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Contents

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## REVIEW



## MINIREVIEWS



## ORIGINAL ARTICLE

### Basic Study

**Contents**

*World Journal of Stem Cells*

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*REVIEW*

# Dysfunctional stem and progenitor cells impair fracture healing with age

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## Abstract

Successful fracture healing requires the simultaneous regeneration of both the bone and vasculature; mesenchymal stem cells (MSCs) are directed to replace the bone tissue, while endothelial progenitor cells (EPCs) form the new vasculature that supplies blood to the fracture site. In the elderly, the healing process is slowed, partly due to decreased regenerative function of these stem and progenitor cells. MSCs from older individuals are impaired with regard to cell

Government.

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number, proliferative capacity, ability to migrate, and osteochondrogenic differentiation potential. The proliferation, migration and function of EPCs are also compromised with advanced age. Although the reasons for cellular dysfunction with age are complex and multidimensional, reduced expression of growth factors, accumulation of oxidative damage from reactive oxygen species, and altered signaling of the Sirtuin-1 pathway are contributing factors to aging at the cellular level of both MSCs and EPCs. Because of these geriatric-specific issues, effective treatment for fracture repair may require new therapeutic techniques to restore cellular function. Some suggested directions for potential treatments include cellular therapies, pharmacological agents, treatments targeting age-related molecular mechanisms, and physical therapeutics. Advanced age is the primary risk factor for a fracture, due to the low bone mass and inferior bone quality associated with aging; a better understanding of the dysfunctional behavior of the aging cell will provide a foundation for new treatments to decrease healing time and reduce the development of complications during the extended recovery from fracture healing in the elderly.

**Key words:** Fracture healing; Aging; Bone; Angiogenesis; Mesenchymal stem cells; Endothelial progenitor cells

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**Core tip:** Bone fractures in the elderly are a significant issue, due to the prevalence of the problem, the difficulty of treatment, and the severe consequences of the extended healing period. The delay in fracture healing with advanced age has been attributed to both the decreased number and function of mesenchymal stem cells that regenerate the bone and the inferior performance of endothelial progenitor cells that direct angiogenesis. Some suggested avenues for potential treatments include cellular therapies, pharmacological agents, treatments targeting age-related molecular mechanisms, and physical therapeutics.

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## INTRODUCTION

Aging is the dominant risk factor for fractures, primarily due to low bone mass and poor bone quality in the elderly[1]. While persons 65 years or older currently account for 13% of the United States population[2], they account for more than 50% of hospital admissions with a musculoskeletal injury which are primarily fractures[3]. Fractures in the elderly population are associated with a unique set of geriatric-specific management challenges. In addition to treatment for a fracture, elderly patients are more likely to be simultaneously treated for additional medical or surgical issues which affect healing and outcomes. In addition, low bone mass and poor bone quality impart technical difficulty in achieving stable internal fixation with plates, screws, nails and wires in surgically treated fractures[4-10]. For example, studies have demonstrated that arthroplasty is typically necessary to avoid predictable healing failure that results from loss of surgical fixation and fracture reduction in elderly fractures of the shoulder, elbow, and hip[4,5,11-13]. In addition, periprosthetic fractures that occur around hip and knee replacement prostheses are increasing exponentially and will continue to increase with the aging population[14-16]. These fractures are particularly challenging for orthopaedic surgeons and healing failure can result in amputation and complete lifelong immobility.

Successful fracture healing requires that both the mineralized tissue and vasculature regenerate simultaneously to repair the highly vascularized bone (Figure 1). In fact, the processes of bone tissue regeneration and angiogenesis have significant

interactions between them during fracture healing. In secondary fracture healing, *i.e.*, in the absence of rigid fixation, the healing process begins when a hematoma forms soon after the injury with subsequent acute inflammation at the fracture site. Inflammatory cytokines as well as growth factors are released to signal the recruitment of mesenchymal stem cells (MSCs) to the injury[17,18]. Resident and infiltrating macrophages also influence the homing and localization of MSCs[18]. The recruited MSCs are multipotent, mesodermally derived cells that are capable of proliferating and differentiating into various cell types including osteoblasts and chondrocytes[19]. Recent evidence supports that the MSCs that home to the fracture site for repair derive primarily from the local periosteum[20,21]. Once the MSCs have reached their target site, circulating growth factors such as bone morphogenetic proteins (BMPs) induce their differentiation into osteoblasts and chondrocytes to initiate the formation of a cartilaginous callus bridge between the bone fragments[21]. Subsequently, the chondrocytes become hypertrophic and undergo endochondral ossification. Both osteoblasts and hypertrophic chondrocytes express high levels of vascular endothelial growth factor (VEGF), a key mediator of angiogenesis and a requisite component of fracture healing[22,23]. VEGF modulates bone repair through the induction of endothelial progenitor cells (EPCs) to increase blood vessel density, providing access for nutrients and cells to the site. With an established vasculature, newly formed osteoblasts begin to replace the soft cartilaginous callus with a stronger osseous one, effectively uniting fragmented bones. Over time, the osseous callus is remodeled into vascularized lamellar bone with a central bone marrow cavity at the diaphysis.

Advanced age is a risk factor for impaired fracture healing[24,25] with increased morbidity and mortality[26-28] as well as increased costs. Increased age has been correlated to healing complications in the tibial shaft[29], clavicle[30], femoral neck[31], and floating knee injuries[32]. Delayed fracture healing, evidenced by a longer time to regain the mechanical strength and mineral content in the bone, has been observed in rodents[33-35]. In general, delayed fracture healing in elderly patients is thought to result from a lower capacity for MSC differentiation and impaired angio-/vasculogenesis[25]. These phenomena were observed by Lu *et al*[36], who assessed the molecular, cellular and histological progression of tibia fractures in juvenile, middle-aged and elderly mice and reported delayed chondrocyte differentiation and maturation, vascular invasion, and bone formation in the older animals[36]. The extended healing time may play a role in the development of serious complications that emerge during prolonged immobilization and the consequent high mortality rate with fractures in the geriatric population[37,38].

In this review, we describe the dysfunctional behavior of aging MSCs and EPCs that contribute to impaired fracture healing in the elderly (Figure 1). Although the causes of delayed fracture healing with advanced age are complex and multifactorial, we highlight the reduction in growth factor expression, effects of reactive oxygen species (ROS), and the role of the sirtuin-1 (SIRT1) signaling pathway as significant factors in aging at the cellular level in MSCs and EPCs. Finally, we discuss potential treatments to enhance bone fracture healing that may be beneficial for elderly patients.

## MSC IMPAIRMENT WITH AGE

One of the factors for diminished fracture healing in the elderly is the altered behavior of MSCs with respect to number, proliferation, migration ability, and differentiation potential with age[20]. In bone marrow and adipose tissue from different species such as non-human primates[39], humans[40-42], mice[43-45], and rats[46] there was a pronounced age-dependent difference in the number of MSCs based on the colony forming unit (CFU) assay; MSCs from younger individuals were more numerous as they formed up to 50% more CFUs than older individuals[40-44,46]. MSCs have also been characterized by their positive expression of surface markers such CD90, CD44, and CD73. In a study on human marrow-derived MSCs, Stolzing *et al*[45] found that young cells expressed more CD90, CD105, and Stro-1 and old cells expressed more CD44. The effect of aging on cell surface markers was also observed by Yu *et al*[39] in MSCs isolated from the bone marrow of rhesus macaques. The MSCs from young and middle-aged individuals had a higher percentage of CD90+ cells than the MSCs derived from older individuals, whereas, the MSCs from older individuals had a higher percentage of CD44+ cells.

The proliferative potential of MSCs also declines with age. The doubling times in MSCs isolated from human bone marrow was 0.9 and 1.7 days in cells from younger and older individuals, respectively[40]. This increase in cell doubling time with age was

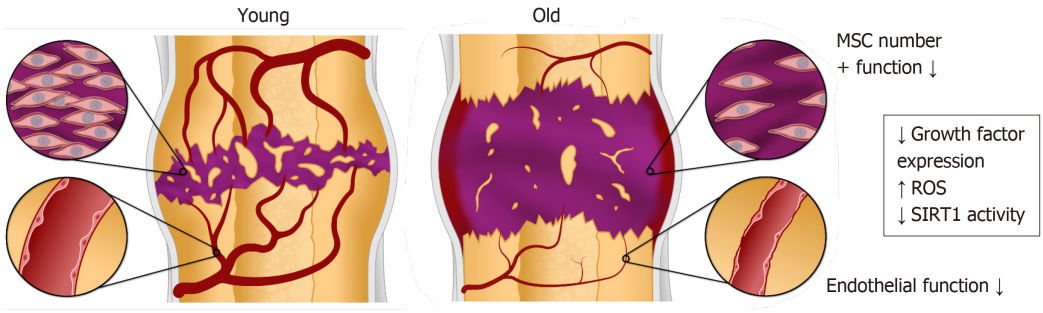


**Figure 1 Fracture healing is impaired with advanced age, including delays in both bone and vascular regeneration due to dysfunction of mesenchymal stem cells and endothelial cells.** MSC: Mesenchymal stem cell.

also observed in MSCs isolated from adipose tissue; cell doubling times increased from approximately 2.6 d in MSCs from younger individuals to 3.8 d in MSCs from older individuals[41,42]. The proliferation rate was also reduced in MSCs isolated from mouse bone marrow by 20% in older animals[44].

The age of the patients not only affects the number and proliferative potential of MSCs but also their ability to migrate to the site of injury, which plays an important role in their regenerative function. It was observed that MSCs from older rats showed lower motility on uncoated filters than those from younger animals[47]. In a different study, twice as many bone marrow-derived MSCs from younger rats migrated towards the chemokine SDF-1 as those from older rats[48]. The decrease in the motility or migration potential of old MSCs may be due to their decreased expression of chemokine receptors[48,49]. In an interesting study on the effect of age on bone marrow microenvironment and migration of MSCs, Yang *et al*[50] found that co-culture with bone marrow aspirate from old mice reduced the migration of an MSC cell line. The authors also found that the bone marrow aspirate from older mice expressed less SDF-1[50]. Together, these studies suggest that the reduced migratory potential of MSCs from in older individuals may be due reductions in both the MSC expression of chemotactic receptors and in chemotactic cytokines secreted by the older tissue. All of these factors together might contribute toward reduced migration of MSCs to the fracture site in elderly patients leading to poor fracture healing.

An important distinguishing feature of MSCs is their ability to differentiate to the osteogenic and chondrogenic lineages, among others. Various groups studying the age-related changes in differentiation potential of MSCs have concluded differently. Several groups have reported that the osteogenic differentiation potential of the MSCs isolated from either bone marrow or adipose tissue is reduced as age advances[41,42,45,51,52]. Zhang *et al*[43] reported that osteogenic differentiation capacity of bone marrow-derived MSCs from mice increases in an age-dependent manner to 18 mo of age and decreases rapidly thereafter. In contrast to these studies, other groups found the MSCs maintained their differentiation potential even in aged donors[53,54]. There is also disagreement in the literature on whether age has an effect on the chondrogenic potential of MSCs. Some groups have reported an age-related reduction in their chondrogenic potential[41,42,52]. In other studies, the chondrogenic potential of MSCs was not affected with advanced age[45,51,55]. However, in all cases, the isolated MSCs were cultured and differentiated *in vitro* where they lack the microenvironment of the native tissue which might be different as the donors age. Conflicting findings in the literature with respect to differentiation potential of MSCs isolated from older individuals require further studies which take tissue microenvironments into consideration to understand any changes in differentiation.

A decline in the expression of growth factors that induce MSC chondrogenic and osteogenic differentiation have been proposed to contribute to impaired fracture healing with age. For example, expression of BMP-2 and Indian hedgehog were at significantly lower levels in the fracture calluses of older rats[56]. Additionally, the response of MSCs to growth factors like BMP-2 may be attenuated with age. As an example, markers of osteogenesis in canine MSCs increased in all animals when treated with BMP-2 in culture, but the increase was less robust in cells from older animals[57]. Similarly, pediatric human iliac crest MSCs were more responsive to exogenous BMP-2 than adult MSCs from the same anatomic location based on the *in vitro* expression of osteogenic markers[58].

The accumulation of ROS is another factor that may affect MSC function in the aged population, resulting in oxidative damage to DNA, structural lipids and proteins as well as cellular senescence[46]. Oxidative stress has been shown to increase during fracture healing[59-61], however the effect of ROS on MSCs during fracture repair in

aging is unclear. In a developmental model of bone formation, chondrogenesis was enhanced by ROS in the developing limb bud, where a cartilage template precedes long bone formation[62]. High levels of ROS have also been associated with hypertrophic chondrocytes that are undergoing endochondral ossification *in vitro*[63]. Furthermore, the addition of an antioxidant to cell culture media inhibited chondrocyte hypertrophy, while elevated ROS stimulated chondrocyte hypertrophy[63]. Osteogenesis through intramembranous ossification, on the other hand, is inhibited by elevated levels of ROS[64-66] and intracellular ROS levels have been observed to dramatically decrease upon osteogenic differentiation due to the upregulation of antioxidant enzymes superoxide dismutase 2 (SOD2) and catalase[66].

Among the molecular regulators of aging, SIRT1, a NAD-dependent histone deacetylase, is of particular importance. SIRT1 expression and activation decrease with age, which modifies a wide range of cellular processes, including MSC proliferation and differentiation. For example, SIRT1 knockdown in human marrow- and adipose-derived MSCs resulted in reduced proliferation *in vitro*[67]. Additionally, MSCs isolated from Sirt1 knock-out mice showed reduced differentiation toward the osteogenic lineage[68]. while Sirt1$^{+/-}$ female mice had reduce bone mass and increased marrow adipogenesis[69]. Differentiation to the chondrogenic lineages were also inhibited in MSCs isolated from Sirt1 knockout mice[68] and with SIRT1 knockdown[70].

## IMPAIRED EPCS WITH AGING

Blood supply is critical for fracture healing. Formation of sufficient vasculature at the fracture sites provides oxygen and nutrients for cell survival and proliferation. Aging has negative effects on angiogenesis which can lead to delayed healing or non-union of fractures[36,71]. Vascular changes such as the decline in endothelial function are reliable markers for aging[72-75]. Highly proliferative EPCs, also described as late outgrowth EPCs or endothelial colony forming cells (ECFCs), are believed to play an important role in maintenance of the viable endothelial layer in the vascular system[76-78].

Aging decreases endothelial cell (EC) proliferation and migration, as well as the expression of EC growth factors and their cognate receptors[79-81]. Aging is also a major cause for endothelial dysfunction and microvascular hypermeability[82,83]. The mechanisms underlying age-related endothelial dysfunction likely involve increased oxidative stress and alterations in molecular pathways affecting common aging processes. Importantly, EPC dysfunction and senescence contribute to oxidative stress[84].

Age related mitochondrial dysfunction is a likely candidate to explain this endothelial progenitor dysfunction. Mitochondria-derived production of ROS results in increased oxidative stress in ECs. Attenuation of mitochondrial oxidative stress in a genetically modified mouse model of overexpression of human catalase in mitochondria improved endothelial function[85]. Conversely, genetic deletion of the mitochondrial antioxidant proteins, mitochondrial SOD and glutathione peroxidase 1, exacerbated age-related vascular dysfunction[86,87]. Age-related oxidative stress may also be caused by increased activity of NADPH oxidase in ECs[88]. Increased oxidative stress in aged ECs inactivates nitric oxide (NO)[88,89]. Impaired bioavailability of NO negatively affects cell division and survival, mitochondrial function and cellular energy metabolism, and EPCs[90].

SIRT1 is an important molecular regulator in ECs[91] in addition to its role in MSCs. SIRT1 expression and activity decreases with aging in the vasculature. Accordingly, pharmacological activation of SIRT1 significantly improves endothelial function in aged mice[92]. Similarly, cleavage of SIRT1 by cathepsin in EPCs mediates stress-induced premature senescence[93].

Age is also a limiting factor for mobilization of EPCs including ECFCs[94-96]. Thus, it appears that the decrease in number and/or function of ECFCs, a homogenous population of EPCs, may be a major driver for failed fracture repair in elderly patients. Previous studies suggest age-related EPC dysfunction may be reversible by anti-aging intervention[97]. Preclinical studies also showed that the serum factors derived from young rats have beneficial effects on EPCs isolated from aged ones[98,99].

In addition to their role in fracture healing, MSCs share properties with pericytes and are important for vascular network formation[99]. Pericytes have an important role in angiogenesis and could be a novel therapeutic target because of their involvement in regulation of capillary permeability, EC proliferation and extracellular matrix generation[100,101]. In fact, age-related loss of pericyte coverage of microvessels contributes to function and structural impairment of microcirculatory network[100]. Interestingly, when adipose derived mesenchymal and endothelial stem cells are

brought in close contact, a Wnt signaling specific mechanism favors osteogenic versus adipogenic differentiation[102,103]. It remains to be elucidated if treatment targeting pericytes could enhance bone healing in aging.

Dysfunction of aged ECs and EPCs lead to endothelial senescence and apoptosis and directly interfered with angiogenesis in aging[82,104-106]. Age-related changes in circulation factors might also contribute to impaired angiogenesis in aging. Pro-angiogenic endocrine factors, growth hormone, insulin like growth factor I, platelet derived growth factor (PDGF) and VEGF, which regulate multiple aspects of angiogenic processes, decline with aging[95,107-109]. This may be explained by reduced expression of, and responsiveness to, HIF-1alpha during aging[110]. Impaired angiogenesis also results in age-related decline in vessel density, impaired adaptation to hypoxia, and ischemia[111].

Impaired angiogenesis during fracture healing creates an ischemic environment at the fracture site and disrupts the interactions between the blood supply and MSCs that are required for bone healing. In a mouse model of fracture accompanied by vascular damage, ischemia significantly decreased the callus size, and the cartilage and bone formation, leading to delayed union[112]. Similar results have been seen in *in vitro* culture of MSCs in hypoxic environments. Hypoxia was found to be linked to reduced osteogenic potential of MSCs, evidenced by the down regulation of many osteogenic markers[113] and osteogenic pathways such as RUNX2[114]. Hypoxia has also been found to inhibit hypertrophic differentiation of chondrocytes and endochondral ossification[115]. Thus, a disruption to the angiogenesis process due to aging may have profound effects on MSC behavior at the fracture site, leading to delayed fracture healing.

## POTENTIAL TREATMENT OPPORTUNITIES FOR IMPROVED FRACTURE HEALING IN AGING

### *Cell-based therapies*

Successful management of bone fractures in the elderly may require special measures not commonly indicated in younger individuals. As native MSCs and EPCs may be compromised with respect to number and/or function with advanced age, delivering these cells to the fracture site is one potential avenue to accelerate fracture repair.

Bone tissue engineering has been investigated intensively for three decades, but efforts to date have not yielded a cell-seeded implant which can be used clinically. Most tissue engineering approaches target intramembranous or direct bone formation, but this approach has had poor outcomes because the cells must initially survive in an avascular hypoxic environment before the invasion of vasculature. Without vasculature, nutrient delivery and waste removal are severely compromised in the center of the implant, causing cell necrosis and failure of cell-seeded implants[113,116]. A relatively new technique to address this issue exploits the tendency of MSCs to undergo a process resembling hypertrophy when cultured under standard chondrogenic differentiation conditions[117,118]. In this regenerative strategy, bone tissue is generated *via* the endochondral ossification pathway, where a cartilaginous template is first formed and later remodeled into mature bone. One advantage of endochondral bone tissue engineering is that the chondrogenic cells function much better than osteogenic cells in low-oxygen environments such as the avascular region of a bone defect[113,119]. Therefore, the chondrogenic cells are maintained in the implant site until the vasculature invades, at which time the hypertrophic cells induce bone formation, as in secondary native fracture healing. Because the cells undergo a process that resembles hypertrophy, they release an array of growth factors for vascular and bone formation that are spatially and temporally controlled. The feasibility of this technique has been demonstrated using embryonic stem cell[120], marrow- and adipose-derived MSCs[121-129], and the murine, chondrocytic cell line ATDC5[130]. Recently, fracture healing through endochondral ossification using hypertrophically primed MSCs in a collagen construct was demonstrated in a weight bearing femoral defect model in rats[131]. In fact, endochondral ossification has been shown to be a better alternative than intramembranous bone regeneration by Thompson *et al*[132], where the chondrogenically primed MSCs supported greater repair of a cranial critical-sized defect (CSD)[132].

Another cell-therapy approach to improve bone healing is to enhance angiogenesis with ECFCs. Currently, implantation of ECFCs has been tested in animals and is currently being investigated in human clinical trials for other indications, such as myocardial infarction, ischemic stroke, liver cirrhosis, and diabetic foot[133]. Our group[134] and others[135-140] have recently shown the utility of using ECs for bone repair. ECFCs were selected based on their proliferative potential, expression of CD31 and

CD309, as well as their ability to take-up acetylated low-density lipoproteins[141].

ECFCs can induce neovascularization at the bone defect site, and stimulate fracture repair and bone regeneration in young rats[134]. ECFCs ($10^6$ cells) were seeded into a type I collagen sponge and transplanted into the bone defect during fracture surgery. The data showed that ECFCs induced more new blood vessels compared to the unseeded type I collagen controls[134]. Furthermore, new bone was formed within the defect area when implanted with ECFCs, but no bone was observed in the controls[134]. Histological examination showed that osteocytes, osteoblasts, and osteoclasts were observed in newly formed bone tissues in ECFC treated animals at 6 weeks[134]. These data suggest that ECFCs can increase neovascularization and stimulate new bone formation in the damaged bone area with a CSD that normally fails to heal.

In another study by our group, hydroxyapatite and tri-calcium phosphate (HA/TCP) scaffolds loaded with ECFCs ($10^6$ cells) were placed into the fibula defect. Histological examination showed significantly greater newly formed bone in HA/TCP scaffolds loaded with ECFCs than that observed in the HA/TCP scaffold only animals[134], suggesting that ECFCs may migrate and further enhance bone regeneration inside the scaffold.

### *Pharmacological agents*

Because the endogenous concentrations of bone anabolic agents that facilitate fracture repair can be significantly reduced in the elderly, one obvious remedy hypothesized for enhancement of their healing process has been to supplement the patient's natural levels of bone anabolic agents. Recognizing that BMP-2 can potently augment the rate of bone fracture repair, Medtronic Inc. has explored the local application of BMP-2 (Infuse) to a fracture surface to accelerate the healing process. While success has been documented for enhancement of recovery from open tibial shaft fractures[142,143], dental and facial reconstruction surgeries[144,145], and spinal fusion procedures[146,147], the same methodology cannot be applied when fracture surfaces cannot be physically exposed. Thus, those fractures that do not require surgical intervention cannot be treated with BMP-2 dosing through local delivery. Moreover, repeated administration of a bone anabolic agent is not possible with this strategy, since the fracture surface is usually only accessible during the initial reconstruction/stabilization surgery.

A second method to augment endogenous levels of osteogenic agents was explored by Eli Lilly and Co. when they examined the use of parathyroid hormone (Forteo) to accelerate the repair rate of tibial[148-150] and hip fractures[151,152]. While measurable improvement in the healing process was documented in many patients, the phase 3 trial failed to reach its clinical endpoint due to concerns over the induced hypercalcemia that was observed as therapeutically effective concentrations of drug were approached. Although the potentially deleterious consequences of the hypercalcemia forced discontinuation of the clinical trial, the results suggested that a more targeted form of parathyroid hormone might succeed if it could concentrate that drug at the site of the fracture and reduce its concentration in healthy tissues.

