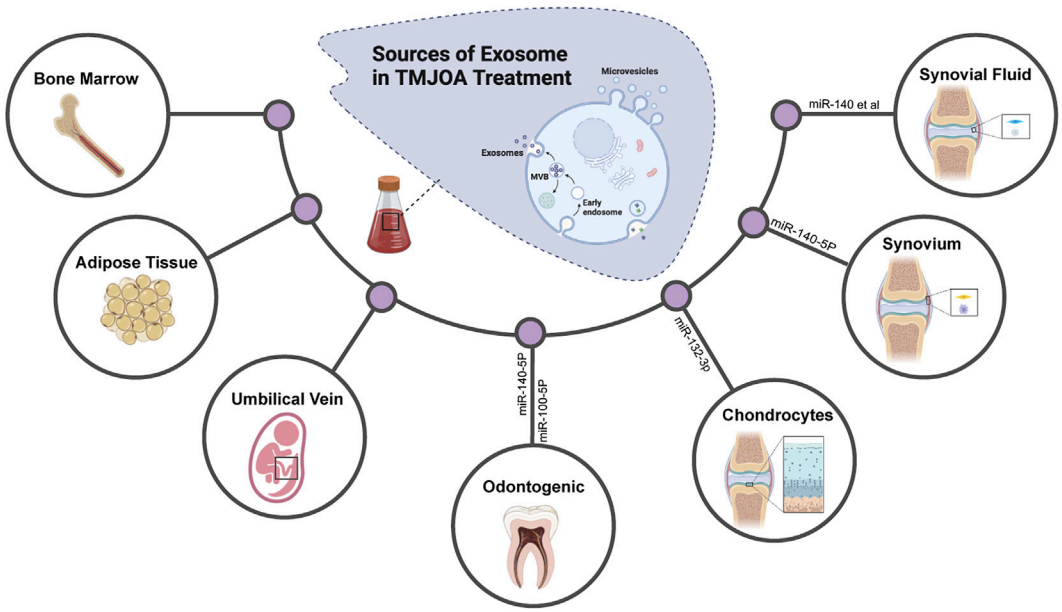

FIGURE 3
Exosomes derived from different tissues and cells are applied for TMJOA treatment. Diversified exosomes are obtained and applied in OA treatment to have a deeper understanding of the occurrence, development of OA, and to find more novel targets in molecular mechanisms of TMJOA treatment.

MSCs-derived exosomes inhibited apoptosis and balanced the anabolic and catabolic processes of chondrocytes to prevent cartilage from damage by exosomal-miR100-5p-mediatied inhibition of mTOR-autophagy pathway (Ribeiro et al., 2016). The TMJ cavity is a highly complicated environment involving a variety of different cells. Undoubtedly, exosomes secreted by these cells play an important role in the regulation of microenvironment homeostasis.

Chondrocytes-derived exosomes

The chondrocyte is the only cell type of cartilage and is critical in the maintenance of cartilage homeostasis. The effect of primary chondrocytes-derived exosomes on TMJOA has been confirmed (Ni et al., 2019; Wu et al., 2019; Zheng et al., 2019; Liu S.-S. et al., 2022; Nikhil and Kumar, 2022). Liu noticed exosome-like structures in abnormal calcified cartilage together with the decrease of matrix Gla protein and the increase of tissue-nonspecific alkaline phosphatase, CD63 and pyrophosphatase/phosphodiesterase-1 in TMJOA (Ni et al., 2019). After local injection of the exosome inhibitor, the process of calcification was inhibited. They speculated it was a new way in preventing and treating TMJOA to inhibit degenerative chondrocyte-derived exosomes. Chondrocytes-derived exosomes positively affected proliferation of chondrocytes and exhibited significant wound closure promotion due to roles in intercell communication. During the repair of cartilage injury, bilayered cryoge and chondrocytes-derived exosomes had a synergistic effect (Liu Q. et al., 2022). Furthermore, Zheng investigated the proteomics of primary chondrocyte exosomes and found the 2409 proteins of exosomes were involved in mitochondrial damage or dysfunction and immune system process. They attributed the protective effects of chondrocyte-exosomes on osteoarthritis to mitochondrial dysfunction elimination and M1-proinflammatory macrophages infiltration decrease with a concomitant M2- anti-inflammatory macrophage increase in cartilage (Nikhil and Kumar, 2022). It could be inferred from Liu' results that the exosomes released by articular chondrocytes inhibited catabolism and increased mRNA levels of ACAN and COL2A as a facilitator of cell communication (Zheng et al., 2019). Chondrogenic progenitor cells (CPCs) have MSC characteristics with strong potential of cartilage differentiation and self-renewal ability (Liu et al., 2020). EVs secreted by CPCs from MRL/MpJ superhealer mice (MRL-EVs) had shown superior therapeutic capability in attenuating OA compared with control mice-EVs. MRL-EVs played a vital role in stimulating the proliferation and migration of chondrocytes (Koelling et al., 2009). After miRNA-seq analysis of exosomes, AMPK signaling, regulation of autophagy, and insulin signaling were observed to be associated with differentially expressed miRNAs and miRNA 221-3p was highly enriched in MRL-EVs.

