

*Sex considerations in Regenerative Rehabilitation strategies for the treatment of knee osteoarthritis*

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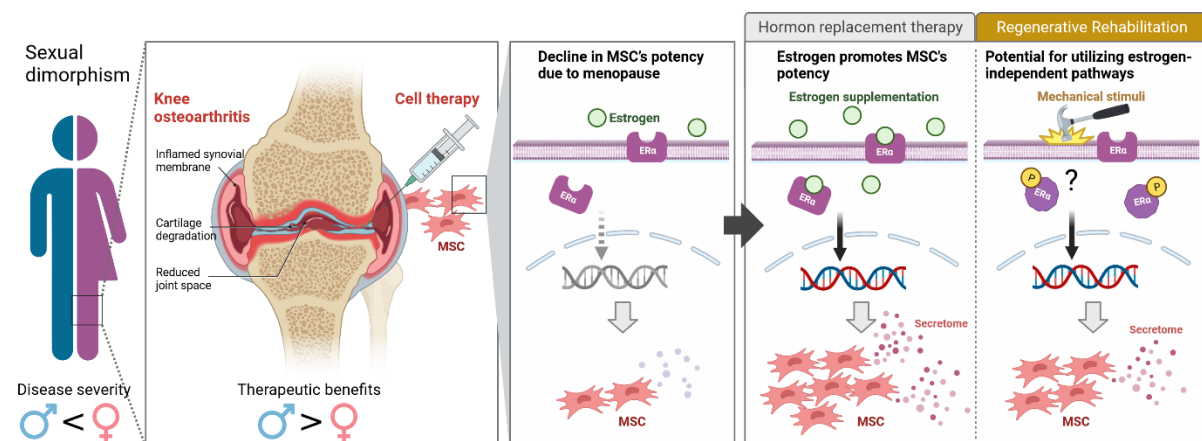
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## ABSTRACT

Regenerative rehabilitation for knee osteoarthritis (KOA) has emerged as a promising field aimed at harnessing the regenerative potential of stem cell therapeutics to maximize functional recovery. Here, we outline recent advancements in the field of regenerative rehabilitation in the treatment of KOA and we highlight sex-specific considerations that we contend will be important for translation to the clinic. According to a systematic review, the currently available evidence suggests that sexual dimorphism in the efficacy of regenerative rehabilitation approaches for the treatment of KOA may be attributed, at least in part, to the functional decline of female mesenchymal stem cells (MSCs) over the lifespan, particularly after menopause. These declines are likely to be accompanied by poor clinical outcomes. While evidence is far from adequate, physical therapeutics have emerged as a means to promote estrogen signaling in MSCs, potentially reversing menopause-related MSC dysfunction. This study calls for actions to dissect the effects of menopause, together with physical therapeutics, on stem cell therapeutics towards the development of effective regenerative rehabilitation approaches.

## KEYWORDS

Regenerative rehabilitation, knee osteoarthritis, mesenchymal stem cell, sexual dimorphism



## Graphical summary of main concepts.

### Regenerative rehabilitation for knee osteoarthritis considering sexual dimorphism.

While females experience a higher severity of knee osteoarthritis, cell therapy has been shown to elicit greater therapeutic effects in males. This difference might be linked to the reduced estrogen levels in post-menopausal females. Such a decrease in estrogen could lead to weakened signaling through the estrogen receptor alpha within mesenchymal stem cells and chondrocytes. We propose that regenerative rehabilitation is a promising strategy to address this disparity. Such an approach leverages the advantages of mechanical stimuli, potentially together with estrogen supplementation, to promote estrogen receptor alpha-mediated signaling. This figure was created using BioRender.com.

## MAIN TEXT

### 1. INTRODUCTION

Regenerative rehabilitation refers to an integrated approach that combines regenerative medicine techniques with rehabilitation strategies to improve the function and quality of life for individuals with various pathological conditions, including knee osteoarthritis (KOA)(1, 2). KOA is a degenerative joint disease characterized by the breakdown of cartilage in the joints, leading to pain, stiffness, and decreased mobility(3). The intersection of regenerative rehabilitation and KOA lies in the potential of regenerative medicine techniques, including mesenchymal stem cell (MSC) therapeutics, to promote tissue regeneration in osteoarthritic joints. For example, physical stimulation promotes secretome release from MSC(4), which underscores the pivotal role of rehabilitation on the regenerative capacity of stem cell-based therapeutics to maximize patient functional recovery.