Looking to the future, a large number of peptide and protein hormones that are commonly released at the site of a wound have been reported to exhibit bone anabolic activity. These include FGF2[153-156], PTHrP[157-159], PDGF[160-162], Prostaglandins[163-165], IGF[166], VEGF[167,168] and others. Because virtually all of these stimulants are known to have multiple anabolic activities that can cause undesirable changes in healthy tissues, it is unlikely that any will prove useful as bone fracture repair drugs unless they can be applied locally to the fracture surface or targeted to the same fracture surface following systemic administration. Hopefully, with the design of new bone fracture homing ligands, such fracture targeted anabolic agents can be developed for less invasive therapies of fractures in the elderly.

### *Therapies targeting age-related molecular mechanisms*

Aging at the cellular level is associated with increased ROS production and decreased endogenous antioxidant levels, leading to accumulation of oxidative damage and cellular senescence. Therefore, antioxidants have been studied as a therapy to improve a variety of health outcomes, including fracture healing. Antioxidants vitamin E[169,170], melatonin[171,172], and N-acetylcysteine[173] have all been shown to promote fracture healing in animal models. Cellular senescence itself can induce chronic inflammatory disease in mice, and depletion of senescent cells by so called senolytic agents can reduce systemic inflammation and extend life span in small rodent by 37%[174-178]. Importantly, targeting cellular senescence prevents age-related bone loss in mice[179]. Therefore, it appears to be promising to identify ways to reduce the generation and maintenance of senescent progenitor cells. This widely overlooked aspect is of particular interest because a high number of senescent cells with detrimental functions are to be expected during aging and aging-associated inflammatory conditions.

Another potential target for improving fracture healing in aging is SIRT1[91]; as described above, it appears to be involved in the age-related decline in both MSC and EC function. Furthermore, crosstalk between SIRT1 activity and ROS production plays a crucial role in the aging process[180]. SIRT1 expression and activity decreases with aging. Accordingly, pharmacological activation of SIRT1 improves the survival of aged MSCs upon transplantation[21] and also significantly improves endothelial function in aged mice[92]. Similarly, cleavage of SIRT1 by cathepsin in EPCs mediates stress-induced premature senescence[93]. Most notably, pharmacological activation of SIRT1 increased bone mass in mouse models of osteoporosis[181].

### *Physical therapeutics*

Physical therapeutics are non-invasive and non-pharmacological treatments that cause physiologic cascades in the body to affect measurable change in molecular and tissue function, leading to improved functional outcomes[182-184]. Movement therapies and therapeutic modalities are two approaches frequently used in the clinic that should be considered within the context of fracture healing and aging.

Movement approaches may include ambulation and therapeutic exercise, both of which mobilize physiological responses secondary to mechanical loading. While the effects of these treatments have not been fully explored in humans, it has been shown that mechanical loading of cells *in vitro* can impact gene expression and bone-derived mesenchymal cell (MSC) differentiation into three types of tissue: Fat, bone, and cartilage[185]. Even short durations of compression can cause an increase in differentiation and calcium mineralization in certain cultures[186]. Another benefit of mechanical loading during cyclical compression, which mimics gait, of human MSCs is an improvement in oxygenation of the fracture's hematoma that benefits cellular metabolism and the ability to heal[187]. One study investigated the effects of functional mechanical loading on large bone defect regeneration *in vivo*. Bone CSDs in rat femora were stabilized using either stiff or compliant fixation plates that allowed compressive loading during ambulation. Findings demonstrated that functional transfer of axial loads during segmental bone repair enhanced bone formation and regeneration[188]. Meanwhile, a retrospective cohort study observed that early ambulation/mobilization of elderly patients with fractures improve outcomes faster than those who delay mobilization[189]. In contrast, immobility was associated with higher mortality and lower function[190].

Several therapeutic modalities are used clinically to facilitate fracture healing, including whole-body vibration (WBV) and pulsed ultrasound. Recent systematic reviews suggest WBV is a safe and effective treatment. Pre-clinical trials with ovariectomized rats have shown those with diminished estrogen respond better to WBV than those with normal levels[191-193]. Interestingly, a study observed an increase in osteogenic potential of bone marrow with WBV during a period of hindlimb unloading compared to those with no treatment; this increase was expounded upon later during re-ambulation and concurrent WBV[194]. Pulsed ultrasound may offer another option for fracture healing in the elderly. Research has shown low-intensity pulsed ultrasound to decrease osteoclastic gene expression[195] decrease MSC adipocyte differentiation[195], and foster MSC's commitment to osteogenesis[196,197]. However, according to recent systematic reviews, there is a low level of evidence to support its use in the early phases of fracture healing in elderly humans[198] and in those undergoing distraction osteogenesis[199].

## CONCLUSION

Bone fractures in the elderly are a significant issue, due to the prevalence of the problem, the difficulty of treatment, and the severe consequences of the extended healing period. The delay in fracture healing with advanced age has been attributed to the decreased number and function of MSCs that regenerate the bone and the inferior performance of EPCs that participate in angiogenesis. The causes of cellular aging and the concomitant decline in functionality are wide-ranging, but provide some intriguing indications of potential targets for speeding fracture healing in older individuals. In the future, cell therapies that supplement the inadequate native cellular response with MSCs or ECFCs; bone anabolic pharmacological agents, particularly in combination with strategies to localize their delivery to the bone fracture; drugs that reduce oxidative stress, cellular senescence, or activate SIRT1; and/or physical therapeutics may prove effective in promoting fracture healing in the elderly.

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REVIEW

# Physical energies to the rescue of damaged tissues

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## Abstract

Rhythmic oscillatory patterns sustain cellular dynamics, driving the concerted action of regulatory molecules, microtubules, and molecular motors. We describe cellular microtubules as oscillators capable of synchronization and swarming, generating mechanical and electric patterns that impact biomolecular recognition. We consider the biological relevance of seeing the inside of cells populated by a network of molecules that behave as bioelectronic circuits and chromophores. We discuss the novel perspectives disclosed by mechanobiology, bioelectromagnetism, and photobiomodulation, both in term of fundamental basic science and in light of the biomedical implication of using physical energies to govern (stem) cell fate. We focus on the feasibility of exploiting atomic force microscopy and hyperspectral imaging to detect signatures of nanomotions and electromagnetic radiation (light), respectively, generated by the stem cells across the specification of their multilineage repertoire. The chance is reported of using these signatures and the diffusive features of physical waves to direct specifically the differentiation program of stem cells *in situ*, where they already are resident in all the tissues of the human body. We discuss how this strategy may pave the way to a regenerative and precision medicine without the needs for (stem) cell or tissue transplantation. We describe a novel paradigm based upon boosting our inherent ability for self-healing.

**Key words:** Stem cells; Physical energies; Mechanical forces; Electric fields; Electromagnetic fields; Electromagnetic radiation; Photobiomodulation; Damaged tissues

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**Core tip:** Rhythmic oscillatory patterns permeate the entire universe and sustain cellular dynamics. Our cells encompass a seemingly infinity of rhythms, unfolding at the nanomechanical and electric level in the microtubular network. Essential signaling molecules are shown to behave as chromophores, supporting the absorbance and emission of light. Photobiomodulation is a rapidly growing area of inquiry for both deciphering novel signaling mechanisms and affording unprecedented clinical applications. The deployment of the diffusive features of physical energies is leading to a regenerative/precision medicine, based upon the reprogramming *in situ* of tissue-resident stem cells, without the needs for cell or tissue transplantation.

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## INTRODUCTION

We are immersed in and we are a part of the oscillatory nature of the universe. In today's physical age, on the threshold of the 4th Industrial revolution, most basic issues will be about electronics, machines, and the future of what we call artificial intelligence (AI). Science is increasingly looking at cell biology with the eyes of physics and electronics, providing compelling evidence that life is embedded within oscillatory patterns that create coherent rhythms, now recordable at cellular, subcellular, and even molecular levels. In addition to expressing rhythmically their molecular dynamics, cells are able to organize their decisions and fate by detecting and deploying the physical energies that permeate nature, including extremely weak mechanical vibrations (nanomotions), magnetic fields, and electromagnetic radiations (light).

As in the universe, in biological organisms, rhythmic oscillations and synchronization of oscillatory patterns are an essential requisite for recognition and connectedness. Sophisticated approaches, including atomic force microscopy (AFM)[1-4], scanning tunneling microscopy (STM)[5,6], terahertz field microscopy (TFM)[7], and hyperspectral imaging (HSI)[8-10] are now providing a dynamic picture of the cellular environment at a nanoscale level, showing that mobile elements of the cyto- and nucleo-skeleton are dancing with patterns that display features of coherence, short- and long-range signal propagation, networking, and memory. Tubulin dimers, and microtubules are now emerging as the constituents of a highly dynamic web, acting both as a source for the generation and the context for the interplay of physical energies[5,6]. These energies include mechanical forces[11-13] as well as the production of electric and very likely electromagnetic fields, with radiation characteristics[5,6,14], and even the occurrence of electromagnetic radiation (light), as a result of biophysical dynamics of a number of molecules increasingly regarded as chromophores[15-17]. To this end, the list of intracellular chromophores is now progressively increasing, including flavins, flavoproteins, and cytochromes[18-22], which are thought to be involved in the generation of reactive oxygen species (ROS) and nitric oxide[19,23-25], behaving as major pleiotropic conductors in cell biology.

Although it is not clear to what extent chromophores are expressed in mammalian cells compared to insects, there is now evidence for the presence of different members of the opsin (a group of cis-retinal dependent G-protein coupled receptors) family in mammalian cells, controlling crucial downstream signaling pathways involving members of the family of transient receptor potential cation channels (TRPs)[26-28]. TRPs are a superfamily of multiple members, which have been shown to be selectively activated by defined wavelengths of light, playing a major role in cellular dynamics[29-33], as photoentrainment and modulation of cellular circadian rhythms[34].

These new achievements in science pose the more general issue of how and to what extent signaling molecules may be viewed as both generators and sensors of physical energies. They also highlight the particular relevance of the identification of frequency region selectivities for inducing defined morphological and functional paths, by precisely tuning the delivery at the cellular or tissue level of specific patterns/

signatures of frequencies, wave forms, and pause intervals for each energy alone (mechanical, electric-electromagnetic or light) or in combinatorial modes.

Within such a dynamic landscape, signaling molecules, like small peptides, based upon their intrinsic helix-turn-helix repeated modules, may be viewed as oscillatory entities[7], walking onto microtubular and microfilament routes in close association with molecular motors[35]. The cellular environment acquires notation of an intracellular niche whose characteristics are forcing Scientists to revisit their knowledge and interpretation of crucial issues that include the biomolecular recognition patterning, the inherent meaning and implication of cell polarity, the modalities through which cellular information is built and unfolded, and the determination of complex cellular decisions and fates. Accordingly, the use of innovative approaches, such as the Resonant Recognition Model (RRM), has led to the conclusion that DNA can also be viewed as an oscillatory entity resonating with electromagnetic frequencies spanning from THz to KHz[36]. RRM relies upon the finding that the function of proteins may be controlled by periodic distribution in the energy of their delocalized electrons, affecting protein dynamics, or protein-DNA interplay, a fundamental step in DNA remodeling and epigenetic control operated by a wide variety of transcription factors[37]. To this end, RRM also postulated that protein conductivity could be associated with defined spectral signatures, resulting from electromagnetic radiation/absorption patterns generated by the flow of electric charges through the protein backbone[37,38]. Interestingly, spectral signatures postulated on the basis of RRM have been verified and supported by experimental evidence[5,39]. Another advantage in the use of RRM is the chance of excogitating novel peptides with unprecedented spectral features and bioactivities[40].

The overall scenario is emerging of an intracellular environment where complex nanoarchitectonics are fashioned within a dynamic assembly of microtubules and microfilaments. These elements can now be regarded as a bioelectronic circuit embedding a multitude of signaling molecules that, besides interacting only with lock-and-key modalities, may also behave as actuators capable of generating phase coherent oscillatory patterns where the building blocks of information arise from the facilitation or dumping of the transfer of physical forces.

Here, we will discuss these issues with particular regard to stem cell biology and the use of physical energies to control stem cell decisions and afford somatic cell reprogramming. We will highlight the relevance of mechanobiology, and the possibility to use mechanical waves to elicit self-repairing mechanisms and tissue rescue in a number of pathological conditions. We will describe the effectiveness of radioelectric fields in enhancing the differentiating potential of stem cells, even reversing their senescence patterning. We will address the various facets of using electromagnetic radiation (light) of defined wavelengths to orchestrate selectively stem cell commitment and tissue repair. We will describe the innovative use of AFM and HSI to decipher the cellular emission of vibrational patterns, in terms of mechanical vibration (AFM) or electromagnetic radiation (HSI), corresponding to specific signatures of growth regulatory and differentiation processes. We will highlight the potential for exploiting the diffusive features of these energies and convey vibrational signatures in the form of nanomechanical motions and/or light patterns to the stem cells *in situ* to afford their reprogramming where they already are, resident in all tissues of the human body. We will finally discuss how this strategy will involve the development of novel interfaces between the human body and machines, as well as AI, paving the way to a precision regenerative medicine without the needs for (stem) cell or tissue transplantation, a novel paradigm based upon boosting our inherent ability for self-healing.

## CELLULAR MICROTUBULES: A NETWORK OF OSCILLATORS THAT SYNC AND SWARM

There is increasing evidence that cells and subcellular domains are mechanosensitive. Mechanobiology is a growing area of interest that deals with the mechanical processes in biological systems. It ranges from cellular mechanics to molecular motors and single molecule binding forces. In addition to tuning the stiffness and shape of cell scaffolding and substrates, mechanical cues and mechanosensitivity are attracting much attention as they represent the context for sensing a wide variety of different stimuli, including osmotic changes, gravity, electromagnetic fields, (nano) motions falling both in an audible range (sound), or even fashioned at subsonic or ultrasonic levels.

The frequency-dependent transport of mechanical stimuli by single microtubules and small networks has been recently studied in a bottom-up approach, using

optically trapped beads as anchor points[41]. When microtubules were interconnected to linear and triangular geometries to perform microrheology by defined oscillations of the beads relative to each other, a substantial stiffening of single filaments was detected above a characteristic transition frequency of 1-30 Hz, depending upon the molecular composition of the filament itself[41]. Below such frequency range, filament elasticity was only controlled by its contour and length persistence. This elastic pattern showed networking features, with the longitudinal momentum being facilitated through linear microtubular constructs *in vitro*, while the lateral momentum was dumped so that the linear construct behaved as a transistor-like, angle dependent momentum filter[41]. These *in vitro* experiments also showed that the overall geometry of the microtubular network was a remarkable cue, since closing the construct circuitry by imposing a triangular shape resulted in stabilization of the microtubular elements in term of the overall molecular architecture and direction of oscillation. These findings suggest that within intact cells microtubular dynamics may afford generation and fine tuning of mechanical signals with a stronger degree of force generation and/or filtering and more flexibly than expected[41]. The complexity in the deformation pattern of microtubules is now prompting further studies to unravel their mechanics through sophisticated atomistic approaches[42].

A major feature of microtubular networks is their ability to exhibit synchronization patterns and even manifest a collective behavior. Synchronization may be viewed as a form of self-organization that occurs in multiple natural and technological systems, from spontaneously excitable cells, like pacemaker cells and neural cells, to coupled lasers, metallic rods, or even robots. On a molecular scale, the observation that simple mixtures of microtubules, kinesin clusters, and a bundling agent assemble into structures that produce spontaneous oscillations, suggests that self-organized beating may be a generic feature of internally driven bundles[43]. These synthetic cilia-like structures exhibit self-assembling at high density, leading to synchronization and metachronal traveling waves, reminiscent of the waves seen in biological ciliary fields[43]. From governing motility in simple protists to establishing the handedness of complex vertebrates, highly conserved eukaryotic cilia and flagella are essential for the reproduction and survival of many biological organisms. Likewise, the emergence of synchronization patterns in eukaryotic microtubules may be essential in the generation and spreading of nanomechanical and electric signaling orchestrated by these nanowires. Despite the fact that synchronization of oscillatory patterns appears to result from intrinsic properties of microtubules under critical, timely/spatial bundling conditions, the intimate mechanism by which individual components coordinate their activity to produce synchronized oscillatory patterns remains unknown.

Another form of self-organization is swarming insects, flocking birds, or schooling fish, where individuals also move through space exhibiting a collective behavior without remarkably changing their internal state(s)[44]. In their pioneer work, Sumino *et al*[45] have shown that an artificial system of microtubules propelled by dynein motor proteins self-organizes into a pattern of whirling rings. They found that colliding microtubules align with each other with high probability. As a function of increasing microtubular density, the alignment ensued in self-organization of microtubules into vortices of defined diameters, inside which microtubules were observed to move in both clockwise and anticlockwise fashion[45]. Besides exhibiting these spatial traits, the phenomenon also evolved on timely bases, since over time the vortices coalesced into a lattice structure. The emergence of these structures appeared to be the result of smooth, reptation-like motion of single microtubules in combination with local interactions (collision dependent nematic alignment)[45]. These discoveries have put forward the issue of previously unsuspected universality classes of collective motion phenomena that are mirrored even at the subcellular level, where microtubules have shown the capability, at least *in vitro*, to behave as swarming oscillatory elements, whose phase dynamics and spatial/temporal dynamics are coupled.

The possibility that microtubules may not only generate and propagate mechanical signals but that they may also be implicated in electric signaling acting as biological nanowires is suggested by the fact that tubulin has a large dipole moment. As a result, microtubules will exhibit a large cumulative dipole moment, imparting features of electrostatic polarity and functional directionality[46]. Within the microtubules, tubulin dimers have highly electronegative C-termini, attracting electrically positive counterions, a mechanism that may account for the observed amplification of ionic signaling[47-49]. These hypotheses have received significant support from the observation that microtubules were able to modify remarkably the electric conductance in solutions with varying concentrations of microtubules made by different concentrations of tubulin and tubulin dimers at a frequency range of alternating electric fields between 1 kHz and 10 MHz[46]. A consistent increase in

solution conductance was observed at an alternating current frequency of 100 kHz, this effect being directly proportional to the concentration of microtubules in solution. Like mechano-transduction, the alternating current frequency dependent response of microtubules-containing electrolytes was also finely regulated, exhibiting a concentration independent peak in the conductance spectrum at 111 kHz, an observation that suggests the presence of intrinsic electric signaling properties of microtubules in aqueous environments[46]. Intriguingly, these properties did not result from conductance patterns elicited by their building blocks, since tubulin dimers exhibited a completely different behavior by decreasing solution conductance at 100 kHz under similar conditions[46].

The microtubular wall is interspersed by nanopores formed by the lateral arrangement of tubulin dimers. The application of patch clamp technique to *in vitro* generated two-dimensional microtubular sheets revealed that voltage-clamped sheets generated cation-selective oscillatory electrical currents whose magnitude depended on the holding potential, ionic strength, and composition[50]. The oscillations progressed through various modes including single and double periodic regimes and more complex behaviors with prominent fundamental frequencies. In physiological potassium concentrations, oscillations represented remarkable changes in conductance that were also affected by the prevalent anion[50]. Current injection elicited voltage oscillations, showing excitability similar to action potentials[50], suggesting a functional role of wall nanopores in the handling of the electrodynamic capabilities of microtubules.

Microtubules have also been shown to form bundles, particularly in neurons. Intriguingly, bundles of brain microtubules have been recently reported to behave as bio-electrochemical transistors that form nonlinear electrical transmission lines[51]. These bundles were shown to generate electrical oscillations and bursts of electrical activity similar to action potentials[51], indicating that electrical oscillatory patterns represent an inherent microtubular feature. These findings may have remarkable biomedical implications in the unfolding of both neuronal and non-neuronal functions. These may include the fine tuning of cytoskeleton-regulated ion channels and may even play a role in higher brain functions, including memory and consciousness.

The biomedical implications of considering the microtubular network as a bioelectronic circuit are further inferred by other observations showing that exposure to alternating electric fields between 100–300 kHz of strength approximately 1.0–2.5 V/cm is able to arrest cell mitosis[52]. This finding has led to a Food and Drug Administration approved treatment for glioblastoma multiforme[53], with the electric field effects on microtubules being considered as the main underlying mechanism of action[52,54,55]. This clinical outcome prompts strong motivation to pursue additional studies aimed at further elucidating the electric signaling features of mammalian microtubules. For this purpose, contributions due to the dipole moments, charges, van der Waals, and solvation energy have been taken into account to dissect and explain microtubular energy balance[56], and optomechanical approaches have been proposed for monitoring microtubule vibration patterns[57]. Moreover, alterations of collective terahertz oscillations have been found to be induced in tubulin by anesthetics, correlating with their clinical potency[58]. This observation may have implications for anesthetic action and post-operative cognitive dysfunction.

There is now evidence that resonance modes not only occur in microtubules at the (nano) mechanical level but can even be detected at the level of their electric conductivity. Even more intriguingly, mechanical and electromagnetic resonance modes can coexist and affect each other within the microtubular network. STM, coupled with an *ad-hoc* designed cell replica developed to deliver electromagnetic fields of defined frequencies to microtubules growing on platinum nanoelectrodes, has shown that tubulins, tubulin dimers, and microtubules exhibited electric conductivity profiles resonating only with specific electromagnetic frequencies applied to the *in vitro* system[5]. STM analysis also provided evidence that the resonant tunneling currents elicited by microtubules occurred in response to electromagnetic fields applied within a MHz range[5].

These findings indicate that microtubules can generate specific electromechanical oscillations as a consequence of a resonant response to defined electromagnetic frequencies produced or delivered within their environment[5]. These observations further support the idea that microtubules may act as an intracellular bioelectronic circuit. Consonant with such perspective are (A) theoretical calculations considering the microtubules as elements generating electric fields of high frequency and radiation features[14]; and (B) experimental assays demonstrating that even a single brain microtubule behaves as a nanowire harboring "memory states" depending on its protein arrangement symmetry, coupled with conductivity state embedded in the microtubule itself, equitable to a memory switch device with a near-to-zero hysteresis

loss[59] (Figure 1).

## BIOMOLECULAR RECOGNITION PATTERNING

The microtubular network and its sync and swarming behavior may help develop a novel hypothesis on biomolecular recognition within the intracellular environment. The "key-and-lock" dynamics, while fitting the description for the interaction of few molecules in aqueous solutions, fails to adequately describe and predict the collective behavior of a high number of different signaling players that cohabit the intracellular environment and share overlaying space and time domains of interaction to afford integrated cellular decisions. In addition, the time needed for cellular proteins to create productive interaction through intracellular diffusion mechanisms would be highly unpredictable on large-scale colliding bases. The high speed and fine coordination of molecular interplay within complex cellular decisions, including stem cell differentiation, cannot be solely explained on the basis of molecular diffusion and collision within the intracellular environment. At this level, a diffusive mechanism would become hampered and highly unpredictable, due to the synthesis and accumulation of a wide variety of glycosaminoglycans, such as hyaluronan, imparting the features of an aqueous gel dynamically modifying its composition and diffusive properties in response to cell metabolism.