Synoviocytes-derived exosomes

Synovial inflammation is observed on magnetic resonance imaging of OA affected joints (Wang et al., 2020). There is increasing evidence that synovial inflammation is positively correlated with TMJOA severity (Roemer et al., 2010), and persistent low-grade synovial inflammation exacerbates cartilage damage (Raghu et al., 2017). Kato analyzed effects of synoviocytes-derived exosomes on chondrocytes and HUVECs (Kato et al., 2014). Compared with resting synoviocytes-derived exosomes, exosomes from IL-1β stimulated synoviocytes significantly promoted matrix catabolism and inhibited anabolism of chondrocytes. Migration and tube formation activity of HUVECs were improved. These findings indicated that SFCs exosomes represented a novel mechanism in the pathogenesis of osteoarthritis, which implied exosomes might be used as a therapeutic strategy for TMJOA. Dysregulated angiogenesis deteriorates the cartilage degradation, bone destruction and synovitis (Kato et al., 2014). Feng Yaping reported HMGB1 increased VEGF and HIF-1α in synovial fibroblasts of TMJOA and conditioned medium obtained from High-mobility group protein 1-treated TMJOA SFCs promoted the migration and tube formation of HUVECs (Chavakis et al., 2007). In addition, it was observed that synoviocytes-derived exosome-mediated cartilage repair was achieved by improvement in cell activity and migration ability as well as reduction of ECM degradation, of which synoviocytes-derived exosomal-lncRNA H19 suppressed the miR-106b-5p/TIMP2 axis (Feng et al., 2021). It was confirmed that the expression of miRNA-126-3p was sharply reduced in synovial fluid exosomes from OA patients. Exosomes derived from miR-126-3p-overexpressing synovial fibroblasts enhanced chondrocytes proliferation and suppressed chondrocytes apoptosis. What's more, the exosomes significantly constrained the inflammation in chondrocytes by decreasing the IL-1β, IL-6, and TNF-α (Tan et al., 2020).

Synovial tissue maintains the basic composition and volume of synovial fluid. SFCs secrete synovial fluid, which in turn provides a low friction environment and nourishes surrounding tissues. Recently, many studies have focused on analyzing and comparing the synovial fluid derived exosomes differences between osteoarthritis patients and healthy people to find new molecular targets and related mechanisms for the treatment of osteoarthritis (Kolhe et al., 2017; Zhou et al., 2021). It has been proposed that miRNA contents differ between OA patients and healthy people. Moreover, there is a high gender-specific differential expression of miRNA in synovial fluid-derived exosomes in patients with OA (Zhou et al., 2021). Chondrocytes treated with OA-derived EVs had down-regulated expression of anabolic metabolism and elevated expression of catabolic metabolism and inflammatory molecules. Previous studies demonstrated that synovial fluid-derived exosomes of OA patients possessed the characterization of the proinflammatory profile to M1 macrophages. The exosomes upregulated the IL-1β expression and induced the release of chemokines and promoted the production of MMP7 and MMP12 (Kolhe et al., 2017).