Studies to date have recognized the significance of accounting for sexual dimorphism in the studies of KOA. As an example, post-menopausal women exhibit both a higher incidence and more severe disease presentation of KOA(5). Moreover, hormonal differences, biomechanical factors, and inflammatory responses unique to females significantly impact disease onset, severity, and treatment outcomes(6). Building upon these premises, a few studies have begun to address the sexual dimorphism by analyzing the effects of regenerative therapeutics according to sex(7, 8). The findings indicate that both the stem cells themselves and the surrounding host tissue microenvironment contribute to the sexual dimorphism in the treatment of KOA.

In this perspective, we outline recent advancements in regenerative rehabilitation identified through systematic review and the need to consider sexually-dimorphic responses across the translational spectrum. We first summarize the existing knowledge regarding pre-clinical and clinical regenerative rehabilitation trials designed that employed stem cell-based regenerative therapeutics. We then summarize current evidence regarding sex-dependent behavior of donor stem cells in the knee joint. The overview provided in this study brings us one step closer towards establishing effective and personalized regenerative rehabilitation for individuals with KOA.

### 2. MAIN TEXT OF REVIEW

#### 2.1 Why regenerative rehabilitation for the treatment of KOA?

KOA represents a major public health burden, affecting more than 30 million older individuals in the United States alone. The number of years lived with KOA-induced disability has increased by 34% from 1990 to 2020(9), suggesting that the impact of KOA on society is rapidly expanding. Despite its profound impact on elderly populations, progress in developing effective interventions for KOA has been slow, with current therapeutic strategies primarily focusing on symptom management and compensatory approaches to improve physical function.

Articular cartilage health is largely dependent on the activity of chondrocytes, the major cell type in cartilage. Chondrocytes play an essential role in maintaining cartilage integrity and homeostasis through nutrient exchange, extracellular matrix synthesis, and response to mechanical loading(10). Owing to their low cell density (~2% of the total volume of cartilage), chondrocytes minimally rely on cell-to-cell contact as a means of directing signal transduction and cellular phenotype(11). Instead, chondrocyte phenotype and behavior largely depend on input signals from the surrounding microenvironment(12). Consequently, the disrupted interaction between chondrocytes and their microenvironment is a leading cause of KOA(12,

13). As such, opportunities for disease-modifying therapeutics for treating KOA involve strategies to manipulate chondrocyte microenvironment, including mechanical and thermal microenvironment that has been shown to influence articular chondrocyte extracellular matrix synthesis and cartilage integrity(14-17). Based on this premise, we have been investigating the disease-modifying effects of varying doses exercise in the setting of KOA. Studies have revealed that, while intense level exercise accelerated development of KOA, physiological moderate level exercise restored cartilage integrity(18-21). However, the chondroprotective effect of moderate exercise is still far from adequate, with articular cartilage exhibiting a degenerative histological profile not comparable to young, healthy controls. This underscores the need to develop therapeutic strategies to manipulate chondrocyte unfavorable microenvironment in the setting of KOA in order to promote the chondroprotective effects of moderate exercise.

One potent strategy to manipulate unfavorable cellular microenvironment is to leverage the advantages of stem cell-based therapeutics in combination with rehabilitation programs. Among the stem cell therapeutics, MSCs have emerged as a cell type with great potential for cell-based articular cartilage repair in individuals with KOA(23). These therapeutic effects are attributed, at least in part, to MSC engraftment and/or trophic factors secreted from MSC(24). MSCs have the capacity to synthesize and secrete a variety of bioactive molecules, including growth factors, cytokines, and extracellular vesicles, which can influence their microenvironment and interact with neighboring host cells(25). Notably, researchers have recently begun to recognize the effects of rehabilitation programs, including physical stimuli, as a means to promote the regenerative potential of stem cell therapeutics. For instance, application of mechanical loading promote chondrogenic potential and secretome release of MSC(4).

Regenerative Rehabilitation is the integration of principles and approaches from rehabilitation and regenerative medicine with the ultimate goal of developing innovative and effective methods that promote the restoration of function through tissue regeneration and repair(1, 2). The most significant feature of regenerative rehabilitation is not merely optimizing rehabilitation protocols after cell therapy, but synergistically enhancing outcomes by combining cell therapy with rehabilitation treatments. Essentially, this growing field seeks to ensure that transplanted cells behave appropriately within the human body, using the body as a natural bioreactor. Regenerative rehabilitation also encompasses stimulating the tissue-resident somatic stem cells to enhance tissue regeneration and repair, even without concurrent cell transplantation therapies.