The growing discernment of a microtubular role in tuning intracellular and intercellular communication may offer a clue to formulate novel hypotheses on the mechanisms underlying the astounding speed at which cellular fate is devised. The vast majority of signaling proteins exhibit helix-turn-helix modules, where the helices can be reckoned as oscillating springs, and the turns can be viewed as inter-oscillator linkers. A single peptide becomes a vibrational element capable of phase-resonant oscillatory patterns[7]. TFM has been exploited to detect protein vibrations, midget motions essential for Life[7]. These observations suggest that, like violin strings or pipes of an organ, proteins can vibrate in different patterns within our cells[7]. Cell proteins not only diffuse through water, but they can "walk" onto microtubular tracks availing of kinesins and dyneins motors as their molecular machines[35]. Signaling peptides can be therefore regarded as a multitude of oscillatory devices using molecular machines to move along the microtubular net, with the microtubules acting themselves as multi-level connections affording efficient phase synchronization between multiple oscillators.

The resonant behavior described in microtubules[5] holds promise for remarkable impact in further elucidation of biomolecular recognition patterning. The chance of using a selective frequency region to induce defined morphological patterns in microtubules has shown that mechanical patterns can be precisely orchestrated through the remote application of electromagnetic fields[5]. Therefore, the finding that local density states in tubulin dimers, microtubules, and possibly other proteins can be modified by changing the frequency of their electromagnetic exposure entails that unfurling of protein structure into rhythmic resonance patterns may result as a relevant inherent mechanism sustaining both intracellular, and intercellular communication. Dissecting the resonance patterns intervening within clusters of signaling molecules, and between such molecules and the microtubular networks, and providing suitable methods to investigate the establishment of collective behavior among oscillators that undergo both sync and swarming will likely represent a novel paradigm for investigating the onset and spread of informational processes in biological systems.

Despite continuous progress in investigating this complex matter, the intimate origin of the observed resonant behavior remains largely elusive, particularly if the correspondence between electromagnetic and mechanical oscillation assessed *in vitro* is translated into an *in vivo* setting.

During electromagnetic exposure of protein and protein complexes, in case of electromagnetic resonance, photons would be expected to find domains in the protein structure amenable for both electric and magnetic absorption. Protein cavities would appear as domains arranged for the interaction of the electric and magnetic components of an incoming electromagnetic field. Although nanotopography within these cavities may be suitable for electric resonance, the issue as to whether these cavities may act as sensors generating resonances in the presence of an electromagnetic field remains an open and difficult to answer question.

A remarkable challenge is represented by the fact that, different from simplified *in vitro* systems, the intact cellular level electromagnetic and nanomechanical oscillations are supposed to be highly interconnected with none of their resonance-elicited responses separately emerging as electrical, magnetic, or mechanical. Within this

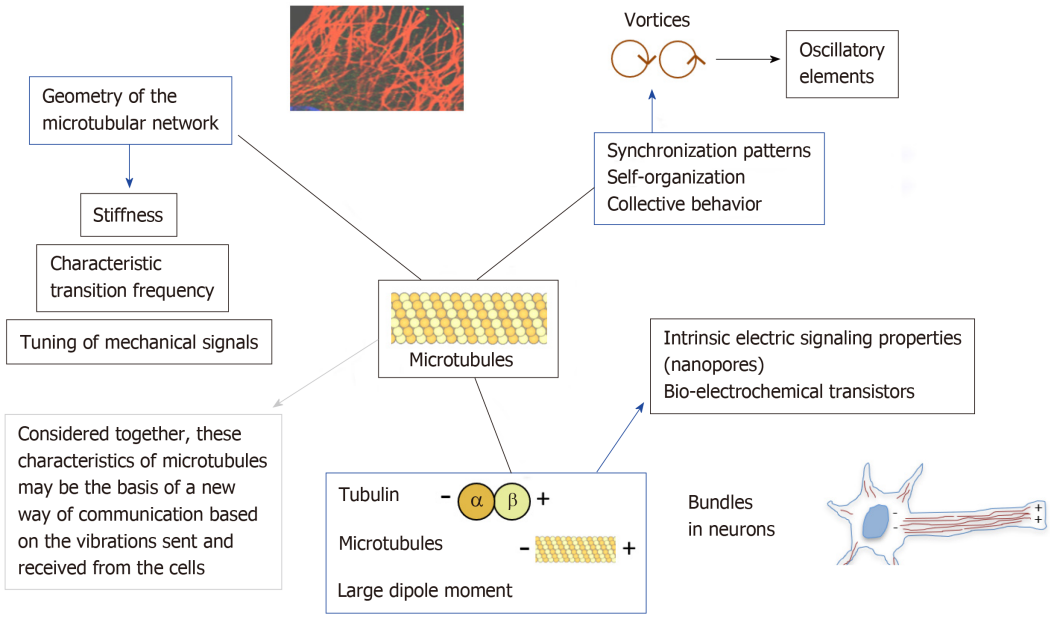


**Figure 1 Cellular microtubules: A network of oscillators that sync and swarm.** Microtubules are emerging as major players in crucial cellular activities, on the basis of a number of interrelated characteristics. These include: (A) The transfer of mechanical waves, changing their stiffness, and the transmission of longitudinal and lateral momentum on the basis of the frequency of their oscillation and the geometry afforded by their timely 3D assembly and disassembly within the cells; (B) The onset and propagation of electric fields and signaling, depending upon the large dipole moment of tubulin, developing both electrostatic polarity and functional directionality, and upon the lateral arrangement of tubulin dimers to create nanopores, interspersing the microtubular wall, and generating cation-selective oscillatory electrical currents; (C) The generation of bundles, as shown in brain microtubules, behaving as bio-electrochemical transistors forming nonlinear electrical transmission lines; (D) The ability to resonate mechanically in the presence of electromagnetic fields of defined frequencies, retaining memory states coupled with conductivity states, like a memory switch device; and (E) The property of synchronizing their oscillatory pattern and swarming into vortices, affecting the vibrational features of signaling peptides moving across the microtubular network by the aid of molecular motor machines, thus modulating biomolecular recognition patterning.

context, a significant step forward may be provided by the recent invention of an atomic resolution scanning dielectric microscopy capable of seeing a single protein complex operating live at resonance in a single neuron without touching or adulterating the cell[6].

Overall, while our view of intracellular and intercellular connectedness is dramatically evolving over time, a novel paradigm is emerging, which considers the cellular and subcellular structures as senders and receivers of electromagnetic and nanomechanical fields. Unfolding this new paradigm may lead to the use of physical energies to orchestrate complex cellular decisions. Translating this perspective at the level of stem cells would result in unprecedented implication in precision regenerative medicine, as discussed below.

## PHYSICAL ENERGIES TO THE RESCUE OF DAMAGED TISSUES: CAN TISSUE RESIDENT STEM CELLS BE A TARGET?

### *Targeting stem cells with mechanical vibrations*

Mechanical signals minutely travel inside and across our cells, with our molecular players and the cyto-nucleo-skeleton behaving as sender and receiver of patterns manifesting with different frequencies, wave form and intensity, as well as pause intervals that are also essential in shaping the ensuing cellular responses. Complexity of biological systems is now emerging through a new landscape made of symmetry breaking, switching among various oscillatory patterns, synchronization of oscillators into clusters, and the swarming of oscillatory clusters that exhibit a collective behavior without necessarily altering their internal states. These dynamics are deeply inbred in a wide variety of cellular nanomotions, including those in the mitotic spindle, the bidirectional organelle transport, and the trafficking of molecular motors and signaling molecules. Within this environment, shaping of mechanical signaling has the advantage of bridging nanomotions with the generation and transfer of information processes across long distances in short time frameworks.

Can this growing body of knowledge be translated into a biomedical application? A positive answer came from the pioneer studies of Rubin *et al*[60], showing that low-

level mechanical stimuli could be delivered to afford strengthening and normalization of anabolic activity in long bones[60-62]. These authors showed that brief daily treatments with mechanical vibrations of high-frequency and very low magnitude could inhibit adipogenesis, while promoting osteogenesis *in vivo*[63] as well as in isolated human stem cells *in vitro*[64,65], up to the point of maintaining the osteogenic potential in bone marrow stem cells, and advancing trabecular bone formation during reambulation[66]. Mechanical vibrations can also be deployed to modulate nuclear signaling and afford significant transcriptional responses. In this regard, an important role is played by the so-called "Linker between Nucleoskeleton and Cytoskeleton", a mechanosensitive network of molecules implicated in chromatin remodeling and epigenetic modification upon nuclear transfer of extremely low-magnitude mechanical signals[67-69]. Based upon consideration that an electromagnetic field may originate from the electrical polarity of microtubules that form the mitotic spindle, a model enabling calculation of such electromagnetic field coupled to acoustic vibrations of the mitotic spindle has been recently developed to describe mitotic spindle kinetics[70], still a poorly understood phenomenon of critical relevance in both physiological and pathological (*i.e.*, cancer) states.

These findings clearly indicate that modulation of cell mechanosensitivity has relevant biomedical implication and may pave the route for developing novel approaches to stem cell biology and regenerative medicine.

### Shock waves

Shock waves are mechanical pressure waves with defined wave profile used in clinical practice since more than 30 years[71]. Low-energy shock wave therapy (SWT) has been successfully used *in vivo* to treat problematic wound-healing disturbances[72-74], tendinopathies, and non-healing bone fractures[75-77]. The ability of SWT to promote tissue regeneration has been initially ascribed to the activation of angiogenic pathways sustained in a paracrine fashion by local release of trophic mediators[78-81]. These findings have fostered extensive investigations to extend further the potential clinical contexts susceptible for SWT application and to elucidate the underlying mechanistic bases. The application of extracorporeal or epicardial shock waves to a porcine model of myocardial infarction has led to a significant improvement of vascularization with increased number of capillaries and arterioles at the infarct border zone, ameliorating ischemia-induced dysfunction and left ventricular function[82,83]. Subsequent studies in infarcted mice indicated that the ability of SWT to prevent left ventricular remodeling and failure through the induction of angiogenesis involved a complex circuitry, encompassing the mechanical stress-induced release of the antimicrobial peptide LL37, its ability to form complexes with nucleic acids, and the release of RNA/protein complexes converging to the activation of Toll-like 3 receptors[84].

The possibility that the angiogenic action of SWT may occur through stem mobilization from the bone marrow has been suggested in studies providing evidence that the beneficial effect of SWT in a hindlimb ischemia model was associated with the mobilization of endogenous endothelial progenitor cells into the systemic circulation[85,86]. Recent studies in an animal model of chronic myocardial ischemia, using wild-type mice receiving bone marrow transplantation from green fluorescent protein donor mice, demonstrated that besides local angiogenesis, cardiac SWT was also inducing the recruitment of bone marrow resident endothelial cells to the damaged myocardium[87]. This response was associated with enhanced expression of the chemoattractant stromal cell-derived factor 1 in the ischemic myocardium and serum. *In vitro* analyses revealed that the ability of SWT to induce endothelial cell proliferation, their enhanced survival, and capillary sprouting was dependent on both vascular endothelial growth factor (VEGF) 2 and heparan sulfate proteoglycan[87]. Besides affecting the release of stored VEGF reservoir bound to heparan sulfate proteoglycan, facilitating VEGF binding to its receptors, SWT has been shown to induce angiogenesis by acting at the transcriptional level, triggering the gene and protein expression of VEGF and endothelial nitric oxide synthase[88]. The tight dependence of these responses upon a mechano-sensing/transduction mechanism could be inferred by the finding that (A) SWT enhanced the phosphorylation of caveolin-1; (B) it increased the expression of *HUTS-4*, which represents β-1 integrin activity; and (C) knockdown of either caveolin-1 and β-1 integrin suppressed SWT induced enhancement of human umbilical vein endothelial cell migration *in vitro*[88].

These molecular findings can also be viewed as a reverse story - from the bed to the bench side - as they provide a mechanistic underpinning on a number of studies that were earlier conducted in patients with severe coronary artery disease, showing that SWT was able to ameliorate myocardial ischemia in patients with severe coronary artery disease[89]. Accordingly, a double-blind and placebo-controlled study demonstrated that SWT improved chest pain and myocardial function without any

complication or side effects in patients with severe angina, leading to the conclusion that SWT was an effective, safe, and non-invasive option for these patients[90]. Following these initial clinical studies, the molecular dissection of mechano-transduction and signaling patterning primed by SWT served as a driving force for further expanding the clinical application of SWT. In a human study, low-energy cardiac SWT was found to suppress left ventricular remodeling and enhance myocardial function in patients with acute myocardial infarction, suggesting that extracorporeal SWT may represent a safe, effective adjunctive therapy to primary percutaneous coronary intervention[91].

A remarkable perspective for using SWT in regenerative medicine approaches is now emerging from an increasing variety of studies conducted *in vitro* in isolated stem cells as well as in animal models of tissue damage. Evidence includes the ability of SWT to promote the osteogenic differentiation in adipose- and bone marrow-derived mesenchymal stem cells[92-94] and the proliferation and migration of rat adipose derived stem cells[95]. Shock wave treatment induced ATP release, increased Erk1/2 and p38 MAPK activation, and enhanced proliferation *in vitro* in different stem cell types, including C3H10T1/2 murine mesenchymal progenitor cells and primary human adipose tissue-derived stem cells (hADSCs)[81]. Purinergic signaling-induced Erk1/2 activation was found to be essential for the proliferative effect of shock waves, which was further confirmed by *in vivo* studies in a rat wound healing model, where shock wave treatment induced proliferation and increased wound healing in an Erk1/2-dependent fashion[81]. Interestingly, converging genetic and molecular evidence has been provided that the miR-138-FAK-ERK1/2-RUNX2 machinery can be generally activated by extracorporeal SWT in tendon-derived and adipose derived stem cells, promoting efficient osteogenic differentiation irrespective of the stem cell origin[93]. *In vivo*, SWT promoted the induction of endogenous neural stem cells and functional improvement after spinal cord injury in rats[96]. Recruitment and induction of endogenous stem cells were also at the basis of the capability of SWT to ameliorate voiding function and improve the innervation and vascularization in a rat model of diabetic bladder dysfunction[97] (Table 1).

Exogenous stem cell transplantation is often hampered by the invasiveness of delivery in certain districts, such as the central or peripheral nervous system, and by other limitations that need to be addressed, including the ideal stem cell source, the timing and modality of delivery, and uncertain fate, especially after transplantation in complex tissues.

In this regard, SWT is emerging as a feasible, efficacious, non-invasive, safe, and cost-effective tool to elicit mechanical signaling and multiple interconnected downstream patterning that converge in optimization of the intrinsic potential of tissue rescue, through the activation of rapid release of trophic mediators, as well as the recruitment and induction of endogenous tissue resident and/or mobilized stem cells.

### *Cellular nanomotions: Vibrational signatures to direct stem cell fate*

It is now clear that cytoskeletal and organelle biology is tightly coupled and that complex connections are dynamically featured at the level of cellular membranes, from the endoplasmic reticulum and the Golgi up to the plasma membranes. The rhythmic patterning and the unfolding of synchronization and swarming modes within the microtubular network are fast remodeling dynamics of the intracellular environment and behave as a vibrational system capable of force generation leading to rhythmic membrane movement.

The nanomechanical oscillatory features of subcellular structures, including the cellular membranes, govern essential biological processes in living cells, including, for example, organelle translocation, nanovescicular assembly and motion, as well as the trafficking and release/cellular exchange of signaling molecules[98]. AFM is based upon the use of a scanning probe that detects local mechanical features, nano-architectonics, and even thermal or electrical characteristics with a particular probe (tip) positioned very close to a target. The extremely close vicinity between the tip and the target sample allows conducting fine analyses even within a nanoscopic environment. The possibility to use AFM in an aqueous milieu makes this system suitable for characterizing living cells, or their subcellular components, with subnanometer resolution grades. These features have made possible to apply AFM to the analysis of cellular/subcellular nanomotions under physiologic conditions, sensing or conveying weak forces with extreme sensitivity[99].

In yeasts or bacteria, cell growth, morphogenesis, and metabolic activity are coupled with characteristic nanomotions that coalesce at the cell surface with defined vibrational blueprint[99]. To this end, a novel area of inquiry has been developed and referred to as "sonocytology", which is based upon the initial observation that nanomechanical motions detected from these small cells could be transformed into

**Table 1 Low-energy shock wave therapy studies**

| Low-energy shock wave therapy | Conditions | Biological effects | References number |
|---|---|---|---|
| *In vivo* studies | Wound-healing disturbances, tendinopathies, and non-healing bone fractures | Activation of angiogenic pathways with local release of trophic mediators | [72-77] |
| | Myocardial infarction in animal models | Improvement of vascularization at the infarction border zone; Mobilization of endogenous progenitor cells from bone marrow into the systemic circulation and to the damaged myocardium; Increase in VEGF gene and protein expression with endothelial cell proliferation | [82-88] |
| | Human severe coronary artery disease or severe angina | Improvement of myocardial ischemia and chest pain | [89-90] |
| | Human acute myocardial infarction | Suppression of left ventricular remodeling and enhancement of myocardial function | [91] |
| | Spinal cord injury in rats | Induction of endogenous neural stem cells and functional improvement | [96] |
| | Diabetic bladder dysfunction in rat model | Improvement of voiding function; Enhancement of innervation and vascularization | [97] |
| *In vitro* studies | Adipose- and bone marrow-derived mesenchymal stem cells | Induction of osteogenic differentiation | [92-94] |
| | Murine adipose derived stem cells | Stem cell proliferation and migration in an Erk1/2-dependent fashion | [81,95] |

audible sounds, following accurate amplification of AFM cantilever vibration, providing a thorough mechanistic analysis of cellular activity[99]. This approach can also be extended to the analysis of complex adaptive behavior in eukaryotic cells. For instance, *in vitro* cardiogenesis, the process of differentiation of stem cells into spontaneously beating cardiomyocytes, entails a major remodeling of the microtubular network, and overall of the cyto- nucleo-skeleton, which will be reflected in remarkable changes in nanomechanical patterning recordable at the level of cellular plasma membrane. In this regard, we have shown and patented for the first time the possibility of using atomic force microscopy (AFM) to afford a nano-mechanical characterization of cellular activity, detecting defined signatures corresponding to the cellular healthy or non-healthy status or to specific differentiating pathways[100]. In particular, we found that stem cells express nanomechanical patterns that can be harvested by AFM and processed into vibrational signatures of their commitment along defined lineages[100]. Our ongoing work is based upon the development of high-fidelity multifrequency mechanical transducers capable of conveying back such signatures to undifferentiated stem cells to direct their commitment towards specific fates. Differently from SWT, this strategy would allow orchestration of the differentiating potential of stem cells on the basis of specific nanomechanical codes, instead of relying upon non-specific, empirically designed, and high-intensity mechanical waveforms.

### *Electromagnetic fields*

A significant biomedical deployment of the "nanoworld" described above is the chance of using physical energies to modulate cellular dynamics and fate. In this regard, we first provided evidence that extremely low-frequency pulsed magnetic fields acted on adult ventricular cardiomyocytes to induce the expression of endorphin genes and peptides[101], playing a major role in intracellular calcium[102] and pH[103] handling, in the regulation of myocardial growth[104-106] and the orchestration of stem cell cardiogenesis[107-109]. In mouse embryonic stem (ES) cells, extremely low-frequency pulsed magnetic fields induced the transcription of cardiogenic and cardiac specific genes and proteins, ensuing into a high-throughput of spontaneously beating cardiomyocytes[110].

We found that a radioelectric field of 2.4 GHz, the same frequency used in wireless fidelity technologies, can be conveyed *in vitro* to stem and somatic cells *via* an *ad hoc* designed radio electric asymmetric conveyer (REAC)[111]. Thanks to its probe, tissue or cell exposure to REAC induce local microcurrents that are attracted and conveyed back to the treated targets without depth limit[111]. The sum of these microcurrents

elicited in the patient's tissue target *in vivo*, or in isolated cells *in vitro*, are concentrated by the asymmetric conveyer-probe of the device, optimizing tissue or cellular bioelectrical activity[111]. This innovative approach proved effective in the modulation of stem cell biology at multiple intertwined layers, including the transcription of stemness genes, the expression of tissue-restricted genes and proteins, and the commitment or terminal differentiation along different lineages. In mouse ES cells[111], as well as hADSCs, REAC exposure optimized the expression of pluripotency and multipotency, respectively, and primed a consistent increase in the yield of stem cells committed along myocardial, skeletal muscle, and neuronal fates[111,112]. Interestingly, following REAC exposure, even human skin fibroblasts could be committed to the same lineages[113]. This observation shows the feasibility of directing human somatic cells to fates in which these cells would never spontaneously appear. This approach did not require methods that so far cannot be easily translated into a clinical practice, such as the use of lentiviral vectors for target gene delivery or the somatic cell reprogramming by cumbersome non-integrating technologies. In addition, REAC-mediated reprogramming of somatic cells involved a biphasic effect on the transcription of stemness genes - a rapid overexpression followed by a down regulation[113] - mimicking the embryogenetic patterning, where the onset of multi-lineage commitment follows, and requires, the transcriptional shutdown of these genes[114-117]. This observation not only may account for the relatively high yield of commitment observed in each lineage (about 10%-15% of the REAC exposed cell population was oriented towards cardiogenesis, skeletal myogenesis, or neurogenesis)[113], but it implies that the transcriptional inhibition of the stemness genes would avoid freezing of the REAC treated cells into an embryonic-like state, which may potentially evolve into malignant cells.

Another breakthrough coming from analysis of biological effects produced by REAC conveyed radioelectric fields was the observation that this treatment proved effective in reversing human stem cell senescence[118]. In fact, a significant decrease in the number of hADSCs expressing senescence-associated β-galactosidase, a marker of cellular senescence, could be observed following REAC exposure throughout long-term cell culture, extended up to the 30$^{th}$ passage[118]. At the 30$^{th}$ passage in culture, REAC-treated hADSCs showed a remarkable overexpression of the *TERT* gene, encoding the catalytic core of telomerase. This effect was paralleled by an increase in telomere length and telomerase activity, with complete restoration of the ability to differentiate along multiple lineages[118]. The antisenescence effect of REAC also involved the activation of a telomerase-independent route, as shown by the increase in the transcription of *Bmi-1*, a pleiotropic inducer of stemness genes and proteins, which, accordingly, were found to be upregulated even at the latest 30$^{th}$ passage in the exposed cells[118].

These observations may also be relevant at the biomedical level. It is now generally accepted that the progressive senescence of tissue resident stem cells across our life span may be responsible for the impairment in tissue self-healing potential. Moreover, from a cell therapy perspective, the strategy of prolonging stem cell culture to yield a high number of transplantable elements, involves the paradox of promoting cellular senescence, thus mocking the initial aim of the cellular expansion of increasing the change of post-transplant tissue recovery.

The "time machine" effect elicited by an electromagnetic energy on stem cell chronobiology may not only prompt innovative approaches for tissue rejuvenation, but it may provide the opportunity of affording (stem) cell expansion procedures without undesired senescence of the cultured cells.