Subchondral osteocytes-derived exosomes

Subchondral bone supports the surface cartilage and bears the mechanical load. The crosstalk between the cartilage and subchondral bone is proceeding in an orderly manner, conducted in an exosome-dependent pattern (Domenis et al., 2017). Once the balance of the interaction is disrupted, cartilage breaks down and subchondral bone remodels abnormally, exacerbating the progression of OA (Wu et al., 2022). TMJ is one of the most flexible joints in the body and the subchondral bone of TMJ has an outstanding ability to withstand multidirectional forces. Sun discovered a new mode of osteoclast-osteoblast communication. MiR-214-enriched exosomes secreted by osteoclasts were specifically transferred into osteoblasts *via* ephrinA2/EphA2 axis and suppressed osteoblast function (Sanchez et al., 2005). Moreover, there was an obvious promotion of bone formation after osteoclast-targeted miR-214-3p inhibition (Sun et al., 2016).One In coculture, researchers found that chondrocytes endocytosed the osteoblast derived exosomes in osteoarthritis sclerotic subchondral bone and upregulated catabolic genes and downregulated chondrocyte-specific genes. Wu demonstrated miR-210-5p suppressed the oxygen consumption of chondrocytes and altered cellular bioenergetics, which could be a potential target for therapeutic intervention in OA (Li et al., 2016). It is suggested that targeting the exosomal-miRNAs-transfer of osteoclasts to chondrocytes is an entirely new treatment strategy. In early-stage osteoarthritis, an upregulation of exosomal-osteoclast-derived microRNAs drove the progression of the disease. However, blockage of osteoclast-originated exosomes retarded osteoarthritis progression, mechanistically, *via* increasing the resistance of chondrocyte to matrix degeneration, endothelial cell angiogenesis and axon sensory innervation (Xu et al., 2021). To explore the potential osteogenesis of the exosomes from osteoblasts, Ge isolated EVs from MC3T3 and presented osteogenesis-related proteins and pathways through the protein profile. Eukaryotic initiation factor 2 pathways played an important role in osteogenesis and represented a potential therapeutic avenue to tackle OA (Liu et al., 2020). Mineralizing osteoblasts-derived exosomes significantly promoted osteogenesis and influenced miRNA profiles in bone marrow stromal cells, which activated the WNT pathway by increasing β-catenin and dampening Axin1 (Ge et al., 2015).

Adipose tissue-derived exosomes

Adipose tissue-derived exosomes are applicated in the treatment of various diseases (Xu et al., 2019; Zhou Q.-F. et al., 2020; Wei et al., 2020). Intra-articular adipose tissue functions to cushion the shock and acts as one of major sources of cytokines, active mediators as well as regenerative cells in repair. At present, research of adipose-derived exosomes for the treatment of OA mainly focused on adipose tissue-MSCs-derived exosomes (Koh et al., 2012; Ribeiro et al., 2016; Scheja et al., 2019). Sembronio et al. (2021) compared standard OA treatment with hyaluronic acid injections with the new TMJOA therapy of microfragmented adipose tissue injection using the Lipogems technology by a randomized clinical trial. Notably, pain reduction and mouth opening significantly improved in both groups. And the statistical analysis showed that the microfragmented adipose tissue injection group had a statistically significant advantage in the success rate of procedure compared with the hyaluronic acid injections group. Considering the number and secretion capacity of adipocytes, we speculate that their role in osteoarthritis is also critical because they may work as a graft in synovial, secretes exosomes and locally serve as a source of MSCs for a long time. However, a lot of investigations into adipocytes-derived exosomes are still needed to shed light on molecular mechanisms underlying pathogenesis.

Other cell and tissue-derived exosomes

As early as in 2005, microparticles derived from T cells and monocytes were clearly reported to induce the synthesis of matrix metalloproteinases and inflammatory mediators in fibroblasts in a dose-dependent manner (Cui et al., 2016). These results provided evidence for vesicles derived immune cells promoting the destructive activity of SFs. It was reported that Sox9-containing-exosomes of monocytes stimulated with IL-4 or IL-13 upregulated COL2A and ACAN, promoted the differentiation of primary chondrocytes (Distler et al., 2005). Abnormality of the tendon was related to OA progression, which indicated that tendon repair might be another treatment for injury.40 In recent years, some studies have evaluated the role of tendon-derived exosomes in osteoarthritis. In the transwell system, paracrine factors released by tenocytes induced MSCs to the tenogenic differentiation in a TGF-β dependent manner and the inhibition of TGF-β pathway eliminated the effect (Bai et al., 2020).