## **2.2 Clinical and pre-clinical evidence of regenerative rehabilitation designed to treat KOA**

Towards establishment of regenerative rehabilitation designed for individuals with KOA, we recently undertook a series of studies to understand the rigor with which the efficacy of cellular therapies are being tested in individuals with KOA(26). Meta-analysis of 35 studies published by 2017 investigating the transplantation of MSCs revealed not only that MSC implantation decreases pain levels, but that those studies that included a rehabilitation component were associated with improved self-reported physical functioning(26). Importantly, we found that none of the studies stratified patients according to the presence of a rehabilitation program. A major conclusion of this work is that future studies to evaluate the potential synergy of physical therapeutics and MSC transplantation in the treatment of individuals with KOA are much needed. The lack of studies designed to assess patient outcomes according to the presence of a rehabilitation program has yet to be addressed even in recently published clinical trials(27-29).

While clinical trials as summarized by meta-analysis have provided preliminary evidence in support of a regenerative rehabilitation paradigm to enhance patient outcomes(26), the limited success in cartilage regeneration efforts highlights the importance of understanding the mechanisms to enhance the regenerative potential of MSC-based therapeutics. With this in mind, we next summarized currently available evidence from pre-clinical studies that used combinative approach of rehabilitative exercise together with stem cell-based therapeutics. We found only three studies that assessed the effects of regenerative rehabilitation approaches combined with exogenous stem cell-therapeutics for treating cartilage diseases using pre-clinical models(30-32) (**Table 1**). These studies used osteochondral defect model, and no study implemented pre-clinical model of KOA. As a rehabilitative approach, these studies implemented gentle treadmill exercise, low-intensity pulsed ultrasound, or temporal joint distraction. All three studies demonstrated that the rehabilitative approaches promoted therapeutic effects of bone marrow-derived MSC on osteochondral repair compared to no rehabilitation group. The large methodological heterogeneity and lack of strong evidence for regenerative rehabilitation *in vivo*, particularly those designed for the treatment of KOA, represents a critical knowledge gap and area for future investigations.

### **2.3 Intersection of Regenerative Rehabilitation and sex: why sex matters?**

Currently, several factors have been shown to influence therapeutic potential of stem cells therapeutics and/or rehabilitation programs, including donor age, source of stem cell isolation, and lower limb physical function (33, 34). However, one factor that is all-too-often overlooked in regenerative rehabilitation studies is sex. It is well established that females display more severe KOA compared to male counterparts, and this disparity only increases after menopause(5). Despite this, our systematic review revealed that most clinical trials of MSC therapeutics for treating KOA do not consider sex as a biological variable(26). A few studies pointed out the sex-dependent cartilage adaptation to stem cell therapeutics. For instance, females displayed lower improvement following stem cell transplantation compared to male counterparts, as determined by cartilage integrity and self-reported physical function (8, 35, 36). These studies underscore the need to consider regenerative rehabilitation strategy according to sex.

While the underlying mechanisms are still unclear, accumulating evidence indicates that the sex-dependent cartilage adaptation may be attributed, at least partly, to sex hormone receptor-mediated signaling(37, 38). Estrogen serves protective effects of osteoarthritic cartilage through binding to estrogen receptor in a ligand-receptor manner(39). In addition, accumulating evidence has demonstrated that MSC express estrogen receptors, and estrogen promotes proliferation and differentiation capacity of musculoskeletal stem cells(40).

Intriguingly, recent studies have highlighted the novel role of estrogen receptor alpha (ER $\alpha$ ) as a mechano-receptor mediating the chondrocyte mechano-responses(37, 38). ER $\alpha$  is expressed in articular cartilage in both sexes across different animal species(41, 42). However, ER $\alpha$  levels in MSC and cartilage decrease with aging and with the onset of menopause. These declines may be further accelerated in the setting of KOA(37, 41, 43). ER $\alpha$  as a mechano-receptor is particularly of interest in the development of regenerative rehabilitation strategies given the mechano-sensitive nature of chondrocytes. While physiological levels of mechanical loading significantly enhance expression of ER $\alpha$ , excessive mechanical loading inhibits expression of ER $\alpha$  in osteoblasts(44, 45). Other physical therapeutics, such as whole body vibration, are also capable of enhancing ER $\alpha$  level and its downstream signal in osteoblasts or MSCs(46, 47). These findings suggest that estrogen receptor-mediated signaling may be a convergence point of stem cell therapeutics and physical therapeutics, particularly in the treatment of KOA in post-menopausal females.