In separate studies, we found that the antisenescence action of REAC was counteracted by 4-methylumbelliferone, a powerful inhibitor of type-2 hyaluronan synthase (HAS2)[119]. The main implication of this finding lies on the fundamental role of HA in maintaining cell polarity and on the possibility of using electromagnetic energy as a tool to optimize cell polarity at the stem cell level. The intracellular role of HA is highlighted by many interrelated observations: (A) The cardiovascular differentiation of ES cells is abrogated by suppression of HAS2[120]; (B) Embryogenesis itself is suppressed by HAS2 knockout due to lethal cardiovascular abnormalities[121]; (C) Intracellular HA acts as docking anchor for hyaluronan binding proteins (hyaladherins), mainly including protein kinases and tissue-restricted transcription factors, favoring targeted phosphorylation steps that are essential for transcriptional efficiency[121-123]; (D) Most of HA mediated interactions encompass molecular motors and are executed at the level of microtubules, providing a dynamic environment that sustains and directs cell polarity[124]; (E) Akin to its pleiotropic functions, HA has been used in the form of mixed ester of butyric and retinoic acids to induce a cardiogenic program of differentiation in mouse ES cells[125]; and (F) In human mesenchymal stem cells, *in vitro*, as well as *in vivo* models of myocardial infarction[126-128], even affording efficient myocardial repair *in vivo* without stem cell transplantation in infarcted rat

hearts[129].

Compelling evidence relates impairment in cellular polarity to stem cell senescence, or the development of an oncogenic risk[124]. Senescent stem cells in *Drosophila* exhibited reduced self-renewal capability as a consequence of centrosome misorientation and altered cell polarity within their stem cell niche[124,130]. The relevance of preserving cell polarity in biological systems is further highlighted by the results of targeted mutation of tumor suppressor p53 in mammary stem cells, where symmetric division and oncogenesis develop in tight association with cell polarity loss[131].

Collectively, these observations point at maintenance of cell polarity as to an underlying attribute for an optimal health. The fact that the antisenescence effect of REAC treatment depended upon intracellular HA availability indicates that proper delivery of electromagnetic fields may represent a tool for optimizing cell polarity in cells and tissue. Such a possibility entails the perspective of conveying radioelectric fields to afford a "one component (cell polarity) - multiple target (stem cell pluripotency, reprogramming, and rejuvenation)" strategy of boosting our self-healing potential.

Deepening the interest for the use of electromagnetic energy in cell biology, the REAC approach also proved effective in inducing the neurological and morphofunctional differentiation of PC12 cells, a rat cell line of pheochromocytoma, retaining metabolic characteristics of Parkinson's disease[132]. The REAC effect included the transcriptional up-regulation of *neurogenin-1*, β3-*tubulin*, and *nerve growth factor*, a set of neurogenic genes, and increased the number of both β3-tubulin and tyrosine hydroxylase expressing cells. The induction of a neurogenic phenotype was associated with the appearance of neuron-like cells[132]. Worthy to note, the differentiating effect of REAC was paralleled by a decrease in the number of PC12 tumor cells, while our previous studies showed no decrease in viability of normal human skin fibroblasts and hADSCs. On the whole, these observations suggest that the REAC treatment may be beneficial in the handling of Parkinson's disease, and further studies are currently ongoing by our Group on this direction (Table 2).

### Photobiomodulation

The origin of the term Photobiomodulation (PBM), considered as the possibility of using light to afford modulation of biological processes and tissue healing in various pathological conditions, probably dates back to 1967, when the Hungarian scientist Endre Mester tried to replicate an experiment performed at that time by McGuff in Boston[133,134]. The latter had been using a beam from ruby laser to eliminate a tumor previously implanted in a rat. Mester was not aware that the system built for him was delivering a ruby laser of only a minimal fraction of power of the laser used by McGuff. As a result, Mester failed to affect the implanted tumor but, he came up with hair regrowth and wound healing at the site of the tumor implantation[135-138]. Initially referred to as "low level laser therapy (LLLT)", this kind of approach was successfully applied over time to afford wound healing and counteract inflammation and pain in orthopedic diseases. Following the recent consensus that light-emitting diodes could successfully replace the use of coherent lasers, the term LLLT has been employed to identify generally a "low level light therapy". Since 2015, the acronym LLLT has been replaced by the term PBM, based upon the difficulty of expressing "low level" as a defined quantity range.

The biological responses elicited by PBM raise the major question as to whether our cells may also use and process electromagnetic signals by themselves, instead of only sensing an exogenously applied electromagnetic radiation (light). A positive answer to this question came from the seminal discovery of Guenter Albrecht-Buehler in 1992, when he analyzed the cellular behavior of Baby hamster kidney cells inoculated sparsely on one face (s-face) and grown as a confluent layer on the opposite face (c-face) of the same thin glass film, using specially prepared substrates to make the confluent cells preferentially oriented and lined up along parallel stripes[139]. Albrecht-Buehler found that after 7 h and in the absence of visible light most of the cells on the s-face traversed with their long axes and with defined angles the direction of the whorls of the confluent cells on the opposed c-face[139]. This cell behavior was abolished by a thin metal coating, absorbing visible and infrared light across the range of wavelengths. On the contrary, the orienting pattern was maintained after coating of the s-face by a thin silicone coating of the glass, which strongly absorbed in the blue end of the visible spectrum but was transparent for red and infrared light[139]. These findings indicated that cells are capable of detecting the orientation of each other by the generation and processing of signals carried by electromagnetic radiation penetrating a thin glass but not a thin metallic film. Moreover, the results from these studies provided evidence that the wavelength of such radiation was likely in the red to infrared range. The author concluded that "the ability of cells to detect the direction of others by electromagnetic signals points to a rudimentary form of cellular

**Table 2 Electromagnetic field studies**

| Electromagnetic fields | Conditions | Biological effects | References number |
|---|---|---|---|
| Extremely low-frequency pulsed magnetic fields | Adult ventricular cardiomyocytes | Induction of the expression of endorphin genes and peptides; Control of intracellular calcium and pH homeostasis; Regulation of myocardial growth; Orchestration of stem cell cardiogenesis | [101-109] |
| | Mouse embryonic stem (ES) cells | Induction of cardiogenesis, cardiac gene and protein expression, ensuing into a high-throughput of spontaneously beating cardiomyocytes | [110] |
| Radioelectric field of 2.4 GHz (REAC) | Mouse ES cells, hADSCs and human skin fibroblasts | Optimization in the expression of pluripotency/multipotency; Increase in commitment along myocardial, skeletal muscle, and neuronal fates, with a biphasic effect on the transcription of stemness genes | [111-117] |
| | hADSCs | Reduction of senescence-associated β-galactosidase expression; Overexpression of the *TERT* gene associated with an increase in telomerase activity; Overexpression of the *BMI1* gene; REAC effects counteracted by chemical inhibition of type-2 hyaluronan synthase | [118-120] |
| | PC12 cells, a rat cell line of pheochromocytoma | Induction of the neurological and morphofunctional differentiation; Up-regulation of neurogenic genes; Decrease in PC12 cells | [132] |

ES cells: Embryonic stem cells; hADSCs: Human adipose tissue-derived stem cells; REAC: Radio electric asymmetric conveyer.

"vision"[139]. Subsequent studies performed by Albrecht-Buehler were published in 2005, showing that near infrared light scattering in dynamic cells was exploited to afford long-range attraction in culture, even when cells were initially seeded in single non-aggregated elements and located randomly onto the tissue culture dish[140]. Nevertheless, cells were able to detect each other within a certain range and move together to form aggregates. In this study the author described a valid assay to calculate the value of range, which resulted to be much larger than one cell diameter and remarkably dependent upon the measured intensity of near-infrared light scattering produced by the cultured cells[140]. For the first time, this milestone study showed that "near-infrared light scattering by the cells mediate a long-range attraction between them, which does not require physical contact and enables them to detect each other's presence".

More than 20 years later, light has been conveyed in the form of PBM to afford wavelength-dependent selective regulation of differentiation and proliferation in hADSCs. In these stem cells, PBM with blue (420 nm) or green (540 nm) light was found to promote osteoblastic differentiation[141]. The effect was mediated at the transcriptional level, recruiting a gene program of osteogenesis, including RUNX2, osterix, and the osteoblast protein, osteocalcin. The 420 nm and 540 nm wavelengths were more effective in stimulating osteoblast differentiation compared to red or near infrared PBM[141]. Intracellular calcium was higher after exposure to light of 420 nm and 540 nm, an effect that could be inhibited by capsazepine (CPZ) and SKF96365 (SKF), which also inhibited osteogenic differentiation[141]. These effects were likely mediated by the activation of light-gated calcium ion channels, as it was inferred by the ability of both CPZ and SKF to suppress the hADSC response to blue or green light[141]. Members of the superfamily of TRPs channels appear to be the class of light-gated ion channels responsible for the differentiating action of blue and green PBM. Consistent with this view, TRPs are present in almost all known living forms[142]. In particular, the vanilloid TRP sub-class (TRPV) was identified as including the receptor TRPV1, specific for capsaicin[143]. TRPV1, exogenously expressed in *Xenopus*, has been shown to be activated by red and even to a greater extent by green light[144]. It is now evident that multiple TRPVs play a relevant role in many biological responses, including pain, inflammation, and regulation of pressure and heat[145]. Opsins are currently believed to represent the major group of intracellular light-sensitive proteins

(chromophores) involved in selective photon absorption (especially blue and green photons) and subsequent activation of TRPs[146,147]. In particular, melanopsin, which relies upon 11-cis retinal isomerization (peaking at 479 nm), is potentially expressed in hADSCs and has been shown to form a pigment maximally sensitive to blue light (with a peak at 479 nm), supporting activation of G(q/11) and G(i/o) signaling cascades, ultimately promoting TRPs-mediate raise in intracellular calcium[22]. Melanopsin has also been targeted by therapeutic devices utilizing blue light to treat jet-lag, affective disorders, depression, and insomnia[148-150]. The fact that SKF, a non-selective TRP inhibitor, was more effective than the selective TRPV1 inhibitor CPZ in abrogating the effects of green and blue light on hADSC osteogenesis[141] suggests that TRPV1 may not be the only light-gated ion channel involved in the osteogenic patterning triggered by blue or green PBM in hADSCs.

Further compounding the complexity and selectivity of PBM in stem cell biology is the finding that red (660 nm) or near-infrared (810 nm) light was able to stimulate, while PBM with blue (415 nm) or green (540 nm) light was found to inhibit the proliferation of hADSCs[151]. In these experiments, PBM with blue/green light produced a CPZ-inhibitable increase in intracellular calcium, and in the amount of ROS, while red/near-infrared light produced a comparable lower increase in intracellular calcium and ROS levels[151]. Moreover, the slight raise in intracellular calcium elicited by red/near-infrared PBM could not be blocked by CPZ. At the same dose of irradiation (3 J/cm$^2$), blue/green light decreased cellular ATP, lowering both mitochondrial membrane potential and intracellular pH, which may account for a significant increase in ROS, while red/near-infrared PBM had the opposite effect[151]. In the same study, the possibility that the blue/green light may have decreased hADSC proliferation by activating TRPV1 ion channel and increasing calcium and ROS was inferred by the observation that TRPV1 was expressed in hADSCs, and CPZ itself, as well as the antioxidant N-acetylcysteine, abolished the inhibition of proliferation induced by blue/green PBM. These findings also highlighted the subtle differences between the effects produced by blue and green PBM, being their action superimposable in reducing hADSC proliferation and intracellular ATP, while the blue light triggered a more pronounced increase in ROS and drop in mitochondrial membrane potential, as compared with green PBM[151]. It was hypothesized that different sensitivities of mitochondrial chromophores may at least account for these differential responses, with red/near-infrared light conversely increasing intracellular ATP and only inducing low levels of ROS.

Taken together, these findings point at the possibility of deploying the diffusive features of PBM to afford a fine tuning of stem cell dynamics and suggest that the ability of PBM to promote tissue repair previously observed *in vivo* may have involved *in situ* reprogramming of tissue-resident stem cells.

Various forms of PBM have been proved effective in ameliorating the outcome of acute stroke in both animal models[152-157] and humans. In a number of, although not all, controlled clinical trials enrolling patients with ischemic stroke associated with measurable neurological defects, 810 nm laser light conveyed to shaved head induced a significant and long-lasting neurological improvement, especially for patients with moderate and moderate-to-severe stroke[158-160].

Application of near-infrared light in animal models of traumatic brain injury (TBI) has been consistently shown to rescue neurological performance and reduce the size of brain lesions in different research laboratories[161,162]. In some studies, the favorable effect of PBM was selectively induced at defined wavelengths, with 665 nm and 810 nm proving the most effective, and with lack of improvement at 730 nm and 980 nm[162]. Based upon this window of selectivity, the chromophore cytochrome c oxidase has been proposed as the putative target responsible for the observed tissue repair[163]. These results were further confirmed in TBI animal models subjected to PBM with either pulsed or continuous wave lasers, with the group treated with 10 Hz pulsed waves exhibiting the most pronounced improvement[164]. The link between these findings and the observed *in vitro* action of PBM on stem cell dynamics is supported by the finding that PBM was found to increase neuroprogenitor cells in mouse dentate gyrus and subventricular zone[165], along with an increase in brain derived neurotrophic factor after 7 d of treatment, followed by a later increase in synapsin-1, underlying a significant enhancement in synaptogenesis and neuroplasticity[166]. Also, learning memory was increased in both mouse and rat models of TBI[167]. These beneficial outcomes were further improved by the combinatorial delivery of PBM and metabolic substrates, including pyruvate and lactate, which were supposed to ameliorate the mitochondrial function[168]. Clinical trials, although still performed in a limited number of patients with TBI, appear to confirm in human subjects the results initially yielded in experimental animal models, as it has been indicated by post-treatment improvement of both language[169] and cognitive performance[170] as well as brain tissue recovery, as assessed by anatomical magnetic resonance imaging and

perfusion single-photon emission computed chromatography[171].

Another major field of potential clinical application of PBM deals with the treatment of neurodegenerative diseases. In animal model of Alzheimer's disease (AD), established through the generation of amyloid beta precursor protein transgenic mice, the number of amyloid beta plaques was remarkably diminished by the application of 810 nm PBM[172]. The behavioral pattern of affected mice was also improved, together with a consistent decrease in the expression of pro-inflammatory cytokines[172]. The rescuing effect of PBM was associated with an increase in mitochondrial function and ATP levels[172]. Despite these encouraging results in experimental animal models, surprisingly no major clinical trials have been conducted using PBM in AD patients. In a small-sample, preliminary (so far only appeared in abstract form) but randomized controlled trial, in patients with moderate-to-severe AD (Mini-Mental State Examination scores ranging 5-24), the combinatorial delivery of PBM through the trans-cranial and intranasal routes afforded a significant improvement (5-point Mini-Mental State Examination score) after 12 wk, with a concomitant improvement in Alzheimer's Disease Assessment Scale - Cognitive assessment[173]. In a recently published study, where patients with AD were subjected to intravascular PBM (final positioning of the optic fiber emitter lined up to the distal site of anterior and middle cerebral arteries), authors reported a significant, long-lasting (up to 7 years) enhancement of cerebral microcirculation and cognitive improvement[174].

Another set of studies performed in both acute and chronic mouse models of pharmacologically-induced Parkinson's disease revealed a remarkable increase in the number of dopaminergic neurons[175]. These studies were confirmed in a tau transgenic mouse model of Parkinson's disease[176]. The only available clinical trial presented so far in the abstract form, enrolling subjects whose age ranged between 18 and 80 years, reported a significant improvement of the investigated indicators of balance, including gait, cognitive function, and speech[177].

Remarkably, LLLT (808 nm) has been recently applied *in vivo* to the tibia and iliac bones of pigs subjected to experimental acute myocardial infarction, leading to a significant reduction in cardiac scarring, with increased density of small blood vessels in the infarcted area, and consistent improvement of heart function[178]. The LLLT action was mediated by an increase in the number of circulating c-kit+ stem cells during the first 48 h post-infarction[178]. These beneficial effects resulted to be a long-lasting outcome of the laser treatment, since they were documented 90 d after the infarct induction[178]. These findings provided the first evidence on the use of light radiation to afford a non-invasive cardioprotection of the heart in the acute phase post myocardial infarction, mediated by endogenous stem cell proliferation and recruitment to the ischemic heart, without resorting to stem cell transplantation strategies (Table 3).

On the whole, experimental and clinical evidence strongly support the feasibility and the remarkable degree of efficacy of PBM in a number of major pathological conditions that so far cannot be solved even by the most advanced pharmacological and/or surgical procedures.

## CONCLUSION

### *Enhancing our self-healing potential*

Cells are increasingly regarded as sensors and transducers of physical energies. Novel and probably unprecedented therapeutic strategies are emerging and will be further deployed in the near future, unfolding (stem) cell biology through the eyes of physics, electronics, and likely bioelectronics (Figure 2). Future approaches will increasingly aim at providing valuable, non-invasive, and cost-effective opportunities for boosting our inherent ability of self-healing. Owing to the diffusive features of mechanical, (electro)magnetic, and light waves, (stem) cell reprogramming may occur in place, without necessarily using transplantation (cells/tissue) procedures to rescue damaged tissues.

We envision the application of high-tech devices, including AFM, TFM, STM, and fast HSI cameras, to acquire specific signatures from mechanical and electromagnetic radiation (light) patterns of stem cells oriented towards defined fates (Figure 3). We also foresee the development of smart actuators, capable of delivering the acquired individual (*i.e.*, mechanical, or electromagnetic) or combinatorial signatures to diseased tissues, selectively driving the fate and rescuing potential of resident stem cells, to afford a precision endogenous regenerative medicine (Figure 3).

### *Physical tissue stimulation and the merging of human body with machines. May*

**Table 3 Photobiomodulation studies**

| Photobiomodulation | Conditions | Biological effects | References number |
|---|---|---|---|
| LLLT | Tumor transplantation in rats | Failure to affect the implanted tumor; Stimulation of hair regrowth and wound healing | [135-138] |
| Cell-generated electromagnetic (light) signals | Baby hamster kidney cells on thin glass film | Cell migration and orientation afforded by endogenous generation and processing of signals carried out by electromagnetic radiation (light) | [139] |
| Near-infrared light scattering | Cell culture | Near-infrared light scattering by cells mediates long-range attraction between them and aggregation within the culture system | [140] |
| PBM with blue (420 nm) or green (540 nm) light | hADSCs | Promotion of osteoblastic differentiation; Overexpression of a gene program of osteogenesis; Increase of intracellular calcium mediated by the activation of light-gated calcium ion channels | [141,142] |
| PBM with red (660 nm) or near-infrared (810 nm) light | hADSCs | Induction of cell proliferation; Maintenance of low ROS level | [151] |
| PBM with blue (415 nm) or green (540 nm) light | hADSCs | Inhibition of cell proliferation; Increase of low ROS level; Lowering of mitochondrial membrane potential and intracellular pH | [151] |
| Various forms of PBM | Acute stroke in animal models | Improvement of the outcome of acute stroke | [152-157] |
| PBM with 810 nm laser light | Human moderate-to-severe stroke associated with neurological defects | Long-lasting neurological improvement | [158-160] |
| Near-infrared light scattering (665 nm and 810 nm) | Traumatic brain injury in animal models | Rescue of neurological performance and reduction of the size of brain lesions; Increase of neuroprogenitor cells in mouse dentate gyrus and subventricular zone; Increase of learning memory; Improvement of mitochondrial function | [161-168] |
| Near-infrared light scattering (665 nm and 810 nm) | Human traumatic brain injury | Improvement of both language and cognitive performance, as well as brain tissue recovery | [169-171] |
| PBM with near-infrared (810 nm) light | Alzheimer's disease in animal models | Reduction of amyloid beta plaques; Decrease in the expression of pro-inflammatory cytokines; Increase in mitochondrial function, and ATP levels | [172] |
| PBM with near-infrared (810 nm) light | Human Alzheimer's disease | Improvement in Alzheimer's Disease Assessment Scale - Cognitive assessment; Enhancement of cerebral microcirculation | [173-174] |
| PBM with near-infrared (810 nm) light | Parkinson's disease in animal models | Increase in the number of dopaminergic neurons | [175-176] |
| PBM with near-infrared (810 nm) light | Human Parkinson's disease | Improvement of the investigated indicators of balance, including gait, cognitive function, and speech | [177] |
| LLLT | Acute myocardial infarction in the pig | Reduction of scarring; Improvement of heart function; Stem cell mobilization and recruitment to the ischemic heart | [178] |

LLLT: Low level light therapy; hADSCs: Human adipose tissue-derived stem cells; PBM: Photobiomodulation.

### *future approaches change the way we are connected to ourselves?*

In the next few years we will probably experience a fundamental transformation in the way we live, work, and relate to each-other. The so-called 4th Industrial revolution will trace the future of electronics, machines, and AI and may even change the way we are connected to and perceive ourselves. Future electronics will be focused more and more on health technologies and will be directed inwards, with respect to human beings, trying to promote self-healing mechanisms. Most of currently available technology has been pointing outwards, being focused of making available

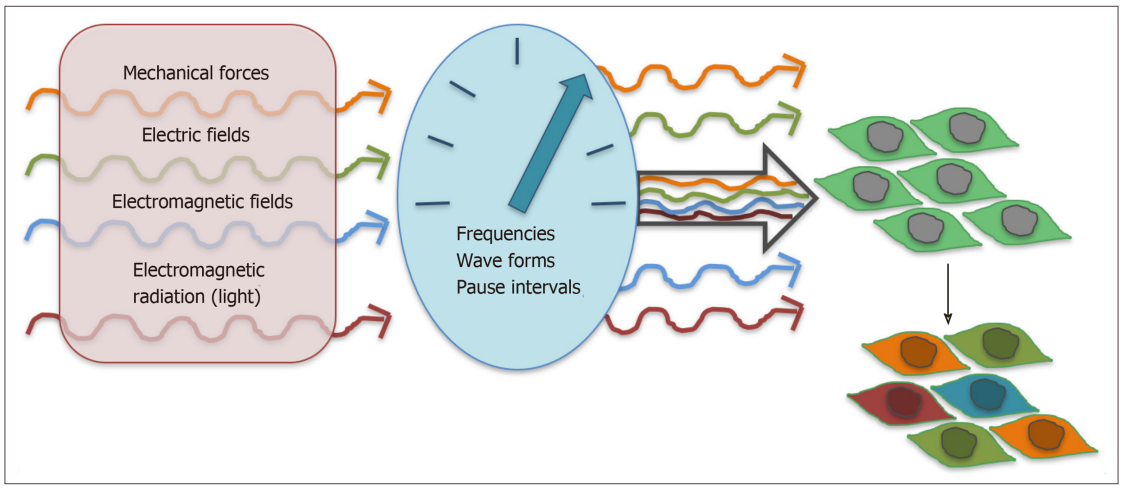


**Figure 2 (Stem) cells as sensors and transducers of physical energies.** A growing number of signaling molecules inside and outside the cell has been shown to behave as mechanosensors/transducers and chromophores (see details in the text). These players modulate complex dynamics controlling multifaceted responses, including proliferation, migration, and differentiation.

"something that accomplishes something".

Emerging future technologies will promote novel interfaces of human beings with machines and AI. What if we may take signatures/codes of our (stem) cell ability to cope with a hostile environment (*i.e.*, oxidative stress, hypoxia) or differentiate along the most complex fates, like the heart, the brain and so on? What if that piece of a stem cell code may be integrated with evolving forms of machines and AI to promote endogenous tissue regeneration? Within this futuristic scenario we may start exploring the synchronistic interchange of who we are and what we are able to become.