Noticeably, since the 1970s, many studies have explored the mechanism of platelet rich plasma (PRP) in tissue repair (Toghraie et al., 2011). It contains a variety of cytokines and active substances, promoting tissue regeneration and healing (Xuan et al., 2020). So far, exosomes have been reported to exist in PRP and participate in related physiological and pathological processes (Dohan et al., 2008). Actually, PRP-derived exosomes have been applied to OA treatment *via* intra articular injection for years in preclinical studies. Zhang incorporated PRP-exosomes into thermosensitive hydrogel (Gel) and assessed its biological activity and the therapeutic effect on OA *in vivo* (Saumell-Esnaola et al., 2022). It promoted BMSCs proliferation, migration, and chondrogenic differentiation, and inhibited chondrocytes apoptosis and hypertrophy to delay the progression of osteoarthritis. Meanwhile, PRP-derived exosomes inhibited the TNF-α release from chondrocytes and presented a potential in alleviating OA *via* WNT/β-catenin pathway (Zhang Y. et al., 2022). Alexander found that citrate-anticoagulated platelet-rich plasma-derived exosomes displayed a higher expression of SOX9 protein and a better inhibition effect on proinflammatory cytokine release compared to hyperacute serum-derived exosomes (Liu et al., 2019). Besides, amniotic fluid (AF) is easily to obtain for application in tissue repair and regeneration. Researchers elucidated (Raghu et al., 2017) commonly expressed exosomal-miRNA of AF-derived exosomes, revealed RNA target genes were associated with senescence, fibrosis, and OA pathways, and suggested it as a therapeutic potential strategy for the treatment of osteoarthritis (Otahal et al., 2020).

To date, exosomes derived from different cells and tissues have exhibited effects on the occurrence, development, prevention, and treatment of TMJOA *in vitro* and *in vivo*. On the one hand, exosomes play a decisive role in controlling cartilage matrix homeostasis by promoting chondrocyte proliferation and migration and inhibiting chondrocyte apoptosis, thus reversing the deterioration of TMJOA, and alleviating the symptoms of TMJOA. On the other hand, the exosome is trigger in promotion of MSCs migration *via* various chemotactic pathways and can stimulate chondrogenic differentiation, and repairs cartilage defects. Besides, it cannot be ignored that they also have the great potential in regulating bone homeostasis to better support cartilage. A large number of studies have shown that changes of exosomes in the state of inflammation, which suggests that exosome-based disease treatment strategies will be effective. In the exosome based TMJOA therapy, they reduce the production of inflammatory factors and inhibit the differentiation of proinflammatory M1 macrophages and increase the ratio of M2 macrophages. However, it is just the beginning and further research is urgently needed to explore a more in-depth mechanism and perfect treatment strategy in TMJOA.

Optimization in exosome-based bioengineering strategies of TMJOA therapy

Owing to the uniqueness and complexity of the TMJ, it is a great challenge to achieve complete restoration of its anatomical,

TABLE 3 Bioengineering materials combined with exosomes for repair and regeneration of cartilage.

Biological material	Composition	Source of exosomes	Retention and release efficiency of exosomes	Delivery	Mechanism	Reference
3D printed scaffold with radially oriented channels	Gelatin methacrylate; Decellularized cartilage ECM	BMSCs	Retention: >56% for 14 days	Implantation in site of defect	Increase chondrocyte migration; Simulation of M2 macrophage polarization; Enhancement of cartilage and subchondral bone regeneration	Chen et al. (2019)
Acellular cartilage ECM with vertically oriented structure	Porcine articular cartilage	Wharton's jelly derived MSCs	Not reported	Implantation in site of defect; Articular injection of exosomes	Promote BMSC and chondrocyte proliferation, BMSC migration and macrophage polarization toward the M2 phenotype	Jiang et al. (2021)
Photoinduced imine crosslinking hydrogel glue	O-nitrobenzyl alcohol moieties modified hyaluronic acids, Gelatin	Induced pluripotent stem cell line	Retention: >90% for 14 days; Release: 1×10^{10} particles/ml/day	Full-thickness defect with an *in situ* formed EHG tissue patch	Promote the migration and proliferation of chondrocytes and hBMSCs; Penetrate into the subchondral bone and formed a seamless interface-cartilage integration ability	Liu et al. (2017a)
Mussel-inspired hydrogel	Alginate-dopamine; Chondroitin sulfate; Regenerated silk fibroin	BMSCs	Release: 87.51% ± 3.71% for 14 days	Injection in site of defect	Promote the recruitment, proliferation and differentiation of BMSCs	Zhang et al. (2021)