## 2.4 Sex-dependent characteristics of stem cells

There is growing interest of sex-dependent differences of donor MSC. By understanding how MSCs from males and females differ in their regenerative potential, rehabilitation protocols can be optimized to harness the strengths of MSC characteristics in a sex-specific manner. This could lead to improved outcomes in terms of tissue healing, functional recovery, and overall rehabilitation success. As such, the following paragraphs focus on sex-dependent stem cell characteristics summarized through systematic review. The current review focuses human samples, since, unlike humans, rodents do not present with a menopausal phenotype(48). Therefore, the translatability of findings from non-menopausal female murine models to menopausal female in humans is unclear(49).

In the case of bone marrow-derived MSC therapy, the sex of the donor influences the quantity and types of growth factors and cytokines secreted(50-53). Nevertheless, existing clinical and pre-clinical trials for stem cell therapeutics have lacked consideration for sex as a biological variable. As a first step to address this knowledge gap, we performed a systematic review with the inclusion criteria of characterizing sex-related differences in adult stem cell population. Exclusion criteria included publication in non-peer review journal, non-original article, language other than English, and cells other than adult stem cells. PubMed and Gender Medicine Database (Gender Med DB) were used for electronic database search of relevant studies published by November 2023. Google Scholar was also used as a complementary search engine. Electronic searches used combined key terms, including “Sex Characteristics,” “Sex Factors,” “Chondrogenesis,” and “Mesenchymal Stem Cells” using Medical Subject Headings terms. The electronic database and google scholar searches yielded 70 studies. After title, abstract, full-text, and citation screening, 11 studies were eligible for the systematic review. The most common reason for exclusion was studies using rodents (mice or rats). We summarized the results of the included studies narratively to provide a clear critical appraisal of the evidence, as recommended by the guidelines on the conduct of narrative synthesis in systematic reviews(54).

Most studies used MSCs isolated from bone marrow, adipose, or skeletal muscle (**Table 2**). There is a large inter-trial variability in study outcomes, including colony formation capacity, growth kinetics (i.e., population doubling time), cell surface markers, transcriptomic profile via microarray, gene expression of cell lineage, chondrogenesis, and secretome profile (**Table 2**). The narrative synthesis revealed that, while sex-dependent differences in colony formation capacity was discordant across two studied investigated(55, 56), female stem cells display higher proliferations compared to male counterparts, as documented by shortened population doubling time(55, 57). In addition, two studies assessed stem cell surface markers of infrapatellar fat pad-derived MSC and the synovial vascular fraction, which consistently revealed that female cells displayed increased Cd73, a MSC surface marker (58), compared to male counterparts(57, 59). The sex-dependent stem cell characteristics were also supported by one study demonstrating that female bone marrow cells displayed higher levels of Rankl and Drak1, two markers of osteoclast differentiation, than male counterparts(60). Further, another study characterized the transcriptomic profile of MSCs across the sexes and revealed that female MSC display alterations in several bioactivity including cell-matrix adhesion and mitochondrial function(61), both of which are well known aging hallmarks and associated with the pathophysiology of KOA(13, 62). When we consider the sex-dependent trajectory of MSC characteristics over time, one large cohort study demonstrated that age-related decline in MSC proliferation and differentiation capacity was predominantly confirmed in females(63). The functional decline of post-menopause MSCs underscores the need to consider both age and sex as biological variables in designing regenerative rehabilitation studies.

Studies have shown that the therapeutic effects of injected and/or transplanted MSC are attributed to MSC engraftment or trophic factors secreted from MSC(24). As such, we finally sought to determine sex-related differences in chondrogenic differentiation and secretome of MSC. Interestingly, our narrative synthesis revealed no sex-dependent chondrogenic capacity of MSC, as documented by three studies demonstrated that female and male bone marrow-derived MSC displayed a similar chondrogenesis capacity(51, 55, 64). In contrast, one study revealed lower chondrogenic capacity of female MSCs(65). On the other hand, another two studies demonstrated that female MSC displayed higher immune suppressive effects than male counterparts(7, 55), suggesting that injected and/or transplanted female MSC displays greater anti-inflammatory effects.

### 3. CONCLUSION

Regenerative rehabilitation holds promise for improving the treatment of KOA. The currently available evidence suggests that sexual dimorphism in the efficacy of regenerative rehabilitation approaches for the treatment of KOA may be attributed, at least in part, to the functional decline of post-menopause MSCs. These declines are likely to be accompanied by poor clinical outcomes. Overall, recognizing and utilizing sex-dependent differences in stem cell behavior post-therapy are pivotal in devising personalized regenerative rehabilitation strategies for KOA. Addressing these distinctions may hold the key to maximizing treatment effectiveness and improving outcomes for individuals with KOA.