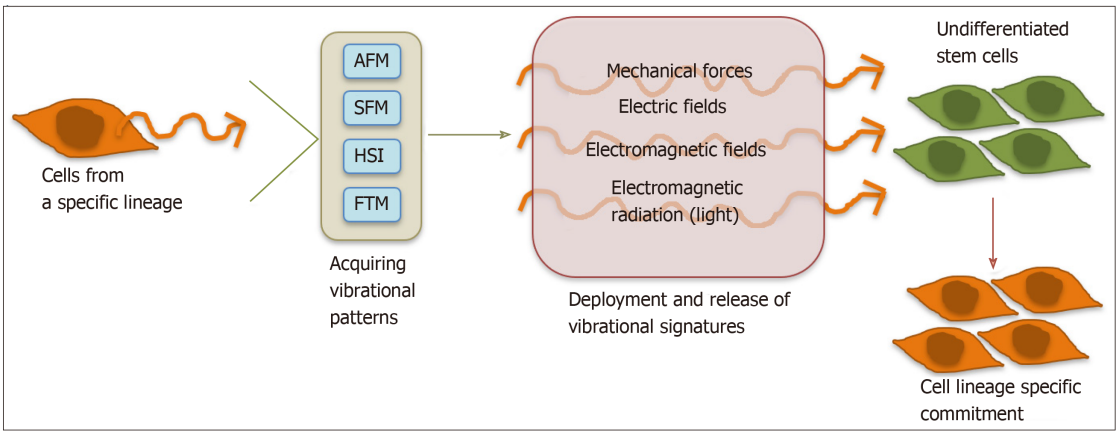


**Figure 3 Harvesting and releasing vibrational signatures to afford commitment of undifferentiated stem cells towards targeted fates.** Atomic force microscopy, terahertz field microscopy, scanning tunneling microscopy, and hyperspectral imaging can be used to harvest detailed oscillatory patterns, including mechanical, electric, and light waves. Computer analysis is then performed to acquire vibrational signatures from the investigated patterns. *Ad hoc* designed transducers are finally developed to provide high-fidelity and timely release of the acquired signatures onto undifferentiated (stem) cells to prime their lineage specific commitment. AFM: Atomic force microscopy; STM: Scanning tunneling microscopy; HIS: Hyperspectral imaging; TFM: Terahertz field microscopy.

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REVIEW

# Effects of various antimicrobial agents on multi-directional differentiation potential of bone marrow-derived mesenchymal stem cells

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## Abstract

Antimicrobial drugs of several classes play an important role in the treatment of bone and joint infections. In addition to fighting pathogenic microorganisms, the effects of drugs on local tissues and cells are also related to the course and prognosis of bone and joint infections. The multi-directional differentiation potential of bone marrow-derived mesenchymal stem cells (MSCs) is essential for tissue repair after local injury, which is directly related to the recovery of bone, cartilage, and medullary adipose tissue. Our previous studies and the literature indicate that certain antimicrobial agents can regulate the differentiation potential of bone marrow-derived MSCs. Here, in order to systematically analyze the effects of various antimicrobial drugs on local tissue regeneration, we comprehensively review the studies on the effects of these drugs on MSC differentiation, and classify them according to the three differentiation directions (osteogenesis, chondrogenesis, and adipogenesis). Our review demonstrates the specific effects of different antimicrobial agents on bone marrow-derived MSCs and the range of concentrations at which they work, and provides a basis for drug selection at different sites of infection.

**Key words:** Antimicrobial agents; Bone marrow mesenchymal stem cells; Osteogenesis; Chondrogenesis; Adipogenesis

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**Core tip:** Bone marrow-derived mesenchymal stem cells (MSCs) are essential for tissue repair (bone, cartilage, and medullary adipose tissue) after local bone and joint infection. The effects of various antimicrobial agents on the three types of differentiation potential (osteogenesis, chondrogenesis, and adipogenesis) of bone marrow-derived MSCs are worth noting. Here in this paper, we collect the latest updates on the use of antimicrobial agents to regulate the differentiation of MSCs.

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## INTRODUCTION

Antimicrobial drugs are referred to as drugs that exhibit an inhibitory or killing effect on bacteria and other pathogenic microorganisms. In clinic, the most commonly used antimicrobial agents are antibiotics, which include natural antibiotics and synthetic antibiotics. Penicillin is a typical natural antibiotic which is produced by fungal metabolism[1]. Synthetic antibiotics, such as quinolones, are the most common type of antibiotics today and play important roles in the treatment of clinical diseases[2]. Antimicrobial agents, in a broad sense, are not limited to antibiotics. Some peptides with antibacterial property and drugs that have been proven to have both antibacterial and other biological functions also fall under the category of antimicrobial agents[3,4]. In addition, extracts of certain plants or Chinese medicines have also been reported to have antimicrobial properties, and they have been speculated to play a role in killing pathogenic microorganisms in clinical and other fields[5]. Similar to bacteria, fungi, viruses, and other pathogenic microorganisms also pose significant challenges to human health, and their corresponding therapeutic drugs also play an important role in clinical and related fields[6,7]. As clinically common diseases, bone and joint infectious diseases can be caused by a variety of pathogenic microorganisms; they cause pain in patients and pose great challenges to clinicians. When using various antimicrobial drugs to treat bone and joint infections, close attention should be paid to the killing effects of these agents on pathogenic microorganisms and to their regulation in local tissues and cells[8]. After using local or systemic antibacterial drugs to treat osteomyelitis and effectively controlling the symptoms of infection, local bone marrow mesenchymal stem cells (BMSCs) diff-erentiate into osteoblasts and lipoblasts, and finally, differentiate into mature bone and adipose tissue to repair locally damaged sites[9]. Similarly, when the symptoms of intra-articular infection are improved, the damaged articular cartilage also needs to be repaired in an environment conducive to chondrogenic differentiation[10]. At this time, the effect of antimicrobial drugs on the differentiation potential of stem cells is crucial. If a drug can promote the differentiation of the stem cells in a direction favorable for tissue repair while also killing the pathogenic microorganisms, the treatment process and the therapeutic effect can be accelerated. On the contrary, if the drug inhibits the differentiation potential of stem cells, it may have undesirable effects on disease treatment.

Considering the multi-directional differentiation potential of bone MSCs and their three most common differentiation directions (osteogenesis, chondrogenesis, and adipogenesis)[11], we review the effects of different classes of antimicrobial agents on these three types of differentiation functions, and hope that it can produce certain ideas for the better drug-mediated treatment of bone and joint infectious diseases.

## EFFECTS OF VARIOUS ANTIMICROBIAL AGENTS ON OSTEOGENIC DIFFERENTIATION

BMSCs are bone marrow-derived cells that play a key role in the renewal and regeneration of osteoblasts. BMSCs can differentiate into bone-forming osteoblasts and have been shown to be a primary source of osteoprogenitor cells[12]. Moreover, BMSCs can be used as bone graft materials to treat bone defects[13]. While the local osseous tissue is damaged by the pathogenic microorganism, BMSCs are activated and differentiate into osteoblasts to complete the repair of local bone dissolution. Failure of BMSCs to completely repair the local bone defects caused by infection may lead to local osteoporosis and even pathological fractures[13]. Therefore, while using various antimicrobial agents to control infection, the consideration of the effect of drugs on osteogenic differentiation of BMSCs is crucial. Drugs with antibacterial properties and osteoinductive ability may play a better therapeutic role in orthopedic infections, such as osteomyelitis; whereas drugs that inhibit the differentiation of stem cells into osteoblasts and destroy the osteogenic microenvironment may adversely

affect the repair of local osseous tissue. In this section, we will review the effects of different antimicrobial agents on osteogenic differentiation, and the overall situation is listed in Table 1.

### *Antibiotics*

**Antituberculosis drugs:** As a representative drug for the treatment of tuberculosis, rifampicin has a strong bactericidal effect on *Mycobacterium tuberculosis*. In addition, rifampicin has also been shown to exhibit anti-Gram-positive bacteria activity and kill the intracellular bacteria hidden in cells, and has a wide range of clinical applications. To demonstrate the potential toxicity of rifampicin and its effects on osteogenic differentiation of osteoblasts, researchers studied osteoblasts treated with different concentrations of rifampicin. The results showed that rifampicin did not cause toxicity to osteoblasts or affect the level of alkaline phosphatase (ALP) in the cells when the concentration of rifampicin did not exceed 10 μg/mL. However, when the drug concentration reached 100 μg/mL and above, the number of osteoblasts and intracellular ALP levels decreased significantly, and the decrease was over 75%[14]. Another study demonstrated that rifampicin is cytotoxic to human bone marrow-derived MSCs at concentrations above 32 μg/mL and inhibited osteogenic differentiation potential of human bone marrow-derived MSCs in a concentration-dependent manner at concentrations ranging from 4-128 μg/mL. The collagen synthesis, mineralization effect, and expression levels of osteogenic genes in MSCs were inhibited to varying degrees with the increase in rifampicin concentration[15].

**β-lactams:** As a representative drug of β-lactam antibiotics, the discovery of penicillin has great significance in the history of human infectious diseases. It has been reported that penicillin, at a conventional blood concentration (30 μg/mL), does not inhibit the osteogenic differentiation process of human bone marrow-derived MSCs[16]. When penicillin was added during the culture of human osteoblasts, cytotoxicity was observed when the penicillin concentration reached 500 μg/mL. At the same time, the differentiation function of osteoblasts was also significantly inhibited after penicillin concentration exceeded 500 μg/mL, and the intracellular ALP level was significantly decreased (above 75%) compared with the control group[14]. Since penicillin cannot tolerate the enzymes produced by a variety of bacteria and is more likely to be destroyed, the probability of clinical drug resistance is increased and the clinical application is greatly limited. Therefore, some antibiotics that are artificially synthesized and can tolerate penicillinase are gradually replacing penicillin and play a greater role in the clinic. Both flucloxacillin and nafcillin are semi-synthetic penicillins that can tolerate penicillinase. It has been reported that flucloxacillin at conventional plasma concentrations (200 μg/mL) does not affect the osteogenic differentiation of human bone marrow-derived MSCs[16]. Nafcillin can still exert its antibacterial effect under acidic conditions, but it has been reported that nafcillin has a strong inhibitory effect on the proliferation and differentiation of human osteoblasts. When its concentration exceeds 10 μg/mL, the ALP level in osteoblasts was drastically reduced[14].

Cephalosporins are an important branch of β-lactam antibiotics and play an important role in the treatment of various infectious diseases. Cefazolin, cefuroxime, cefotaxime, and cefepime are representative drugs of first, second, third, and fourth generation cephalosporins, respectively, and their effects on the differentiation of osteoblasts have been reported. Previous studies showed that cefuroxime does not alter the osteogenic differentiation of human bone marrow-derived MSCs at conventional blood concentrations (50 μg/mL)[16]. Cefazolin and cefepime cause osteogenic inhibition (above 25% and 75%, respectively) at concentrations up to 200 μg/mL, and cefotaxime inhibits the differentiation of osteoblasts (above 75%) at concentrations up to 500 μg/mL[14].

Carbapenems are a new class of β-lactams that are known for their broad spectrum. These drugs have strong antibacterial activity against most Gram-positive, Gram-negative, aerobic, anaerobic, and multi-drug resistant bacteria, and are one of the most important antibacterial drugs employed for the treatment of serious bacterial infections. Imipenem and meropenem are representative drugs that fall under in this category. Studies on the effect of these two drugs on differentiation of human osteoblasts have shown that imipenem does not have a significant effect on the differentiation potential of osteoblasts[14], while meropenem inhibits the differentiation of osteoblasts to a certain extent at concentrations of more than 500 μg/mL[14].

**Macrolides:** Macrolide antibiotics, drugs that inhibit bacterial protein synthesis by blocking peptide acyltransferase in bacterial ribosomes, are a class of drugs with extensive antibacterial spectrum. Azithromycin is a drug commonly used in clinical practice, and has certain inhibitory effects on various bacteria, mycoplasma, and

**Table 1 Effects of various antimicrobial agents on osteogenic differentiation**

| Agent | | Ref. | Cell / animal | Effect | Concentration |
|---|---|---|---|---|---|
| Antituberculosis drugs | Rifampicin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| | | [15] | BMSCs | Inhibition | 4-128 μg/mL |
| β-lactams | Penicillin | [16] | BMSCs | No effect | 30 μg/mL |
| | | [14] | Osteoblasts | Inhibition | ≥ 500 μg/mL |
| | Flucloxacillin | [16] | BMSCs | No effect | 200 μg/mL |
| | Nafcillin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| | Cefazolin | [14] | Osteoblasts | Inhibition | ≥ 200 μg/mL |
| | Cefuroxime | [16] | BMSCs | No effect | 50 μg/mL |
| | Cefotaxime | [14] | Osteoblasts | Inhibition | ≥ 500 μg/mL |
| | Cefepime | [14] | Osteoblasts | Inhibition | ≥ 200 μg/mL |
| | Imipenem | [14] | Osteoblasts | No effect | 0-1000 μg/mL |
| | Meropenem | [14] | Osteoblasts | Inhibition | ≥ 500 μg/mL |
| Macrolides | Azithromycin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| Aminoglycosides | Gentamicin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| | | [16] | BMSCs | Inhibition | ≥ 75 μg/mL |
| | | [17] | BMSCs | Inhibition | 50-200 μg/mL |
| | | [18] | C2C12 | Inhibition | 12.5-800 μg/mL |
| | Amikacin | [14] | Osteoblasts | No effect | 0-1000 μg/mL |
| | Tobramycin | [20] | BMSCs | Inhibition | 300-1000 μg/mL |
| | | [14] | Osteoblasts | Inhibition | ≥ 500 μg/mL |
| Tetracyclines | Tetracycline | [22] | BMSCs | Inhibition | 10 μg/mL |
| | Doxycycline | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| | Minocycline | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| Quinolones | Levofloxacin | [14] | Osteoblasts | Inhibition | ≥ 200 μg/mL |
| | Ciprofloxacin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| Polypeptide antibiotics | Colistin | [14] | Osteoblasts | Inhibition | ≥ 100 μg/mL |
| | Bacitracin | [23] | BMSCs | Promotion | 0.1-10 μmol/L |
| | Vancomycin | [24] | BMSCs | No effect | 0-500 μg/mL |
| | | [24] | BMSCs | Inhibition | 5000 μg/mL |
| | | [14] | Osteoblasts | No effect | 0-2000 μg/mL |
| | | [25] | BMSCs | No effect | 0-20 μg/mL |
| | | [16] | BMSCs | Inhibition | 200 μg/mL |
| Other types of antibiotics | Metronidazole | [16] | BMSCs | No effect | 20 μg/mL |
| | Trimethoprim | [14] | Osteoblasts | Inhibition | ≥ 500 μg/mL |
| | Linezolidone | [14] | Osteoblasts | Inhibition | ≥100 μg/mL |
| | Salinomycin | [26] | BMSCs | No effect | 10 μmol/L |
| Natural peptides | Lactoferrin | [27] | Adipose-derived stem cells | Promotion | 10-100 μg/mL |
| | | [29] | MC3T3-E1 | Promotion | 1-1000 μg/mL |
| | Hepcidin | [30] | BMSCs | Promotion | 0.2 mmol/L |
| | LL-37 | [31] | BMSCs | Promotion | 5-20 μg/mL |
| | KR-12 | [32] | BMSCs | Promotion | 1-1000 μg/mL |
| Chinese traditional drug extracts | Cordycepin | [33] | Adipose-derived stem cells | Promotion | 10 μg/mL |
| | | [34] | BMSCs | Promotion | 10 μg/mL |
| | Tanshinone IIA | [35] | BMSCs | Promotion | 1-5 μmol/L |
| | | [36] | C2C12 | Promotion | 2.5-10 μmol/L |
| | Andrographolide | [37] | Osteoblasts | Promotion | 4.46 or 8.92 μmol/L |
| | Baicalin | [38] | Sprague-Dawley rats | Promotion | 50 mg/kg |
| | | [39] | Osteoblasts | Promotion | 50 μmol/L |
| | Costunolide | [40] | C3H10T1/2 | Promotion | 1 ng/mL |
| | Extract of lithospermum | [41] | C2C12 | Promotion | 30 or 60 μg/mL |

| | | | | | |
|---|---|---|---|---|---|
| | | [42] | C2C12 | Promotion | 2 or 4 μg/mL |
| | Naringin | [43] | Adipose-derived stem cells | Promotion | 0.1 μmol/L |
| | Curcumin | [44] | Adipose-derived stem cells | Promotion | 5-20 μmol/L |
| | Limonene | [45] | C2C12 | Promotion | 2.5-10 μL |
| | Extract of piperaceae | [46] | Sprague-Dawley rats | Promotion | 100 or 200 mg/kg |
| | Eugenol | [47] | Dental pulp cells | Inhibition | 0.1-1 mL |
| | Saikosaponin-A | [48] | BMSCs | Promotion | 10-40 μL |
| | Licochalcone A | [49] | MC3T3-E1 | Promotion | 2.5-5 μL |
| Antifungal drugs | Trichostatin A | [50] | Adipose-derived stem cells | Promotion | 75 nL |
| | | [51] | Periodontal ligament cells | Promotion | 100-400 nL |
| | | [52] | Adipose-derived stem cells | Promotion | 1 μL |
| | Voriconazole | [53] | Osteoblasts | Promotion | 15 or 200 μg/mL |
| | Fluconazole | [53] | Osteoblasts | No effect | 15 or 200 μg/mL |

chlamydia. Studies have shown that azithromycin does not produce cytotoxicity in the concentration range of 0-200 μg/mL; however, it inhibits the differentiation potential of osteoblasts at very low concentrations. When its concentration exceeds 10 μg/mL, the differentiation of human osteoblasts grown in the osteogenic induction environment was significantly inhibited, and the level of intracellular ALP synthesis decreased by more than 75%[14].

**Aminoglycosides:** Aminoglycoside antibiotics are a class of drugs that are effective against Gram-negative bacteria and aerobic bacteria, and gentamicin is a representative drug of this category. Studies have shown that gentamicin inhibits the osteogenic differentiation of human osteoblasts. When the drug concentration is less than 100 μg/mL, the drug does not have a significant effect on osteogenic differentiation. However, when its concentration exceeds 100 μg/mL, gentamicin exhibits osteogenic inhibitory effects. When its concentration exceeds 500 μg/mL, the osteogenic differentiation potential is almost completely suppressed[14]. In another study, a similar phenomenon was observed in bone marrow-derived MSCs. When the gentamicin concentration reached 75 μg/mL, the proliferation and osteogenic differentiation activity of MSCs decreased significantly[16]. In addition, studies have shown that gentamicin can inhibit the osteogenic differentiation of human bone marrow-derived MSCs in a dose-dependent manner within a concentration range of 50-200 μg/mL[17]. The ALP level in the C2C12 cell line was similarly been reduced by gentamicin[18]. Amikacin is a drug commonly used for the treatment of gentamicin-resistant infectious diseases. Its most prominent advantage is that it remains stable and active against the aminoglycoside inactivating enzymes produced by many Gram-negative bacilli. In addition, its effect on osteoblast differentiation is also less severe than that of gentamicin. At an amikacin concentration of 1000 μg/mL, the osteogenic differentiation of osteoblasts is still not significantly inhibited. Osteogenesis inhibition is exhibited only after the amikacin level reaches a very high concentration of 2000 μg/mL[14]. As an aminoglycoside, tobramycin is often used for the treatment of gentamicin-resistant *Pseudomonas aeruginosa* infections. Studies have shown that tobramycin may have a lower cytotoxicity than gentamicin while exhibiting antibacterial effects[14]. However, the effect of tobramycin on osteogenic differentiation is still inhibitory[19]. When the concentration of tobramycin reaches 300 and 500 μg/mL, the osteogenic differentiation potential of human bone marrow-derived MSCs and osteoblasts is inhibited, respectively[14,20].

**Tetracyclines:** Tetracycline antibiotics exhibit a therapeutic effect on a variety of bacterial, rickettsial, chlamydial, and mycoplasma infections. Tetracycline is a representative member of such drugs. In addition to its role in killing various pathogenic microorganisms, tetracycline has been reported to exhibit bone tissue affinity and can, thus, be used for various targeted therapies[21]. Studies related to osteogenic differentiation have shown that 10 μg/mL tetracycline can promote osteogenic differentiation of rat bone marrow-derived MSCs, increase ALP and mineralized nodules, and upregulate the osteogenic gene expression levels in

MSCs[22]. Doxycycline is the most commonly used tetracycline antibiotic, but unlike tetracycline, it exhibits a strong inhibitory effect on osteoblast proliferation and osteogenic differentiation. When its concentration reaches 100 μg/mL, the differentiation of human osteoblasts is severely inhibited[14]. Minocycline is also widely used in clinical practice, and its antibacterial efficacy is relatively strong among tetracyclines. Similar to doxycycline, minocycline significantly inhibited the differentiation potential of osteoblasts (above 75%) at concentrations above 100 μg/mL[14].

**Quinolones:** Quinolones are a class of synthetic antibiotics that are widely used in a variety of clinical infectious diseases due to their excellent and broad-spectrum antimicrobial properties. Levofloxacin is a commonly used quinolone in the clinic. Studies have shown that it does not cause toxicity to human osteoblasts in the concentration range of 0-200 μg/mL, but when the drug concentration reaches 200 μg/mL or more, the differentiation potential of osteoblasts is significantly inhibited (above 75%)[14]. Ciprofloxacin is another representative drug of quinolones, which has poor biocompatibility and significantly inhibits the proliferation and differentiation of osteoblasts at concentrations above 10 μg/mL (above 75%)[14].

**Polypeptide antibiotics:** Polypeptide antibiotics are a class of antibiotics with structural features similar to those of polypeptides, and their main members include polymyxins, bacitracins, and vancomycins. Colistin is one of the more commonly used polymyxin antibiotics. It mainly acts on Gram-negative bacteria and works synergistically with gentamicin. It has been reported in the literature that when the concentration of colistin reaches 100 μg/mL, the differentiation ability of human osteoblasts is inhibited[14]. Bacitracin is a metal peptide antibiotic produced by *Bacillus subtilis* and *Bacillus licheniformis*; it can strongly inhibit Gram-positive bacteria and has antagonistic effects on the development of resistance to *Staphylococcus aureus*. Our previous studies have shown that bacitracin can promote the osteogenic differentiation of human bone marrow-derived MSCs in a dose-dependent manner, thus increasing intracellular ALP, collagen, and mineralization, and upregulating the levels of osteogenesis marker genes. When the concentration of bacitracin reached 100 μmol/L, its ability to promote bone differentiation decreased, but this effect was still stronger than that in the control group[23]. Vancomycin is mainly used for the treatment of methicillin-resistant *Staphylococcus aureus*. There have been several reports on the effects of vancomycin on osteogenic differentiation. The general view is that vancomycin does not adversely affect the osteogenic differentiation of human osteoblasts and human bone marrow-derived MSCs at effective antimicrobial concentrations and higher concentrations[14,24,25]. However, it has also been reported that vancomycin inhibits the osteogenic differentiation of bone marrow-derived MSCs at a concentration of 200 μg/mL[16]. Therefore, further research on the regulation of osteogenic differentiation by vancomycin needs to be conducted to determine whether the effect of this drug on osteogenic differentiation is related to cell type and drug concentration.