structural, and functional integrity. The optimization of exosome-based strategy is a necessary step for TMJOA treatment. Bioengineering is constantly developing and provides an optimized solution in regenerative medicine (Table 3). Helgeland made a systematic review to answer the question of whether scaffold based TMJ tissue regeneration have better outcomes in TMJOA treatment. The overall preclinical evidence indicated that biomaterial scaffolds combined with biological components enhanced the potential for cartilage regeneration in TMJOA (Bellio et al., 2020). Additionally, the optimization of bioengineering technology in exosome based TMJOA therapeutic strategy has aroused the hot interests of researchers. There might be several disadvantages with direct administration of exosome-containing suspension in cartilage regeneration, especially, the difficulty of local exosome retention. Liu developed a photoinduced hydrogel exosome scaffold for a better retention of cargo exosome (Helgeland et al., 2018). In the system, they demonstrated that it retained stem cell-derived exosomes and showed an excellent biocompatibility and cartilage-integration by positively regulating both chondrocytes and BMSCs *in vitro* and promoting cartilage matrix and cell deposition at cartilage injury site. Using a crosslinked network of chondroitin sulfate, alginate-dopamine, and regenerated silk fibroin, an injectable hydrogel with encapsulated exosomes was exploited in superficial cartilage regeneration. Exosomes released by the hydrogels recruited BMSCs into defects *via* the chemokine pathway (Liu X. et al., 2017). These findings revealed the hydrogel coated with exosomes as a promising approach for accelerating cartilage regeneration *in situ* and neo-cartilage extracellular matrix remodeling. Chen designed a 3D printed cartilage extracellular matrix-gelatin methacrylate-exosome delivery scaffold (ECM/GelMA/Ex scaffold) with radial channels and superior cell recruitment capacity. The scaffold not only enhanced cartilage regeneration but also facilitated recovery of subchondral bone. Furthermore, they also found that MSCs exosomes enhanced mitochondrial biogenesis and rescued the mitochondrial dysfunction in degenerated cartilage (Zhang et al., 2021). The controlled exosome release platform with histological biological scaffolds solves the problems of insufficient local exosome concentration and short half-life of exosomes after injection to a large extent, optimizing the exosome based TMJOA treatment strategy.

The dense matrix biological barrier of cartilage makes chondrocyte-targeted drug delivery difficult. Exosomes enter the cell mainly *via* endocytosis, direct membrane fusion, or pinocytosis, 66 and the released contents could exert biological effects. It indicates exosomes have a great potential as a vehicle for drug delivery. Hence, it is an innovation and optimization in TMJOA treatment to modify surface structures for improving the efficiency of exosomes entering cells and to modulate encapsulated contents for strengthening therapeutic effects by genetic engineering technology or direct physicochemical loading. A study demonstrated that exosomes gained MSC targeting capability after fusing exosomal membrane protein Lamp 2b with MSC-binding peptide E7 (Chen et al., 2019). SFMSCs with E7-exosomes entered the middle zone of the cartilage more easily. Additionally, BMSCs-derived exosomes

loading with KGN (a small molecule that can induce MSCs differentiation to chondrocytes) by electroporation efficiently increased COL2 and ACAN and induced higher cartilage differentiation of SFMSCs. *In vivo*, it showed best cartilage repair. ATF4-overexpressing exosomes were developed by introducing the mRNA of ATF4 into exosomes *via* electroporation. It showed these exosomes alleviated inflammation and cartilage degeneration in osteoarthritis mice by promoting chondrocytes autophagy and inhibiting apoptosis (Xu et al., 2021). To achieve a more predictable and desirable clinical response, specific therapeutic miRNA enrichment could be performed through the overexpressing genetic technique. It was suggested that miR-140-5p-overexpressing hSDMSCs derived exosomes enhanced the proliferation and migration abilities of chondrocytes (Wang Z. et al., 2021). They observed the exosomes highly expressed miR-140-5p blocked this side-effect in ECM *via* targeting RalA to enhance SOX9 and ACAN. Exosomes derived from miR-92a-3p-overexpressing MSCs targeted WNT5A to elevate chondrogenesis in MSCs and suppress cartilage degradation in primary chondrocytes (Tao et al., 2017). Besides, hBMSC-derived overexpressing miR-26a-5p exosomes relieved OA and were served as a repressor to retard damage of SFs *via* PTGS2 downregulation *in vitro* (Mao et al., 2018). Generally, utilizing a specific exosomal-miRNA mainly involves these proposed mechanisms, the overexpression of miRNA in cells, the isolation of exosomes containing miRNA, then delivery to chondrocytes in inflammatory microenvironment or TMJOA animal models, and finally targeting a pathogenic gene *via* miRNA. Some studies have shown that the effect of hypoxia-preconditioned exosomes on cartilage repair are superior to that of normoxia-preconditioned exosomes, manifesting in the promotion of chondrocyte proliferation and migration and the inhibition of chondrocyte apoptosis (Jin et al., 2020). TGF-β1-stimulated BMSCs-derived exosomes highly expressed miR-135b and polarized synovial macrophages (SMs) into M2 type to alleviate cartilage destruction. M2 polarization of SMs was significantly reversed by increase of MAPK6 (Zhang B. et al., 2022). Pretreating exosomes with physical or chemical stimulation optimizes exosome-based therapeutic strategies.