### Abbreviations

ER $\alpha$ , estrogen receptor alpha; KOA, knee osteoarthritis; MSC, mesenchymal stem cell.

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65. Scibetta AC, Morris ER, Liebowitz AB et al. Characterization of the chondrogenic and osteogenic potential of male and female human muscle-derived stem cells: Implication for stem cell therapy. *J Orthop Res*. 2019;37(6):1339-49.

## Selected publication

\*Iijima H, Gilmer G, Wang K, et al. Age-related matrix stiffening epigenetically regulates  $\alpha$ -Klotho expression and compromises chondrocyte integrity. *Nat Commun.* Jan 10 2023;14(1):18. doi:10.1038/s41467-022-35359-2.

This study revealed that increased matrix stiffness, a characteristic of aging cartilage, triggers Klotho promoter methylation, downregulates Klotho gene expression, and accelerates chondrocyte senescence. Conversely, exposing aged chondrocytes to a soft matrix restores a more youthful phenotype, suggesting that age-related alterations in extracellular matrix biophysical properties play a crucial role in compromising cellular health during cartilage aging.

\*Guo Y, Stampoultzis T, Nasrollahzadeh N, et al. Unraveling Cartilage Degeneration through Synergistic Effects of Hydrostatic Pressure and Biomimetic Temperature Increase. *iScience.* 2023.

This study revealed that the combined impact of reduced hydrostatic pressure and transient thermal increase, simulated using a customized bioreactor, maximizes chondroprotection. These findings underscore the crucial role of these cues in preserving cartilage homeostasis and propose a refined model of the vicious cycle of cartilage degeneration.

\*Gilmer G, Bean AC, Iijima H, Jackson N, Thurston RC, Ambrosio F. Uncovering the "riddle of femininity" in osteoarthritis: a systematic review and meta-analysis of menopausal animal models and mathematical modeling of estrogen treatment. *Osteoarthritis Cartilage.* Apr 2023;31(4):447-457. doi:10.1016/j.joca.2022.12.009.

This study revealed that post-menopausal animals exhibit worse cartilage outcomes compared to non-menopausal counterparts in knee osteoarthritis models, emphasizing the disproportionate impact on post-menopausal women. Additionally, the research suggests that early initiation and higher doses of estrogen treatment may improve cartilage health, highlighting potential therapeutic considerations for knee osteoarthritis in post-menopausal individuals.

\*\*Wang N, Zhang X, Rothrauff BB, et al. Novel role of estrogen receptor- $\alpha$  on regulating chondrocyte phenotype and response to mechanical loading. *Osteoarthritis Cartilage.* Feb 2022;30(2):302-314. doi:10.1016/j.joca.2021.11.002.

This study revealed that damaged cartilage in osteoarthritis exhibits significantly reduced levels of estrogen receptor  $\alpha$  compared to preserved cartilage in both human and mice samples. The findings suggest an estrogen-independent role of estrogen receptor  $\alpha$  in mediating chondrocyte phenotype and response to mechanical loading, proposing that enhancing ER $\alpha$  levels could represent a novel approach for treating osteoarthritis.

\*\*Wang N, Lu Y, Rothrauff BB, et al. Mechanotransduction pathways in articular chondrocytes and the emerging role of estrogen receptor- $\alpha$ . *Bone Res.* Mar 3 2023;11(1):13. doi:10.1038/s41413-023-00248-x.

This study revealed that estrogen receptor  $\alpha$  (plays a crucial role in modulating chondrocyte response to mechanical loading through a ligand-independent mechanism, aligning with its known effects on mechanotransduction in various cell types. The review positions estrogen receptor  $\alpha$  within mechanotransduction pathways, highlighting its significance in influencing chondrocyte behavior and proposing future research directions to enhance understanding under physiological and pathological conditions.

\*Gilmer G, Hettinger ZR, Tuakli-Wosornu Y, et al. Female aging: when translational models don't translate. *Nat Aging.* Dec 5 2023;doi:10.1038/s43587-023-00509-8.

This study highlights a significant gap in preclinical models, with female patients in age-related studies lacking representation of key aspects such as menopausal phenotypes, pregnancy, and breastfeeding. Recommendations are provided to address this limitation, urging action from researchers, journals, funding agencies, and animal providers.