**Other types of antibiotics:** Metronidazole is a drug commonly used in the treatment of anaerobic infections in the clinic. Studies have shown that conventional plasma concentrations (20 μg/mL) of metronidazole do not affect the osteogenic differentiation potential of human bone marrow-derived MSCs[16]. Trimethoprim (TMP) is a well-known sulfa drug enhancer with an antibacterial spectrum similar to that of sulfonamides. When TMP is combined with a sulfa drug, the combined antibacterial properties of both are greatly enhanced, and the formation of resistant bacteria can be reduced. Studies have shown that TMP does not affect the differentiation potential of osteoblasts in the concentration range of 0-200 μg/mL. However, when the concentration of TMP reaches 500 μg/mL, the osteogenic differentiation of the cells is inhibited[14]. Linezolidone is a bacterial protein synthesis inhibitor and is a fully synthetic oxazolidinone antibiotic. The drug has good biocompatibility and does not affect the viability of osteoblasts between 0-500 μg/mL. However, when the concentration of linezolidone is greater than 10 μg/mL, osteogenic inhibition occurs[14]. Salinomycin is a polyether antibiotic produced by *Streptomyces albus*. Studies have shown that 10 μM of salinomycin does not affect osteogenic differentiation and cellular mineralization of human bone marrow-derived MSCs[26].

### *Natural peptides*

In addition to the use of antimicrobial agents for the treatment of pathogenic microorganisms, which cause infection symptoms, activation of immune cells and secretion of some peptides with antimicrobial effects in the human body also play a decisive role in the elimination of infection. Lactoferrin is an important non-heme iron-binding glycoprotein found in milk, with powerful biological functions, such as

broad-spectrum antibacterial, anti-oxidation, anti-cancer effects, and immune system regulation. It has been reported that lactoferrin promotes the differentiation of human adipose-derived stem cells into osteoblasts in a concentration-dependent manner and also promotes the expression of osteogenic genes[27]. Similarly, other studies have found that lactoferrin promotes the proliferation of MC3T3-E1 osteoblast cells *via* the mitogen-activated protein kinase (MAPK) signaling pathway and promotes the differentiation of MC3T3-E1 into osteogenesis *via* the protein kinase A and p38 signaling pathways[28,29]. Hepcidin is a cysteine-rich polypeptide synthesized and secreted by the liver, which has a wide range of antibacterial and anti-protozoal functions. Studies have found that, in addition to regulating iron metabolism and antibacterial properties, hepcidin also regulates the function of rat bone marrow-derived MSCs. At a concentration of 0.2 mmol/L, hepcidin enhanced the mineralization ability of rat bone marrow-derived MSCs and upregulated the expression of osteogenic genes. The researchers found that this osteogenic differentiation may be related to the activation of the p38 signaling pathway[30]. As an important part of the immune system, antimicrobial peptides (AMPs) can destroy microbial membranes and induce the death of pathogenic bacteria, having the potential to become a substitute for traditional antibiotics. The only natural antimicrobial peptide, cathelicidin (hCAP18/LL-37), was confirmed in 1995 and proved to exhibit antibacterial activity both *in vitro* and *in vivo*. Moreover, in addition to its resistance to pathogenic microorganisms, LL-37 has also been shown to promote the proliferation, migration, and osteogenic differentiation of rat bone marrow-derived MSCs. In the concentration range of 5-20 μg/mL, LL-37 promoted the osteogenic differentiation potential of MSCs in a dose-dependent manner. More importantly, LL-37 at a concentration of 10 μg/mL can reverse the osteogenic inhibition caused by lipopolysaccharide[31]. However, since the peptide chain of LL-37 is too long and too difficult to synthesize, it is inconvenient to use it as a conventional therapeutic drug for bacterial infections and inflammatory diseases. Short-chain AMPs have recently attracted attention due to their lower production costs. Among the LL-37 active fragments of different lengths investigated, KR-12 is the shortest antimicrobial peptide with antibacterial activity. In our previous study, KR-12 stimulated osteogenic differentiation of human bone marrow-derived MSCs within an effective antimicrobial concentration (1-1000 μg/mL). This osteoinductive phenomenon also appears to be concentration-dependent[32].

### *Chinese traditional drug extracts*

Chinese traditional drugs are mainly composed of botanicals (roots, stems, leaves, and fruits), animal drugs (viscera, skin, bone, organs, *etc*.,), and mineral medicines. Since such drugs are often present in a mixture rather than in a monomer form, their pharmacological effects are often studied by extracting the active ingredient of the drug. Similar to the above-mentioned antimicrobial drugs, some Chinese herbal extracts with antibacterial or anti-pathogenic properties have attracted a lot of attention in recent years[33-49]. Compared with traditional antibiotics, these Chinese traditional drug extracts exhibit less side effects and are less prone to drug resistance while exerting antibacterial effects. Among these herbal extracts, some promote osteogenic differentiation of bone marrow-derived MSCs, such as cordycepin, tanshinone, and baicalin[33-36,38,39]. If these extracts can exert stable antibacterial activity and simultaneously induce bone marrow-derived MSCs to differentiate into new osseous tissue by virtue of their osteoinductive properties, the clinical application prospects of these extracts will be more extensive. The Chinese traditional drug extracts that have been reported to regulate osteogenic differentiation and to exhibit antibacterial properties in recent years are also listed in Table 1.

### *Antifungal drugs*

Local and systemic fungal infections are not uncommon, and with the increase in immunodeficiency diseases, such as acquired immunodeficiency syndrome, the harm caused by fungal infections is also more serious. Fungal infections of bone tissue are rare and often accompanied by systemic immunodeficiencies or inhibition. While antifungal agents are used to treat fungal infections, the effects of the drug itself on osseous tissue and osteogenic differentiation are equally noteworthy. Trichostatin A (TSA) is a drug that exhibits a therapeutic effect on mold. It has been found that TSA at 75 nmol/L can stimulate the osteogenic differentiation potential of rat adipose stem cells[50]; some scholars have found similar phenomena in human periodontal ligament cells (HPDLCs). TSA can promote the differentiation of such cells into osteoblasts in a concentration-dependent manner within a concentration range of 100-400 nmol/L[51]. As inhibitors of histone deacetylases, TSA (1 μmol/L) also increases bone formation during osteogenic differentiation of human adipose-derived stem cells[52]. Voriconazole is an antifungal drug commonly used to treat severe invasive infections caused

by fluconazole-resistant Candida. Studies on its effects on osteoblasts have shown that voriconazole at both 15 μg/mL and 200 μg/mL can stimulate osteogenic differentiation of human osteoblasts *in vitro*, whereas fluconazole exhibits no such effect of inducing differentiation[53].

## EFFECTS OF VARIOUS ANTIMICROBIAL AGENTS ON CHONDROGENIC DIFFERENTIATION

As an important seed cell for local cartilage repair, the ability of bone marrow-derived MSCs to differentiate into chondrocytes in the direction of cartilage is essential[54]. After the cartilage tissue is damaged by factors such as trauma, inflammation, and infection, microfracture surgery is an important approach for clinical treatment of local cartilage defects[55]. Surgery can transport MSCs in the medullary cavity to the cartilage defect area and complete the repair of the local defect by dividing the cells into the cartilage direction[56]. During the treatment of joint infections, surgical treatment, such as debridement drainage, and the application of systemic or topical antibiotics are equally important. If the drug can effectively control the infection and promote the differentiation of MSCs into chondrocytes to repair the existing cartilage defects, its clinical application range will be greatly increased, and it will play a more important role in the process of infectious arthritis and tissue engineering cartilage repair. At present, there have been very few studies on the regulation of chondrogenic differentiation by various antibacterial drugs. In this section, we list antimicrobial drugs that have been shown to have an effect on chondrogenic differentiation, and the overall data are listed in Table 2. In the previous section, we discussed the inhibitory effect of doxycycline on human osteoblast differentiation. The effect of this drug on the chondrogenic differentiation potential of human bone marrow-derived MSCs has also attracted attention. It has been reported that doxycycline at 2 μg/mL can enhance the chondrogenic differentiation of MSCs *in vitro*. This phenomenon was further confirmed *in vivo*[57]. Oxytetracycline is another member of the tetracycline antibiotic class, and some scholars have reported its ability to promote cartilage differentiation in ATDC5 cell line (pre-chondrocyte cell line). Studies have shown that oxytetracycline can promote the differentiation of ATDC5 cells into cartilage in a dose-dependent manner within a concentration range of 0.01 to 10 μmol/L[58]. Cordycepin is a natural extract that has been extensively studied in recent years. Its broad-spectrum antibacterial, anti-fungal, and anti-viral capabilities have attracted the attention of the medical community. The positive effect of cordycepin on the osteogenic differentiation potential of various stem cells has been introduced in the previous section, and its regulatory effect on the chondrogenic differentiation of MSCs is also worthy of attention. Studies have shown that 1 μg/mL of cordycepin can promote the differentiation of MCSs into cartilage and increase the expression levels of intracellular cartilage genes. Further experiments have demonstrated that this phenomenon is mediated by the inhibition of Nrf2 and the activation of BMP signaling[59]. Similarly, some scholars have found that lactoferrin promotes early chondrogenic differentiation of ATDC5 cells by the activating Smad2/3-Sox9 signaling pathway while also exhibiting osteoinductive effects, and also inhibits excessive hypertrophy of chondrocytes[60].

Phorbol-12-myristate-13-acetate (PMA) is an antibiotic extracted from penicillium culture and the first antibiotic to treat human diseases. Very low concentrations of PMA (0.1 μmol/L) have a strong inhibitory effect on the chondrogenic differentiation potential of chick embryonic stem cells[61]. TSA exhibits osteogenic induction properties while possessing antibacterial properties. It can positively regulate osteogenic differentiation, but exhibits an inhibitory effect on chondrogenic differentiation. When the concentration of TSA reaches 100 nmol/L, the chondrogenic differentiation of human bone marrow-derived MSCs induced by transforming growth factor-β (TGF-β1) can be inhibited[62].

## EFFECTS OF VARIOUS ANTIMICROBIAL AGENTS ON ADIPOGENIC DIFFERENTIATION

Adult bone marrow contains a variety of cells, such as endothelial-like cells, fibroblasts, macrophages, osteocytes, adipocytes, and MSCs. Among them, adipocytes are the most abundant and can occupy more than 50% of the volume of the bone marrow cavity. In old age, adipocytes can even occupy more than 90% of the volume of the marrow cavity[63]. Bone marrow adipocytes are also involved in bone meta-

**Table 2 Effects of various antimicrobial agents on chondrogenic differentiation**

| Agent | | Ref. | Cell | Effect | Concentration |
|---|---|---|---|---|---|
| Antibiotics | Doxycycline | [57] | MSCs | Promotion | 2 μg/mL |
| | Oxytetracycline | [58] | ATDC5 | Promotion | 0.01-10 μmol/L |
| | PMA | [61] | Embryonic stem cells | Inhibition | 0.1 μmol/L |
| Natural peptides | Lactoferrin | [60] | ATDC5 | Promotion | 1 μmol/L |
| Chinese traditional drug extracts | Cordycepin | [59] | MSCs | Promotion | 1 μg/mL |
| Antifungal drugs | Trichostatin A | [62] | BMSCs | Inhibition | 100 nmol/L |

bolism. In the pathological state of advanced osteoporosis or osteonecrosis, the differentiation of bone marrow-derived MSCs into adipocytes is enhanced, resulting in an increased number of adipocytes and decreased bone mass[64]. As an important component of the bone marrow microenvironment, bone marrow adipocytes not only occupy the non-hematopoietic medullary cavity space, but also have many physiological functions and play an important role in the pathological process of various diseases[65]. Bone marrow-derived MSCs are the main source of bone marrow adipocytes, and their adipogenic differentiation potential plays a vital role in the physiological renewal of adipose tissue in the medullary cavity and the repair of fat necrosis caused by pathological factors, such as infection[66]. During the process of using antibacterial drugs to treat osteomyelitis caused by various pathogenic microorganisms, both the antibacterial properties of antimicrobial drugs and their effects on the adipose tissue repair process are worthy of attention. In this section, we will review the effects of various antimicrobial agents on adipogenic differentiation to provide a reference for clinical use (Table 3).

### *Antibiotics*

Isoniazid is another important member of anti-tuberculosis drugs, and its inhibition of adipogenic differentiation has been reported in the literature. Isoniazid inhibited the adipogenic differentiation potential of 3T3-L1 pre-adipocytes in a concentration-dependent manner, in a concentration range of 0.5-10 mmol/L. A similar phenomenon was also observed in human adipose stem cells[67]. Streptomycin is an aminoglycoside antibiotic, but it is widely used in the treatment of tuberculosis because of its anti-tuberculosis effect. Some scholars have found that 100 μg/mL streptomycin can inhibit the expression of adipogenic genes and the adipogenic ability of human bone marrow-derived MSCs[68]. Spiramycin is a macrolide antibiotic that exhibits antibacterial properties in the body and can enhance the phagocytosis of phagocytic cells. Studies on the effects of this drug on adipogenesis have revealed that spiramycin inhibits adipogenesis both *in vivo* and *in vitro*. Spiramycin at concentrations of 2.5-20 μmol/L inhibited the adipogenic differentiation of 3T3-L1 pre-adipocyte cells in a dose-dependent manner, which was further confirmed in the high-fat diet-induced obese mice model[69]. It is reported in the above study that salinomycin at 10 μmol/L does not affect the osteogenic differentiation potential of human bone marrow-derived MSCs. At this concentration, the adipogenic differentiation activity of MSCs is also not affected[26]. However, we believe that this result does not represent the effect of thalimycin at different concentrations on the osteogenic and adipogenic differentiation of MSCs. Further studies are needed to demonstrate the effect of this drug on the multi-directional differentiation potential of bone marrow-derived MSCs. Geldanamycin is an antibiotic secreted by *Streptomyces hygroscopicus* and has been shown to exhibit antibacterial, antiprotozoal, and antitumor activities. Studies have shown that geldanamycin can inhibit the adipogenic differentiation of 3T3-L1 pre-adipocytes in a dose-dependent manner at very low concentrations (0.001-1 μmol/L). *In vivo* experiments in mice further confirmed the inhibitory effect of geldanamycin on adipogenic differentiation[70].

### *Natural peptides*

The positive regulation of lactoferrin on osteogenic and chondrogenic differentiation has been mentioned in the previous section, and its regulation of adipogenic differentiation is also worthy of attention. More than one study has shown that lactoferrin negatively regulates the adipogenic differentiation potential of cells. Some scholars have found that MC3T3-G2/PA6 cells gradually lose their ability to differentiate into adipocytes under the action of 10-100 μg/mL lactoferrin[71]; the level of adipogenic genes in C1C12 pluripotent stem cells have also been found to

**Table 3 Effects of various antimicrobial agents on adipogenic differentiation**

| Agent | | Ref. | Cell / animal | Effect | Concentration |
|---|---|---|---|---|---|
| Antibiotics | Isoniazid | [67] | 3T3-L1 | Inhibition | 0.5-10 mmol/L |
| | | [67] | Adipose stem cells | Inhibition | 2 or 10 mmol/L |
| | Streptomycin | [68] | BMSCs | Inhibition | 100 μg/mL |
| | Spiramycin | [69] | 3T3-L1 | Inhibition | 2.5-20 μmol/L |
| | Salinomycin | [26] | BMSCs | No effect | 10 μmol/L |
| | Geldanamycin | [70] | 3T3-L1 | Inhibition | 0.001-1 μmol/L |
| Natural peptides | Lactoferrin | [71] | MC3T3-G2/PA6 | Inhibition | 10-100 μg/mL |
| | | [72] | C1C12 | Inhibition | 0.1-10 μmol/L |
| | | [73] | Subcutaneous preadipocytes | Promotion | 10 μmol/L |
| Chinese traditional drug extracts | Cordycepin | [74] | 3T3-L1 | Inhibition | 10-100 μg/mL |
| | Tanshinone IIA | [75] | 3T3-L1 | Inhibition | 2.5-10 μmol/L |
| | | [76] | 3T3-L1 | Inhibition | 1-10 μmol/L |
| | Andrographolide | [77] | 3T3-L1 | Inhibition | 1-5 μg/mL |
| | Baicalin | [78] | 3T3-L1 | Inhibition | 200 μmol/L |
| | | [79] | Atherosclerosis mice | Inhibition | 50 or 100 mg/kg |
| | Oleuropein | [80] | BMSCs | Inhibition | 10 μmol/L |
| | | [81] | 3T3-L1 | Inhibition | 0.1-100 μmol/L |
| | Piperlonguminine | [82] | 3T3-L1 | Promotion | 3-30 μmol/L |
| | Hydroxytyrosol | [83] | BMSCs | Promotion | 1 or 100 mmol |
| | | [84] | Omental pre-adipocyte cells | Inhibition | 30 μg/mL |
| | Shikonin | [85] | 3T3-L1 | Inhibition | 0.5-2 μmol/L |
| | Ursolic acid | [86] | 3T3-L1 | Inhibition | 2.5-10 μmol/L |
| | Alpinia officinarum | [87] | 3T3-L1 | Inhibition | 150-400 μg/mL |
| | Dioscin | [88] | 3T3-L1 | Inhibition | 1-4 μmol/L |
| | Methyl cinnamate | [89] | 3T3-L1 | Inhibition | 12.5-100 μmol/L |
| | Tetrandrine | [90] | 3T3-L1 | Inhibition | 2.5-10 μmol/L |
| | Honokiol | [91] | 3T3-L1 | No effect | 1-10 μmol/L |
| | Licochalcone A | [92] | 3T3-L1 | Inhibition | 5 or 10 μmol/L |
| Antifungal drugs | Trichostatin A | [51] | Periodontal ligament cells | No effect | 400 nmol/L |
| | | [93] | 3T3-L1 | Inhibition | 500 nmol/L |
| Antiviral drugs | Efavirenz | [94] | Pre-adipocytes | Inhibition | 0.5-4 μmol/L |
| | | [104] | SGBS pre-adipocytes | Inhibition | 0.1-5 μmol/L |
| | Zidovudine | [95] | 3T3-F442A | Inhibition | 6-50 μmol/L |
| | | [99] | 3T3-F442A | Inhibition | 1-6 μmol/L |
| | Stavudine | [95] | 3T3-F442A | Inhibition | 3-75 μmol/L |
| | Lamivudine | [95] | 3T3-F442A | Inhibition | 8-200 μmol/L |
| | Nelfinavir | [98] | 3T3-L1 | Inhibition | 20 μmol/L |
| | Efavirenz | [96] | Adipocyte precursor cells | Inhibition | 4 μmol/L |
| | | [97] | Adipocyte precursor cells | Inhibition | 2 or 4 μmol/L |
| | Maraviroc | [96] | Adipocyte precursor cells | No effect | 0.1-4 μmol/L |
| | Nevirapine | [97] | Adipocyte precursor cells | Promotion | 2 or 4 μmol/L |
| | Darunavir | [100] | 3T3-L1 | Inhibition | 0.1-25 μmol/L |
| | Raltegravir | [101] | 3T3-F442A | Inhibition | 1-50 μg/mL |
| | Indinavir | [102] | 3T3-L1 and 3T3-F442A | Inhibition | 10 or 20 μmol/L |
| | | [103] | 3T3-F442A | Inhibition | 1-50 μg/mL |
| | Elvitegravir | [104] | SGBS pre-adipocytes | Inhibition | 0.1-5 μmol/L |
| Antimalarials | Amodiaquine | [105] | 3T3-L1 | Inhibition | 0.1-10 μmol/L |
| | Quinine | [106] | Preadipocytes | Promotion | 5-50 μmol/L |
| | Artemisinic Acid | [107] | Adipose-derived stem cells | Inhibition | 50 or 200 μmol/L |

downregulate under the action of nipple proteins, and instead, the cells differentiate into osteogenesis and cartilage[72]. However, studies have shown that lactoferrin at a concentration of 10 μmol/L can promote the adipogenic activity of subcutaneous preadipocytes, and the associated adipogenic protein levels are also increased[73]. These results suggest that more research on the regulatory effect of lactoferrin on adipogenic differentiation needs to be conducted.

### *Chinese traditional drug extracts*

In Table 1, we list the Chinese traditional drug extracts that have been reported to have antibacterial properties and can regulate osteogenic differentiation in recent years. Among these Chinese traditional drug extracts, cordycepin, tanshinone, andrographolide, and baicalin have also been reported to exhibit the ability to regulate adipogenic differentiation[74-79]. In addition, other Chinese traditional medicines that have antimicrobial effects and have the opportunity to play a role in clinical infectious diseases have also been reported to regulate adipogenesis[80-92]. We summarize the regulation mediated by these Chinese traditional drug extracts on adipogenic differentiation in Table 3.

### *Antifungal drugs, antiviral drugs, and antimalarials*

The promotion of TSA for osteogenic differentiation and inhibition of chondrogenic differentiation have been mentioned earlier in this paper. In a study of its effects on adipogenic differentiation, TSA at a concentration of 400 nmol/L did not promote differentiation of HPDLCs into adipogenic phase[51]. In another study, the researchers concluded that TSA at a concentration of 500 nmol/L inhibited the adipogenic differentiation activity of 3T3-L1 cells by inhibiting the activity of histone deacetylase[93].

Viruses and malarial parasites are not common pathogenic microorganisms of bone and joint infections. However, due to the particularity of the mechanism of pharmacological action, its related therapeutic drugs may have a significant impact on fat metabolism. We summarize the antiviral and antimalarial drugs that have been reported to regulate adipogenesis in recent years and list them in Table 3[94-107].

## CONCLUSION

In order to achieve better results *via* the antimicrobial drug treatment of bone and joint infections, we should pay attention to the elimination of pathogenic microorganisms using various antimicrobial drugs while also taking into account the effects of these drugs on local tissue repair. Bone marrow-derived MSCs are used as core cells for the renewal and repair of local bone, cartilage, and medullary adipose tissue. The regulation of multiple differentiation potentials of MSCs by various antimicrobial agents affects recovery from bone and joint infectious diseases. In the course of clinical drug treatment, only by understanding the effects of antibacterial drugs on the osteogenic, cartilage, and adipogenic differentiation of bone marrow-derived MSCs and rationally selecting the antimicrobial drugs that are most beneficial for controlling infection as well as repairing local tissue according to the pathogens and infection sites involved, can effective treatment against infection with minimum damage to local tissue be achieved.

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MINIREVIEWS

# Effect of aging on behaviour of mesenchymal stem cells

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## Abstract

Organs whose source is the mesoderm lineage contain a subpopulation of stem cells that are able to differentiate among mesodermal derivatives (chondrocytes, osteocytes, adipocytes). This subpopulation of adult stem cells, called "mesenchymal stem cells" or "mesenchymal stromal cells (MSCs)", contributes directly to the homeostatic maintenance of their organs; hence, their senescence could be very deleterious for human bodily functions. MSCs are easily isolated and amenable their expansion *in vitro* because of the research demanding to test them in many diverse clinical indications. All of these works are shown by the rapidly expanding literature that includes many *in vivo* animal models. We do not have an in-depth understanding of mechanisms that induce cellular senescence, and to further clarify the consequences of the senescence process in MSCs, some hints may be derived from the study of cellular behaviour *in vivo* and *in vitro*, autophagy, mitochondrial stress and exosomal activity. In this particular work, we decided to review these biological features in the literature on MSC senescence over the last three years.