Future opportunities and challenges of exosome-based therapeutic strategy in TMJOA

Although exosomes were originally regarded as useless metabolic byproducts, it is well recognized that exosomes, as various carrier of signaling mediators, play a vital role in mediating cell-to-cell communication and in activating immunomodulatory activity. Certainly, numerous studies have shown exosomes are sufficient to treat degenerative diseases, including systemic OA and TMJOA. A correct view of the prospects and existing problems of exosome-based therapeutic strategy is the basis for further research.

Emerging as a trending research area, exosome-based therapeutic strategy in TMJOA has gained much interest because of its unique regulatory ability in TMJ inflammation as well as the low immunogenicity (Wang Y. et al., 2021). Some studies have reported that MSCs show a certain degree of immunogenicity in mediating disease treatment (Qi et al., 2016; Gregory et al., 2005). Compared with MSCs, exosomes have been reported not to express class II human leukocyte antigens and have lower immunogenicity when applicated *in vivo* (Krampera et al., 2006; Stagg et al., 2006; Sun et al., 2009; Geiger et al., 2015; Qi et al., 2016; Yaghoubi et al., 2019; Kang et al., 2020). TMJ is a complex system, and the immune privilege of exosomes maximally ensures it not to be cleared by immune cells when playing the therapeutic role in TMJOA. In addition, at present, few studies have reported the tumorigenic effect of exosomes *in vivo*, which might occur in the MSC-based therapy (Wislet-Gendebien et al., 2012; Funes et al., 2014; Li et al., 2022). Because cartilage is a dense biological barrier staggered by collagenous fiber, the transport property of exosomes is advantageous in TMJOA treatment. Being nano-sized and biocompatible, exosomes can be served as nanocarriers, easily reaching to the cartilage to fuse with chondrocytes. Certainly, the side effects associated with cell-based therapy, such as vascular embolism and pulmonary embolism (De Boeck et al., 2013; Jung et al., 2013), are also rare when exosomes are injected systematically. Furthermore, the less strict storage condition also gives exosomes greater possibilities for therapeutic application. Low temperature cold storage or repeated freezing and thawing does not influence exosome sizes and bioactivities, which shows higher clinical application value compared with cell-based therapies. It takes more time to resuscitate frozen cells to normal functional state, and the activity of resuscitated cells cannot be predicted (Mäkelä et al., 2015). Most importantly, many studies have shown that cell-cell interactions are mainly dependent on exosomes. MSCs-derived exosomes share the same or even more powerful biological effects than MSCs (Zhou X. et al., 2020), such as metabolic regulation of cartilage matrix, inhibition of inflammatory factors, relief of TMJOA pain, and homing of cells to the cartilage defect. Moreover, exosomes modified by genetic engineering have higher organotropism and cartilage-targeting capability. It has involved the comparison of exosomal differences in organs or systems between healthy and sick populations. High-throughput sequencing has confirmed that exosomes in the joint system differ between normal state and TMJOA, which indicates exosomes can be used as biomarkers for the early diagnosis of TMJOA. MiRNA is one of the important cargoes of exosomes. Through literature review, we noticed some exosome-derived miRNAs related with TMJOA treatment. RNAhybrid and miRanda databases were used to predict the target genes of the miRNAs, and Gene ontology (GO) and Kyoto