505 \*\*Selle M, Koch JD, Ongsiek A, et al. Influence of age on stem cells depends on the sex of the bone  
506 marrow donor. J Cell Mol Med. Mar 2022;26(5):1594-1605. doi:10.1111/jcmm.17201.  
507 This study revealed that human bone marrow-derived mesenchymal stem cells from a large donor  
508 cohort exhibited reduced colony-forming capacity, proliferation rate, adipogenic differentiation  
509 potential, and altered surface antigen expression with increasing donor age, particularly in females. The  
510 findings highlight the importance of considering age and sex-related effects on bone marrow-derived  
511 mesenchymal stem cells, suggesting a functional loss and immunophenotypic change in aged women.

**Table 1.** Study characteristics assessing the effects of rehabilitative programs on regenerative potential of stem cell therapeutics in pre-clinical model of cartilage disease.

| Author                    | Pre-clinical model    | Treatment | Rehabilitation program          | Outcomes             |
|---------------------------|-----------------------|-----------|---------------------------------|----------------------|
| Harada Y<br>(2015)(32)    | Osteochondral defects | BM-MSC    | Temporal joint distraction      | Histological scoring |
| Yamaguchi S<br>(2016)(31) | Osteochondral defects | BM-MSC    | Treadmill exercise              | Histological scoring |
| Yamaguchi S<br>(2016)(30) | Osteochondral defects | BM-MSC    | Low-intensity pulsed ultrasound | Histological scoring |

*Abbreviation: BM-MSC, bone marrow-derived mesenchymal stem cell.*

**Table 2.** Studies comparing stem cell characteristics across male and female adults.

| Author                   | Cell/Source | Males |             | Females |             | Characteristics of female cells                                                                      |
|--------------------------|-------------|-------|-------------|---------|-------------|------------------------------------------------------------------------------------------------------|
|                          |             | N     | Age, years  | N       | Age, years  |                                                                                                      |
| Beitzel K (2013)(56)     | BMC         | 52    | 51.4 (12.2) | 32      | 65.0 (8.3)  | Similar proliferation capacity                                                                       |
| Bianconi E (2020)*1(61)  | MSC         | 12    | 31–71       | 33      | 18–69       | Altered broad biological processes including cell-matrix adhesion and mitochondrial function         |
| Fossett E (2012)*2(57)   | IFP-MSC     | 8     | 67 (50–83)  | 6       | 67 (50–83)  | Higher proliferation capacity and altered cell surface marker                                        |
| Jiang Y (2008)*1(60)     | BMC         | 27    | 48 (14–70)  | 53      | 53 (15–79)  | Higher expression of specific cell lineage genes (Rankl and Drak1)                                   |
| Lee H (2019)(64)         | BM-MSC      | -     | -           | -       | -           | Similar chondrogenic differentiation capacity                                                        |
| Mckinnirey F (2021)(7)   | BM-MSC      | 4     | 46.0 (9.8)  | 4       | 45.3 (12.7) | Release secretome with enhanced immunosuppressive capacity and elevated anti-inflammatory mediators  |
| Payne KA (2010)*3(51)    | BMC         | 21    | 39 (16–82)  | 20      | 53 (20–77)  | Reduced GAG production to anabolic stimulation with TGF-beta1                                        |
| Scibetta AC (2019)*3(65) | M-MSC       | 4     | 49 (21–78)  | 4       | 54 (12–92)  | Lower chondrogenic differentiation capacity                                                          |
| Selle M (2021)*1(63)     | BM-MSC      | 95    | 18–93       | 80      | 18–93       | Reduced proliferation capacity and immunophenotypic change over time                                 |
| Siegel G (2013)*3(55)    | BM-MSC      | 20    | 54 (13–80)  | 18      | 50 (13–80)  | Reduced proliferation capacity over time, release secretome with enhanced immunosuppressive capacity |
| Zenic L (2021)*4(59)     | SVF         | 8     | 33–66       | 8       | 33–66       | Altered cell surface marker                                                                          |

Abbreviation: BMC, bone marrow cell; BM-MSC, bone marrow-derived mesenchymal stem cell; GAG, glycosaminoglycan; MSC, mesenchymal stem cell; IFP-MSC, infrapatellar fat pad-derived mesenchymal stem cell; M-MSC, muscle-derived mesenchymal stem cell; SVF, stromal vascular fraction.

\*1 Age is provided as a range.

\*2 Age represent averaged value of males and females. Age is provided as mean (range).

\*3 Age is provided as median (range).

\*4 Age is provided as range across males and females.