**Key words:** Mesenchymal stem cells; Aging; Autophagy; Mitochondrial stress; Extracellular vesicles

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**Core tip:** The point of interest of this work is the behaviour of the mesenchymal stromal cell (MSC) through aging, which can occur over time in the culture (*in vitro*) or in its own physiological niche (*in vivo*). This review defines the current knowledge published in the MSC field that focuses mainly on the mechanisms that influence its senescence *in vivo* and *in vitro* in the last three years. Three cellular mechanisms are of special importance in this review, since they can decisively influence the behaviour of MSC in aging, such as autophagy, oxidative stress and the production of extracellular vesicles.

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## INTRODUCTION

Mesenchymal stem cells (MSCs) are located in specific areas of tissues, called "niches", and are characterized as being in a state of relative quietness, from which they can exit under the proper conditions to obtain the proliferative potential necessary for tissue regeneration[1]. MSCs have sustained interest among researchers by contributing to tissue homeostasis and modulating inflammatory response, all activities accomplished primarily by the secretion of cytokines and growth factors, because their paracrine action is the main mechanism explaining their effects, regardless of source.

Senescence is defined as a mechanism for limiting the regenerative potential of stem cells which is involved with metabolic changes in the oxidative state of the cell, this process that has been also linked to mitochondrial fission and fusion events could indicate association between mitochondrial dynamics and senescence[2]. Furthermore, senescence-associated phenotypes are characterized by increased activity of SA-$\beta$-gal, altered autophagy, and increased G1 cell cycle arrest, reactive oxygen species (ROS) production and expression of p53 and p21[3]. It is now evident that senescent cells secrete dozens of molecules, for which the terms "senescence-associated secretory phenotype (SASP)" and "senescence-messaging secretome (SMS) factors" have been proposed. Premature aging produced by overexpression of mutant *LMNA* called progerin in the rare disease Hutchinson-Gilford Progeria Syndrome is linked to upregulation of SASP by GATA4-dependent regulation *via* MCP-1 in human MSC aging[4]. The secreted factors contribute to cellular proliferative arrest through autocrine/paracrine pathways as well as *in vivo* and *in vitro*[5-8]. SMS factors released by senescent cells play a key role in cellular senescence and physiological aging by activation of cytoplasmic signalling circuitry, so SMS factors secreted in conditioned medium of senescent MSCs induce a paracrine mechanism of premature senescence in young cells[9].

The milestone in MSC investigation will be discovering senescence markers to determine the quality of the *in vitro* cells for cell-based therapies. Madsen *et al*[10] have proposed TRAIL receptor CD264 as the first cellular senescence mesenchymal marker in bone marrow-derived MSCs, because it has the same expression profile of p21 during culture passage and it is not linked to sex[10]. On the other hand, it is a good approach to identify immunogenic markers from age tissue sources, and the first study was developed by Amati *et al*[11], who proposed the angiotensin-converting enzyme CD143 as a marker expressed in adult tissue sources from the screening using bone marrow- and cord blood-derived MSCs (Figure 1B).

## MSCs' BEHAVIOUR *IN VITRO*

After long-term expansion, the phenotype of MSCs keeps stable and cells present similar immunogenic properties to lower passage cells. However, their immunosuppressive properties are reduced[12]. One of the drawbacks of MSCs is the decline in their self-renewal capacity with increased donor age (Figure 1A) and *in vitro* expansion[13-18] (Figure 1B). However, by increasing the number of umbilical cord vein-MSC passages, immunosuppressive effects were promoted as a result of the greater purity of the MSCs and their major compatibility with culture conditions[19]. These results reveal the different implications of the application of high passage MSCs in the clinic, it would help increase their production for therapeutic uses but might interfere with their efficacy. The self-renewal of MSCs decrease is caused by shor-tening telomeres in aged MSCs[14] and this was also demonstrated when overexpression of hTERT bypassed a replicative senescence in hBM-MSCs[20]. Kouroupis *et al*[21] have reported that the number of $CD146^+$ UC-derived MSCs decreased with the *in vitro* age and this is associated with the telomere length. This year, it was discovered that epigenetic changes are implicated in the maintenance of stem cell properties of MSCs, demonstrating that expression of the pluripotency marker Oct4 keeps self-renewal and reverse aging in human hair follicle derived-MSCs through the inhibition of p21

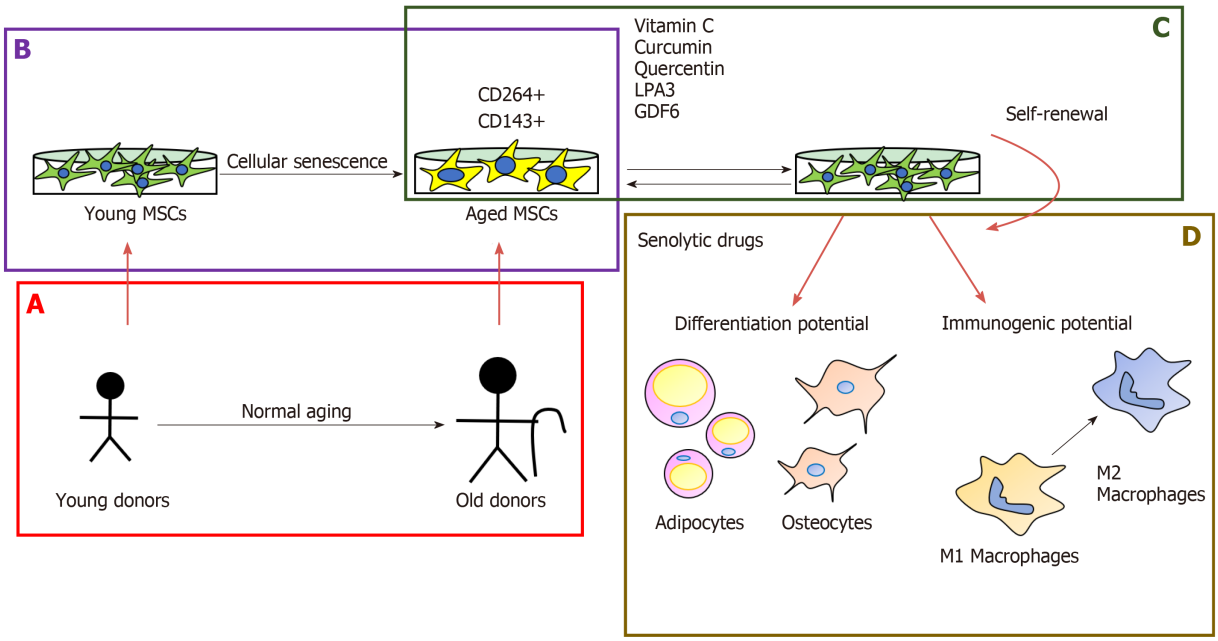


**Figure 1 Effect of aging on self-renewal, differentiation and immunogenic potential from mesenchymal stem cells.** A, B: Stem cell properties of mesenchymal stem cells (MSCs) are limited by age donor (A), and their long-term *in vitro* culture (B); C: Some new agents can ameliorate the effect of cellular senescence on the therapeutic capacity of MSCs; D: Treatment with senolytic drugs affects the behaviour of MSCs. MSCs: Mesenchymal stem cells; LPA: Lysophosphatidic acid.

by DNA methyltransferases[22].

Non-coding RNA can play a role in the cellular senescence in MSCs, though the interfering lincRNA-p21 expression might allow the rejuvenation of aged BM-MSCs from C57BL/6 mice *via* the Wnt/b-catenin signalling pathway[23]. Rn7SK is a conserved small nuclear non-coding RNA, which is overexpressed in senescent adipose tissue-derived MSCs. So, it is directly involved in the decrease of osteogenic differentiation and proliferation[24].

There is an increase in the number of studies about the effect of natural-origin regulators that prevent or ameliorate cellular senescence in MSCs. Vitamin C also has the potential to re-establish the activity of telomerase reverse transcriptase (TERT) in bone marrow-derived MSCs from senescence-accelerated mouse prone 6 (SAMP6) mice[25]. Curcumin improves the proliferation of aged rat adipose tissue-derived MSCs through *TERT* gene expression[26] (Figure 1C). Another option for treating age-related diseases is the use of senolytic drugs, which eliminate target senescent cells and rejuvenate tissues[27]. Grezella *et al*[28] have studied the impact of these drugs on human MSCs, such as ABT-263, quercetin, danazol and nicotinamide ribose, which don't have a positive effect on MSCs because they produce changes in the SASP of human femoral bone marrow MSCs. However, Geng *et al*[29] have proposed quercetin as a geroprotective compound for human MSCs from Werner syndrome. Because it re-establishes the differentiation potential and self-renewal through its antioxidant capacity and growth differentiation factor 6, secreted by young MSCs, it can restore the osteogenic capacity of MSCs from elderly donors[29,30] (Figure 1D).

Human bone marrow MSCs from young donors have a better monocyte polarization capacity than MSCs from old donors[31]. Non-senescent MSCs secrete some bioactive factors, which can ameliorate the replicative senescence through enhanced cell proliferation and osteogenic differentiation potential in prolonged *in vitro* culture[32]. Human umbilical cord blood MSCs stimulate the rejuvenation function in human skin[33]. Lysophosphatidic acid (LPA) is a bioactive small glycerophospholipid derived from cytoplasm that promotes cell proliferation, survival and migration[34]. Complementing those results, Kanehira *et al*[35] have stated that two components of these acids (LPA1 and 3) regulate cellular senescence in MSCs positively and negatively, respectively.

## MSCs' BEHAVIOUR *IN VIVO*

MSCs isolated from the term umbilical cord vein have stronger immunomodulatory capacity than preterm ones. Increased immunological maturity of term umbilical cord

vein MSCs may be the explanation for that[19].

*In vivo* senescence of MSCs is associated with bone-related disease because the cells lost the osteogenic capacity. In the last year, the number of studies based on gene therapy has increased with a view to improving the stem cell properties in the development of cell-based therapies. Non-coding RNA like miR-1292 was proposed as a senescence regulator in human adipose-derived MSCs and delay bone formation *in vivo* by targeting FZD4 *via* the Wnt/b-catenin pathway. It is a good target for the prevention and treatment of osteoporosis[36]. The loss of the *in vivo* osteogenesis potential of aged bone marrow MSCs is mediated by p53 through the miR-17 pathway[37]. In cardiovascular disease, it was found that overexpression of miR-10a in aged human bone marrow MCs activates AKT and improves the angiogenesis in ischaemic mouse hearts[38]. The overexpression of FOXQ1 in UC-derived MSCs regulates the migration and anti-senescence effects[39]. SATB2-modified bone marrow-derived MSCs significantly ameliorate ovariectomy-induced alveolar bone loss *in vivo*[40].

In the last few years, the MSCs from human-induced pluripotent stem cells have had low oncogenic potential and strong immune capacity to regulate T cells[41]. They modulate CD4 and CD8 cells and lead the upregulation of immune genes and downregulation of c-myc and DNA replicative pathways[42].

## AUTOPHAGY IN MSCs

Autophagy increases when MSCs enter the replicative aging state, and p53 contributes an important role in the upregulation of autophagy in this condition[43]. In contrast, suppression of the p53 transcriptional activity produced strong cell death of $H_2O_2$-treated MSCs through autophagy induction[44]. Autophagy is playing an important role in the mammalian stress response because can be modulated by several ways through hypoxia induced stress in different organelles. Autophagy is deeply linked to senescence, and in some experimental models, the onset of senescence is dependent on a preliminary autophagy induction: For instance, the downregulation of IGF-1 protects senescence MSCs from hypoxic condition by growing the level of autophagy, thereby allowing the survival of senescence bone marrow MSCs after myocardial infarction transplantation[45] (Figure 2). Brunk and Termal[46] presented the theory of aging which consisted in accumulation of damage in mitochondrial-lysosomal axis as a result of imperfect autophagocytosis during aging in tissue with limited turnover, and this has remained valid until now, when reversible quiescence is the normal stem cell state throughout life-adds[46-48]. In the opposite, in other contexts the decrease of autophagy provokes senescence, as shown in several types of MSC acute senescence which the autophagy flux is heavily imbalanced, indicating the autophagy counteracts damaged processes, and its decline produces senescence[49]. Reconciling these opposite events would be possible by speculating that MSCs try to lead with stress by inducing autophagy that removes damaged components; in this scenario, autophagy would protect from aging and its malfunction might trigger senescence. However, if autophagy cannot counteract stress-induced damage, it could induce senescence. Hyperglycaemia has been reported to MSC senescence[50]. Chang *et al*[51] researched the role of high-glucose-induced autophagy in MSC senescence publishing that high glucose increased autophagosome formation, which was linked with the development of senescence process in the cell. 3-methyladenine treatment in MSCs prevented their senescence because of increasing apoptosis. However, N-acetylcysteine or Diphenyleneiodonium, an inhibitor of NADPH oxidase, treatments were effective blocking autophagy and senescence through preventing high-glucose-induced autophagy[51].

All these results indicate that hyperglycaemia induces MSC aging and an increase of inflammation through oxidant-mediated autophagy, contributing to MSCs' niche dysfunction. On the other hand, methionine restriction may mediate its anti-aging effects through the induction of macroautophagy/autophagy as well[52].

MSCs are extremely sensitive and very low doses of radiation can induce senescence because of impairing autophagy and their limited DNA repair capacity[53]. Activation of autophagy restored bone loss in aged mice, suggesting that autophagy has a key role in the aging of MSCs, and an increase of autophagy can partially reverse this senescence process and might represent a new potential therapy for clinically treating age-related bone loss[54,55].

MSCs in lysosomal storage disorders (LDS), which impair lysosomal homeostasis, are prone to apoptosis and senescence due to impaired autophagy and DNA repair capacity[56]. Recently, a study showed that novel small molecules can selectively and sensitively respond to acidic pH, promoting lysosomal acidification and inhibiting

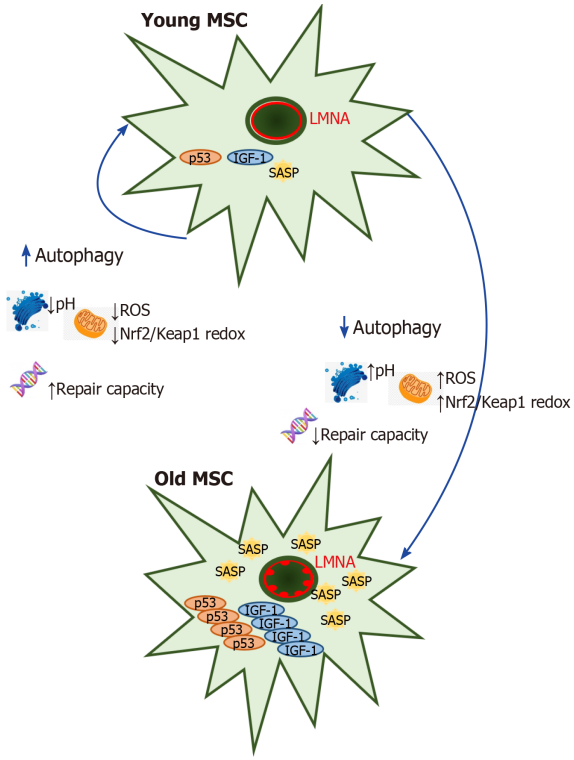


**Figure 2 Autophagy influences senescence in mesenchymal stem cells.** The self-renewal potential of young mesenchymal stem cells (MSCs) is influenced by their autophagy capacity to regulate the good levels of oncogenic factors like p53 and inflammatory signals like senescence-associated secretory phenotype and IGF-1, which produces overexpression of reactive oxygen species in the mitochondria, accumulation of mutations at DNA levels and acidification in the lisosomal apparatus together with an increase of *LMNA* in the nucleus. When autophagy is downregulated by the pathologic process, young MSCs become old MSCs in an accelerated way, losing their self-renewal capacity. MSC: Mesenchymal stem cell; ROS: Reactive oxygen species.

senescence in MSCs through autophagy[57]. Decreased autophagy is one of the mechanisms underlying aging. Yang *et al*[58] demonstrated that reducing autophagy decreases the hypoxia tolerance of senescent MSCs and Yun *et al*[59] demonstrated that high p-Cresol serum concentration caused by chronic kidney failure produced cell senescence through the induction of autophagy response and could be potentially rescued by the administration of melatonin through inhibiting mTOR-dependent autophagy[58,59]. Maintaining optimal levels of autophagy might serve as a new strategy for using MSC transplantation.

## MITOCHONDRIAL STRESS IN MSCs

Oxidative stress is characterized by unregulated production and/or the elimination of reactive oxygen and nitrogen species. The main ROS generation sites, under physiological conditions, are found within the electron transport chain in the mitochondria. MSC differentiation processes ROS are mainly generated from mitochondrial complexes I and III and the NOX4 isoform of NADPH oxidase[60]. The deregulation of ROS generation by CI and CIII can be an important factor for aging and it has been shown that an increase in ROS levels and the resulting oxidative damage are highly correlated with aging[61-63]. Deschênes-Simard *et al*[64] linked the bypassing of senescence in premalignant lesions to a decrease of differentiation, an increase of self-renewal potential and an increase in their dependence of mitochondrial functions. Aged adipose tissue-derived MSCs and their adipogenic differentiation are decreased by downregulation of Sirtuin 1 through miR-34a[65]. Another component, Sirtuin 3 (SIRT3), protects aged human MSCs against oxidative stress through positive regulation of MnSOD and CAT *via* activation of FoxO3a[39]. Huang *et al*[66] have reported that the reduction of ERRalpha-directed mitochondrial glutaminase expression suppresses the osteogenic differentiation in aged mice MSCs. Melatonin reduces endoplasmic reticulum stress (ERS) in the liver and several diseases in the nervous system and lung. It is involved in maintaining stemness during long-time *in vitro* expansion[67]. Yun *et al*[59] demonstrated that MSCs from rats

with chronic kidney disease exhibited greater senescence induced by oxidative stress than normal MSCs, whereas when treated with melatonin, it protected them from $H_2O_2$ and excessive associated senescence. Fang *et al*[68] have reported that it prevents senescence in canine adipose-derived MSCs through activation of Nrf2 with the inhibition of NFK beta and ERS. L-carnitine is a transport of long-chain fatty acids into the mitochondria for degradation by beta-oxidation and it has the potential to increase telomerase activity by changing the methylation status of the human TERT promotor in aged adipose tissue-derived MSCs[69,70]. Wang *et al*[57] postulate that treatment with curcumin gives bone marrow MSCs the ability to survive and this could be attributed to their protection in the mitochondrial function, destabilization of HIF-1α and the activation of the Epac1-Akt signalling pathway. Therefore, they suggest that curcumin influences the preconditioning of MSCs to facilitate cell therapy in the treatment of tissue repair. Oh *et al*[71] propose the role of 17β-estradiol (E2) as a potential target to prevent or treat metabolic disorders in the production of reactive mitochondrial oxygen species induced by glucose (mtROS) through signalling mediated by the oestrogen receptor in MSCs from umbilical cord blood *in vitro*, suggesting that E2 serves as a potent antioxidant. Denu *et al*[72] propose that SIRT3 is a sirtuin involved in aging (it is the main mitochondrial deacetylase) that decreases mitochondrial ROS and promotes an efficient oxidative metabolism. It has been shown that SIRT3 reduces the decrease in function and senescence associated with age in multiple cell types. Then, the increase in nuclear translocation of Nrf2 triggered the positive regulation of SIRT3 and the activation of manganese superoxide dismutase (MnSOD), which plays an important role in the decrease of mtROS levels. During MSC expansion *in vitro*, they experience a replicative senescence that compromises their immunomodulatory and differentiation functions due to increased ROS and oxidative stress in aged stem cells. MSCs accelerate aging and inhibit differentiation in adipocytes and osteoblasts because of the elimination of SIRT3, and because the overexpression of SIRT3 in the last step of the MSC restores its capacity for differentiation and reduces oxidative stress[73]. The study by Yao *et al*[74] attempts to demonstrate that human umbilical cord MSC-derived EVs carrying MnSOD could alleviate oxidative stress in liver tissue *in vivo*.

Oxidative stress is a key process in the induction of cellular senescence according to several studies[75-77]. Afterwards low-grade chronic inflammation during aging and associated pathologies can lead to oxidative stress and rupture of the cells that cause senescence. According to Platas *et al*[78], chronic oxidative stress related to aging or mechanical stress can cause cellular senescence in joint tissues and age-related alterations in the differentiation and function of MSCs.

## MSC-DERIVED EXTRACELLULAR VESICLES

Exosomes and microvesicles are small vesicles included in the term extracellular vesicles (EVs). Recently, it is unravel their function in cell-to-cell communication and their capacity for transporting proteins, signalling lipids and miRNAs which are relieved to target cells *via* endocytosis and membrane fusion. Lately, MSC-derived EVs are being studied for their role in MSC-based cellular therapy. These VEs have the capacity to alter cell or tissue metabolism at short or long distances in the organism. The EVs are influencing tissue responses to infection, injury and disease. MSC-derived EVs could be used for cell-free therapies. However, these therapies might be applied in clinic when parameters as quality, reproducibility and potency of their production can be controlled. In addition, it must be taken into account the MSC-derived EV content is not static, they are produced by MSCs and they are influenced by specific MSC´s niche. So, MSC-derived EVs are altered when MSCs are co-cultured with different types of cells *in vitro* or with tumour microenvironment *in vivo*[79,80]. It has been demonstrated that MSCs can induce tumour growth, and MSC-derived EVs can be very important in the tumour microenvironment transferring information between cells along disease's development. There are some findings supporting a new mechanism, suggesting the contribution of these MSC-derived EVs to tumour growth[81]. So, EVs secreted by MSCs might have therapeutic effects on the reconstruction process through promoting the cell cycle and inhibiting cell apoptosis, as happens in vaginal epithelium[82].

Articles focused on a murine model have shown that a brief interaction of old MSCs with young MSC-derived Evs rejuvenated them and restored their functionality *via* inter-cellular communication. These EVs contained autophagy-related mRNAs through inhibition of AKT in aged MSCs increased the levels of autophagy-related mRNAs in their EVs[83]. MSC-derived EVs are also involved in the transport of anti-immunoinflammatory markers aging depending, confirming variations with aging of

Toll-like receptor 4 pathway activation in rat bone marrow MSCs and containing pro-inflammatory miRNAs (miR-21, miR-155, miR-146 and miR-21) in their MSC-derived EVs[13]. Surprisingly, recent experiments show that the self-renewal power of these EVs is even better than that of the young MSCs. It has been demonstrated that such *ex vivo* self-renewal from old MSCs could increase the donor cohort improving efficacy in transplantation therapies[84].

## CONCLUSION

Aging affects the behaviour of MSCs in different ways depending on several factors, such as their status, source and pathological process. MSCs *in vitro* go into senescence earlier than *in vivo* and the pathological process stimulates their senescence *in vivo*. Despite this, or perhaps because of it, MSCs are an excellent tool to keep exploring in cellular therapy and to study senescence both *in vivo* and *in vitro* and their versatility seems to be extensively to their derived EVs.

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ORIGINAL ARTICLE

Basic Study

# Similarities and differences between mesenchymal stem/progenitor cells derived from various human tissues

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## Abstract

### *BACKGROUND*

Mesenchymal stromal/stem cells (MSCs) constitute a promising tool in regenerative medicine and can be isolated from different human tissues. However, their biological properties are still not fully characterized. Whereas MSCs from different tissue exhibit many common characteristics, their biological activity and some markers are different and depend on their tissue of origin. Understanding the factors that underlie MSC biology should constitute important points for consideration for researchers interested in clinical MSC application.