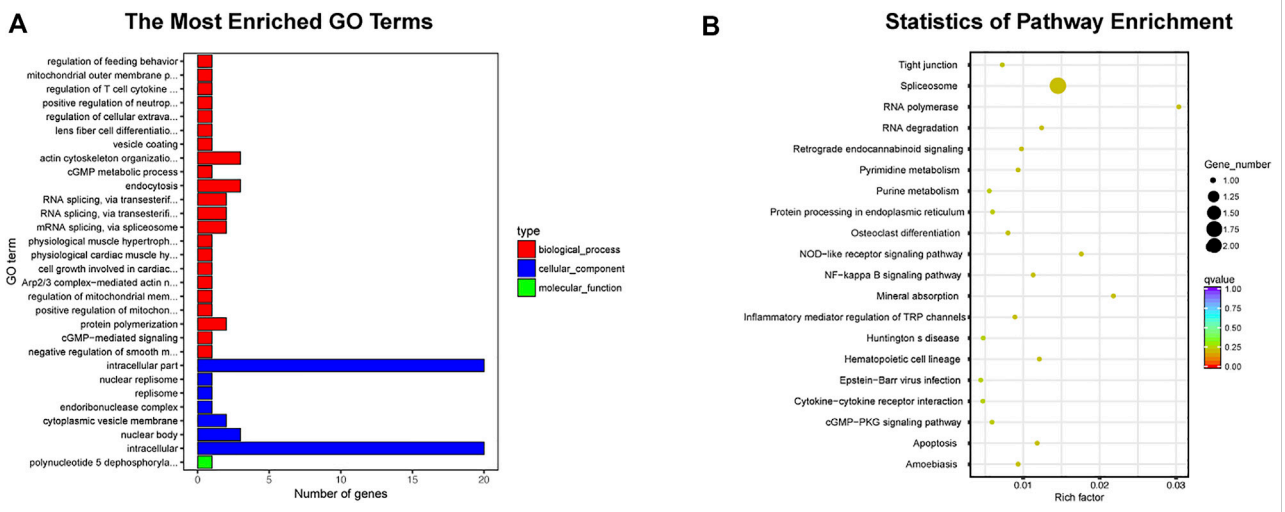

FIGURE 4
Enrichment analysis of exosome-derived miRNAs clearly related to TMJOA. **(A)** GO analysis, 30 enriched iterms are shown. **(B)** KEGG analysis, 20 pathways are shown.

Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed for the identified target genes (Figure 4). It showed 22 biological processes enriched in the GO analysis, including endocytosis, actin cytoskeleton organization, protein polymerization, and other processes. And it displayed 20 signaling pathways obtained by the KEGG analysis, including osteoclast differentiation, inflammatory mediator regulation of TRP channels, apoptosis, and other pathways. The information of enrichment analysis suggested the mechanism and related targets of exosomes in the treatment of TMJOA, which will help us further study the pathogenesis of TMJOA and find out more effective TMJOA therapeutic strategy in the future.

Despite the excellent therapeutic effects of exosomes, there are many issues that need to be addressed. Unlike TMJOA cell-therapy, although results from preclinical studies have demonstrated the chondroprotection role of exosomes, explorations into the exosomes efficacy in treatment are still in the start-up stage. Currently, the research on TMJOA treatment mainly focused on small animals. There is almost no large animal studies or human clinical trials to evaluate exosome-based therapeutic strategy in TMJOA. Insufficient evidence from preclinical research and clinical trials significantly hindered the elucidation of mechanisms and the clinical translation applications. Therefore, future studies are recommended to bridge this knowledge gap and validate the safety and efficacy of exosomes therapy. Meanwhile, the difficulties encountered in the acquisition and the preparation of exosomes are inescapable. Various methods of exosomes separation *in vitro* have been developed, such as ultracentrifugation-based technique, size-based technique, and immuno-affinity action-based technique (Lőrincz et al., 2014). However, the most standardized and optimal operational procedure has not yet been established. The comparability between different studies is poor due to the differences of yield and purity of exosomes. Extracting homotypic exosomes with consistent contents is crucial in precisive therapy and in reduction of adverse effects caused by unintended unknown by-products (Ding et al., 2021). It is urgent to develop an optimal isolation procedure, which maximizes yield and purity of exosomes and minimizes changes of contents and sizes during extraction. In addition, due to the quick turnover of synovial fluid in TMJ cavity, more studies are needed to determine the effective dose and frequency of exosomes injection. Like cell-based therapy, exosome-based therapeutic strategy is also limited by rapid clearance *in vivo* and short effective period in direct injection. Therefore, it is particularly critical to optimize the therapeutic strategy of exosomes in TMJOA *via* tissue engineering approaches. Notably, the cartilage of TMJ is fibrocartilage, which is different from the hyaline cartilage of the most joints of the body. It is made up of various proportions of both cartilaginous tissue and fibrous and has a more complex tissue structure and tensile and compressive strength. Although there are many studies on the treatment of OA, it is still questionable whether these treatment measures are also effective for TMJOA. We should explore the effectiveness of these treatments on TMJOA in a more scientific and rigorous manner. 'A one size fits all' therapeutic scaffold may not achieve the best treatment effects in TMJOA (Embree et al., 2016; Jiang et al., 2021; Jiang et al., 2021; Fan et al., 2022). According to the

characteristics of different fibrocartilage tissue types, layered scaffolds loaded with exosomes exhibit outstanding advantages in the formation of layered tissue structure in cartilage regeneration to simulate the normal fibrocartilage to the maximum extent. Though faced with challenges, exosome-based therapeutic strategy is promising in TMJOA and worthy of further investigations *in vivo* and *in vitro*.

Conclusion

In this review, we summarized the roles of MSCs and exosomes in TMJOA, manifesting in the regulation of cartilage matrix metabolism, the balance of subchondral bone homeostasis, the relief of inflammation, and the effects of immune regulation. Currently, MSC-based therapy is facing many challenges, while exosome-based therapeutic strategy can be a promising novel alternative because of its advantages in cell-to-cell communication in TMJ system. Exosomes, as mini vesicles, deliver nucleic acids and proteins to target tissues or cells and exert therapeutic efficacy in TMJOA. The pathogenesis of TMJOA is complicated and multifactorial. The optimization of the existing exosome-based strategies, such as the combination of tissue engineering scaffolds or genetic modification, more effectively reduce the side effects involved in exosomes treatment and improve cartilage repair and regeneration. However, the translation from experimental research to clinical application of exosomes has been hindered due to insufficient evidence of preclinical and clinical trials. Further research is needed to identify effective constituents of TMJO-target exosomes and explore underlying mechanisms, to investigate therapeutic targets, to evaluate the safety of exosomes application, and finally to establish a consensus in the therapeutic potency of exosome-based therapeutic strategy.

Author contributions

Conceptualization, JW, JnL, and WY; writing—original draft preparation WY; writing—review and editing YW, MH, JnL, XZ, YY, JW, and JqL; supervision, project administration, funding acquisition, JW and JnL; created the table, WY and MH; prepared the figures, WY, YW, MH, JqL, XZ, and YY. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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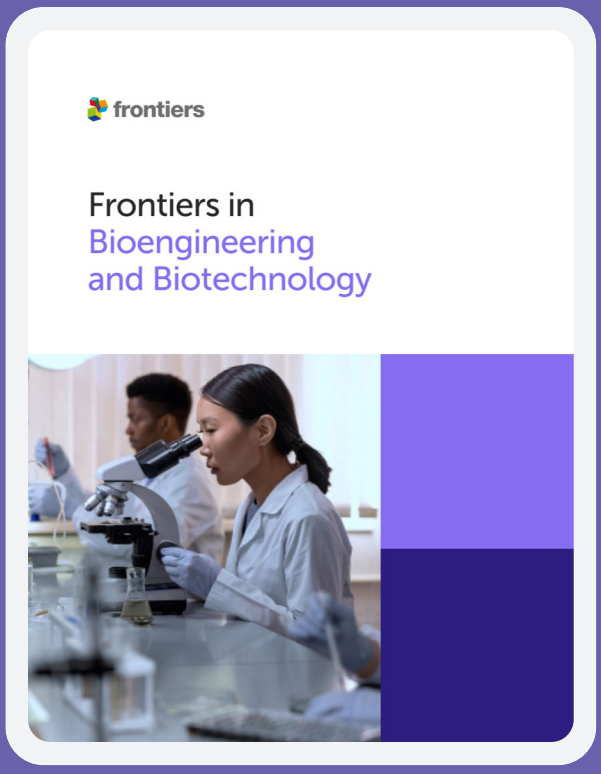

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