### *AIM*

To characterize the biological activity of MSCs during longterm culture isolated from: bone marrow (BM-MSCs), adipose tissue (AT-MSCs), skeletal muscles (SM-MSCs), and skin (SK-MSCs).

### *METHODS*

MSCs were isolated from the tissues, cultured for 10 passages, and assessed for: phenotype with immunofluorescence and flow cytometry, multipotency with differentiation capacity for osteo-, chondro-, and adipogenesis, stemness markers with qPCR for mRNA for Sox2 and Oct4, and genetic stability for p53 and c-Myc; 27 bioactive factors were screened using the multiplex ELISA array, and spontaneous fusion involving a co-culture of SM-MSCs with BM-MSCs or AT-

additional data are available.

**ARRIVE guidelines statement:** The ARRIVE Guidelines Checklist is not applicable for the manuscript, studies were not performed on animal models.

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MSCs stained with PKH26 (red) or PKH67 (green) was performed.

*RESULTS*

All MSCs showed the basic MSC phenotype; however, their expression decreased during the follow-up period, as confirmed by fluorescence intensity. The examined MSCs express CD146 marker associated with proangiogenic properties; however their expression decreased in AT-MSCs and SM-MSCs, but was maintained in BM-MSCs. In contrast, in SK-MSCs CD146 expression increased in late passages. All MSCs, except BM-MSCs, expressed PW1, a marker associated with differentiation capacity and apoptosis. BM-MSCs and AT-MSCs expressed stemness markers Sox2 and Oct4 in long-term culture. All MSCs showed a stable p53 and c-Myc expression. BM-MSCs and AT-MSCs maintained their differentiation capacity during the follow-up period. In contrast, SK-MSCs and SM-MSCs had a limited ability to differentiate into adipocytes. BM-MSCs and AT-MSCs revealed similarities in phenotype maintenance, capacity for multilineage differentiation, and secretion of bioactive factors. Because AT-MSCs fused with SM-MSCs as effectively as BM-MSCs, AT-MSCs may constitute an alternative source for BM-MSCs.

*CONCLUSION*

Long-term culture affects the biological activity of MSCs obtained from various tissues. The source of MSCs and number of passages are important considerations in regenerative medicine.

**Key words:** Mesenchymal stem/progenitor cells; Bone marrow MSCs; Adipose tissue MSCs; Muscle-derived MSCs; Skin-derived MSCs; Cytokines and trophic factors of MSCs; Spontaneous fusion of MSCs

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**Core tip:** A comprehensive characterization of mesenchymal stromal/stem cells (MSCs) with different tissue origin during long-term culture was demonstrated in terms of: basic phenotype strength, stemness and genetic stability, and ability to secrete bioactive factors and affect one another in co-culture. MSCs were phenotypically heterogeneous and showed diverse differentiation potentials and secretion of bioactive factors associated with tissue origin. Bone marrow (BM)-MSCs and adipose tissue (AT)-MSCs expressed stemness markers Sox2 and Oct4 in long-term culture, whereas skeletal muscles (SM)-MSCs and skin (SK)-MSCs did not. All MSCs were stable for p53 and c-Myc expression. AT-MSCs fused with SM-MSCs as effectively as BM-MSCs. Long-term culture affected the biological properties of the MSCs.

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## INTRODUCTION

Mesenchymal stromal/stem cells (MSCs) have attracted close attention in the scientific world since their first isolation from bone marrow by Friedenstein in the 1960s, and have been described as plastic adherent cells with a fibroblast-like morphology. Isolated cells showed a high proliferative capacity and clonal expansion, and after heterotopic transplantation under the renal capsule, were able to form ectopic bone. This observation suggested that the bone marrow environment contained osteogenic precursors, which could potentially provide therapeutic benefits[1,2]. However, the term "mesenchymal stem cells" was proposed by Caplan and introduced to denote a type of cells that originated from adult bone marrow with a natural capacity for multipotential differentiation into diverse types of cells of mesenchymal origin[3].

Although bone marrow is the bestdescribed source of MSCs, over the last decade, many studies have documented the possibility to obtain stem/progenitor cells with the biological MSC characterization of other adult tissues, such as skin[4], placenta[5], cord blood[6,7], cord tissue[8], adipose tissue[9], dental pulp and deciduous teeth[10-12], or even testicles and brain[13,14]. There is evidence that MSCs obtained from different tissues constitute a heterogeneous population of cells, and it became necessary to create standards for MSC characterization. Such standards were proposed in 2006 by the International Society for Cellular Therapy as the minimal criteria for defining mesenchymal stromal cells[15]. However, despite their attractiveness thanks to their ability to differentiate and form different tissues of mesodermal (adipogenic, chondrogenic, osteogenic, or tendon), ectodermal (epithelial, glial, or neural), and endodermal (hepatocytes or islet cells) origin[16], the immunomodulatory capacity exerted by the ability to secrete different factors with pro- and anti-inflammatory properties and/or direct cell-to-cell interactions[17] play an important role in tissue homeostasis and tissue regeneration.

Research on the MSCs of different tissue origin provides insight into their role in tissues and organ homeostasis and the regenerative potential of stem cells and helps in searching for alternative, more accessible sources of such cells[18]. Several studies have documented that adherent cells isolated from different tissues meet the minimal criteria corresponding to the basic MSC phenotype[15], such as the expression of CD73, CD90, and CD105. These three markers were documented to be strongly expressed in human bone marrow-derived MSCs (hBMMSCs)[19], adipose tissue-derived MSCs (hADMSCs)[20], adult dermal-derived skin stromal cells (hADSSCs)[21], and muscle-derived progenitor cells (hMDPCs)[22]. These cells also showed a lack of CD34 and CD45 hematopoietic markers. CD146 is considered to be another MSC marker, because it was observed on the surface of MSCs obtained from different sources[22,23]. Higher CD146 expression increases the potential of cells to migrate *in vitro* and *in vivo*, and a decrease in its expression may be related to a higher osteogenic capacity[24]. Moreover, CD146, along with PDGFRα, is an important receptor required in the regulation of angiogenesis[25,26]. A very interesting finding that emphasized the role of the PDGFRα receptor in muscle regeneration was the discovery of PICs (PW1+ Interstitial Cells), which are myogenic and adipogenic progenitor cells, characterized by the co-expression of PW1/Peg3 and PDGFRα[27]. PW1/Peg3 is a stem cell marker, as well as a factor related to cell apoptosis[28]. Moreover, research suggested that PW1 played an important role in the formation of blood vessels, as confirmed in a mouse model[29].

Assessment of the paracrine potential of MSCs from different sources is very important, because it defines their ability to interact with neighboring cells and their potential to suppress or trigger immunocompetent cells to action. Previous studies have documented that bone marrow-derived MSCs are a source of cytokines and trophic factors and secrete IL-6, IL-8, MCP-1, VEGF, OPG, and TIMP-2; however, the type and level of cytokine secretion varied between BMMSCs and MSCs from the umbilical cord, as established by Park *et al*[30], which is another piece of evidence that the MSC niche affects the paracrine abilities of stem/progenitor cells. In 2013, Ribeiro *et al*[31] described the different suppressive capacities of MSCs isolated from adipose tissue, bone marrow, and umbilical cord blood, showing that adipose tissue-derived MSCs had the strongest immunosuppressive impact on NK and B-cells. Skin-derived progenitor cells were also reported to be able to secrete certain levels of trophic factors, such as VEGF, G-CSF, HGF, IGF-1, or bFGF, which may affect tissue regeneration[32]. There are a limited number of studies on the paracrine abilities of skeletal muscle-derived progenitor cells.

Another important issue discussed in this paper is the assessment of the pluripotent abilities of cells *via* an evaluation of Sox2 and Oct4 mRNA expression associated with cell stemness[33]. Both of these factors are highly expressed in embryonic stem cells (ESCs) and are known from their crosstalk in cell fate regulation. Aberration in Sox2 and Oct4 expression may affect cell proliferation and proper differentiation, which leads to morphological abnormalities[34]. c-Myc is a factor related to cell proliferation and metabolism[35]. Overexpression of the gene coding cMyc leads to uncontrolled cell proliferation and tumorigenesis[36]. As a protein with a suppressive function, p53 regulates Sox2, Oct4, and c-Myc expression and helps to maintain stem cells in an undifferentiated state[37,38]. Long-term *in vitro* culture along with the influence of tissue-specific environment may affect the expression of these genes. Observations on the dynamics of these changes may help to determine the best strategy in MSC manufacture for potential use in cell therapy.

Cell fusion plays important role in tissue regeneration in normal and pathological conditions. In normal biological processes, cell fusion is involved in tissue formation and immune response. The biological potential of cell fusion is a promising tool in regenerative medicine, as MSCs plasticity plays an important role in regeneration[39].

In normal conditions, the regeneration of skeletal muscles involves the fusion of newly emerging myogenic cells with damaged muscle fibres, and cell fusion was also confirmed in skeletal muscle restoration following mechanical injury[40]. In pathological conditions, in patients with Duchenne muscular dystrophy (DMD), delivered bone marrow cells were able to fuse with the patients' skeletal muscles[41]. The best documented regeneration process by cell fusion is liver regeneration by transplantation of bone marrow-derived cells[42]. The ability of MSCs of different tissue origin to fuse *in vitro* may help to select biologically active cells for use in target tissue regeneration.

MSC-based treatment is still provided as experimental procedures. The reason lies in the great diversity of MSCs, depending on their original tissue location, age of donor, methodology of isolation, and culture conditions. All these factors affect the behavior of MSC culture, making the *in vivo* activity of MSCs difficult to predict. Unifying the methodology and understanding the factors that underlie MSC biology should constitute important points for consideration for researchers interested in clinical MSC application.

This paper presents research involving longterm observations of the biology of human MSCs derived from bone marrow (BM-MSCs), adipose tissue (AT-MSCs), skeletal muscle (SM-MSCs) and skin (SK-MSCs), collected post-autopsy (bone marrow) and as post-surgery medical waste (skin, muscle, and adipose tissue) in consideration of alternative stem cell sources. We assessed the maintenance of the basic phenotype of MSCs, their differentiation potential, secretion of cytokines and trophic factors, as well as the mRNA expression profile associated with the pluripotent (Sox2, Oct4), suppressor (p53), and protooncogenic (c-Myc) function of the examined MSCs. Lastly, we studied the ability of MSCs of different tissue origin to fuse *in vitro*. We hope that this paper will enrich knowledge on the biological properties of MSCs in long-term cultures and that it will deliver useful information for researchers working in this area during future studies.

## MATERIALS AND METHODS

### Tissue collection

Bone marrow, 10-12 mL, was collected into heparinized syringes from deceased donors ($n$ = 6), average age 36.3 years (range 23-49 years), during autopsy, 24-48 h after death, with approval from a local Bioethics Committee (KB746/2012). Skeletal muscle ($n$ = 9) and skin ($n$ = 7) tissue was collected from limbs amputated due to critical limb ischemia following surgical procedures, average patient age 65.0 years (range 60–69 years). The research procedure was approved by a local Bioethics Committee (KB201/2016). Adipose tissue was separated from the dermis collected from postoperative tissue ($n$ = 7). Tissue samples were transported in phosphate-buffered saline (PBS) supplemented with a 1% antibiotic-antimycotic solution.

### Mesenchymal stem/progenitor cell isolation

Bone marrow cells were diluted in PBS in a 1:4 ratio. Next, mononuclear cells were isolated on the Lymphoprep gradient during a 30 min centrifugation at 1410 rpm. Mononuclear fraction was collected and washed two times in PBS. Skeletal muscle and adipose tissue was cut into small pieces. In the skin samples, dermis was dissected from epidermis and adipose tissue and cut into small pieces. All tissues were digested in 0.01% collagenase from C. histolyticum (Sigma, Saint Louis, United States) at 37°C. For skeletal muscle tissue, digestion time was 1 h, and adipose and skin tissues were digested for between 20 and 30 min of incubation. Enzymatic activity was neutralized by adding a 1:1 volume of culture media with FBS (BioWest, Riverside, Montana, United States). Next, the digested tissues were passed through a 70 μm cell strainer. Cell suspensions were centrifuged at 1200 rpm for 10 min, afterwards, 0.1% saponin (Sigma, Saint Luis, United States) was added in a pellet in order to remove the remaining erythrocytes during 5 min of incubation at room temperature (RT). Cell suspensions were purified by washing twice in PBS and centrifuged at 1200 rpm for 10 min at RT. Cell cultures were performed in T25 culture flasks. BM-MSCs and AT-MSCs were cultured in a αMEM medium (IITE, Wroclaw, Poland). SK-MSCs and SM-MSCs were cultured in a DMEM F12 (Gibco, Carlsbad, United States) medium. Both culture media were supplemented with 10% FBS (BioWest Riverside, Montana, United States), 1% of antibiotic-antimycotic (Biowest Riverside, Montana, United States), L-glutamine (Biowest Riverside, Montana, United States) and 20 ng/mL bFGF (Sigma, Saint Louis, United States). Undigested remains of skin tissue were placed in separate culture flasks in order to allow the cells to migrate from the tissue and adhere to the plastic surface. Isolated cells were incubated

at 37°C with a 5% $CO_2$ atmosphere, and culture media were changed every three days. Adherent cells were cultured up to 10 passages (P10), and each passage was performed when the culture reached 80% confluence.

### Immunofluorescence staining

Adherent cells isolated from the examined tissues were prepared in 5 × $10^5$ concentrations in 1 mL of culture media, and 100 μL of cell suspension were added onto each well of a 96-well plate and incubated overnight in 37 °C, 5% $CO_2$ in order to allow the cells to reassume their native morphology. Next, the culture media were removed, and the cells in the wells were washed with PBS. Subsequently, 50 μL of 10% buffered formalin solution (Sigma) was added onto the cell layer and incubated for 20 min at RT. After this time, formalin was discarded, and the wells were washed with PBS. Nonspecific binding was blocked by using 100 μL of 10% goat serum, adding a 1% BSA solution to each well, and incubating for 1 h at RT. Primary antibody solutions were added directly after the aspiration of the blocking solution. After 30 min or 1 h of incubation (Table 1), the primary antibody was removed, the wells were washed three times with PBS, and secondary goat anti-mouse (Alexa Fluor 488 nm or 594 nm) or goat anti-rabbit (Alexa Fluor 488 nm or 594 nm) antibody was added and incubated for 40 min. In order to obtain double staining, the secondary antibody was removed, the wells were washed three times with PBS, and the cells were incubated again with another antibody. To visualize the binding of the second antibody, the secondary antibody conjugated with a different fluorochrome from that used for the first visualization. Incubation time and dilution of each antibody is presented in Table 1. For nuclei visualization, DAPI diluted 1:5 (Vector Labs, Burlingame, United States) was added and incubated for 20 min. The cells were washed three times with PBS. Immunofluorescence staining was analyzed using a Leica fluorescence microscope (Wetzlar, Germany), and images were obtained using a microscope camera.

### Flow cytometry

Surface marker expression was analyzed in each passage. Adherent cells were detached with TrypLE (Gibco, Carlsbard, United States) and washed three times in PBS. 50 μL of a 2 × $10^5$ cell suspension in PBS were prepared, and 2 μL of a cytometry dedicated antibody (BD Biosciences) were added (Table 1). The cells were incubated for 30 min on ice in the dark. After incubation, the cells were washed in 1 mL of PBS and centrifuged for 5 min at 200 g. Next, cell pellets were resuspended in 50 μL of PBS and analyzed using an Amnis Cytometer (Merck) with an appropriate compensation template.

### Co-cultures of MSC of different tissue origin

Cells isolated from SM-MSCs and AT-MSCs were stained with PKH67 (green), and cells isolated from BM-MSCs were stained with PKH26 (red), according to the protocol suggested by the manufacturer (Sigma, Saint Louis, United States). On day 0, 5 × $10^3$ of PKH67-stained cells and 5 × $10^3$ of PKH26-stained cells were mixed in each combination, and co-cultures were performed on 24-well plates in DMEM F12 media supplemented with 20 ng/mL bFGF and maintained up to seven days. All co-cultures were performed in duplicate. The fusion was monitored using a Leica AS-438 fluorescence microscope (Wetzlar, Germany), and images were taken every day using a microscope camera. On day 7, the cells were detached from the plate, fixed and permeabilized in the BD Cytoperm/Cytofix agent (BD Biosciences), stained for 20 min with DAPI (Vecta Shield) and analyzed using an Amnis Cytometer (Merck).

### Multilineage differentiation potential of MSCs

Cells (5 × $10^5$) were resuspended in culture media, and 400 μL of cells were added to each well of a 24-well plate and incubated overnight to allow them to reassume their native shape. Next, the culture media were removed, washed with PBS, and 400 μL of osteogenic, adipogenic, and chondrogenic media (Promocell, Heidelberg Germany) were added. The media were changed every four days. Adipogenic differentiation was achieved within 14 d and osteogenic and chondrogenic differentiation was achieved within 21 d. After the differentiation, the media were removed, and the cell layer was fixed for 20 min at RT in a 10% buffered formalin solution (Sigma). Staining was performed to prove MSC differentiation using Alizarin Red S for osteogenesis, Oil Red O for adipogenesis, and Alcian Blue for chondrogenesis (Sigma, Saint Louis, United States).

### Multiplex ELISA for cytokines, chemokines, and growth factors secreted by MSCs

Culture media were collected from the MSC monolayer at each passage up to P10. The media were centrifuged at 1200 rpm for 10 min to remove existing cells and

**Table 1 Antibodies used for immunofluorescence and for flow cytometry analysis**

| Antigen | Company | | Dilution | Incubation time |
|---|---|---|---|---|
| Immunofluorescence staining | | | | |
| CD73 | Thermo Fisher | Weston, Florida, USA | 1:100 | 1 h |
| CD90 | Abcam | Cambridge, UK | 1:100 | 1 h |
| CD105 | Dako | Palo Alto, California, USA | 1:10 | 1 h |
| CD34 | | | 1:100 | 30 min |
| CD45 | | | 1:100 | 30 min |
| CD56 | | | 1:80 | 30 min |
| CD146 | Santa Cruz | Dallas, Tx, USA | 1:25 | 1 h |
| PDGFRα | | | 1:25 | 1 h |
| PW1 | Atlas Antibodies | Bromma, Sweden | 1:100 | 1 h |
| Alexa Fluor 488 nm Donkey anti-mouse | | | 1:700 | 40 min |
| Alexa Fluor 488 nm Donkey anti-rabbit | Thermo Fisher | Weston, Florida, USA | 1:700 | 40 min |
| Alexa Fluor 594 nm Donkey anti-mouse | | | 1:700 | 40 min |
| Alexa Fluor 594 nm Donkey anti-rabbit | | | 1:700 | 40 min |
| Flow Cytometry | | | | |
| CD73 (IgG) APC | BD Pharmingen | San Jose, California, USA | 1:25 | 30 min |
| CD90 (IgG) FITC | | | 1:25 | 30 min |
| CD105 (IgG) PE | | | 1:25 | 30 min |
| CD34 (IgG) FITC | | | 1:25 | 30 min |
| CD45 (IgG) APC | | | 1:25 | 30 min |
| CD56 (IgG) PE | | | 1:25 | 30 min |
| CD146 (IgG) PE | | | 1:25 | 30 min |
| Isotype controls: IgG APC IgG PE IgG FITC | | | 1:25 | 30 min |

cellular debris. Supernatant was collected and stored at -80°C until cytokine panel analysis. Multiplex ELISA was performed using a Bio-Plex Pro™ Human Cytokine Grp I panel 27-Plex dedicated kit (Bio-Rad, Hercules, United States), according to the protocol recommended by the manufacturer (Bio-Rad, Hercules, United States). Supernatant samples from P1, P5, and P10 (P9 for the SK-MSCs) were analyzed. The DMEM F12 (Gibco, Carlsbad, United States) medium with 10% FBS (BioWest Riverside, Montana, United States), 1% of antibiotic-antimycotic (Biowest Riverside, Montana, United States), L-glutamine (Biowest Riverside, Montana, United States), and 20 ng/mL bFGF (Sigma, Saint Louis, United States) was used as control in standards and blanks. The analysis was performed using the Bio-Plex 200 system (Bio-Rad, Hercules, United States) with the Bioplex Manage Software 6.1.

### *qPCR*

The cells from the MSC pellet from the T75 flask were harvested upon reaching 80% confluence and lysed using 350 μL of Buffer RLT Plus (Qiagen) + 0.1% of β-mercaptoethanol (Sigma, Saint Louis, United States), and RNA was isolated and purified with an RNeasy Plus Mini kit (Qiagen Hilden, Germany). Purity of RNA was assessed using 1.5% agarose gel electrophoresis with 0.0025% ethidium bromide (Sigma, Saint Louis, United States). Next, 1 mg of RNA was aspired, and reverse

transcription was performed using iScript IV (Bio Rad, Hercules, United States). Quality of DNA was examined *via* PCR for β-actin presence followed by gel electrophoresis using 3% of agarose with 0.0025% ethidium bromide. qPCR for the expression of mRNA for Sox2, Oct4, p53, and c-Myc was performed using the following TaqMan probes: Sox2 (Hs01053049), Oct4 (Hs00999632), p53(HS00153349), c-myc (Hs00153408), and GAPDH (Hs03929097) (Thermo Fisher, Weston, United States), with GAPDH as the housekeeping gene. Analysis was performed in a ViiA 7 apparatus. The results were calculated using the $2^{-\Delta\Delta CT}$ method and presented as the RQ (relative quantification) value.

As a reference for the pluripotent stem cells, iPSCs were used, which were a kind gift from Professor Kurpisz from the Institute of Human Genetics, Polish Academy of Sciences, Poznań, Poland. The protocol for creating the iPSCs is described by Lewandowski *et al*[43].

### Statistical analysis

All graphs and statistical analyses were made using GraphPad Prism 7. The double-tailed *t*-test for unpaired samples, with Welch's correction for unequal variances, 95% confidence, was used for the assessment of the Multiplex ELISA *P*-value. The double tailed *t*-test for paired samples was used to evaluate the *p*-value of the qPCR results.

## RESULTS

### Phenotype of bone marrow-, adipose tissue-, skeletal muscle-, and skin-derived MSCs

Mesenchymal stem cells from all examined sources expressed the naïve MSC markers CD73 and CD90 in passage P0, with values ranging between 94% and 99% of the population. Between 50% and 65% of the population of BM-, SM- and SK-MSCs and 97% of AT-MSCs expressed CD105 in passage 0. The expression of the proangiogenic markers CD146 and PDGFRα was also observed in passage P0 in the majority of MSCs from all sources; however, their expression was different. The heterogeneity of MSCs was also confirmed by the expression of PW1, the factor associated with one of the atypical stem cell markers, but also with cell apoptosis. The majority of MSCs were isolated from solid tissues, SM-, AT-, and SK-MSCs showed a high expression of PW1; however, the presence of PW1 was not observed on the surface of MSCs isolated from bone marrow in passage 0. A subpopulation of cells that co-expressed the proangiogenic markers CD146 and CD73 characteristic for the MSCs phenotype was detected on each population of MSCs, as confirmed by double immunofluorescence staining (Figure 1).

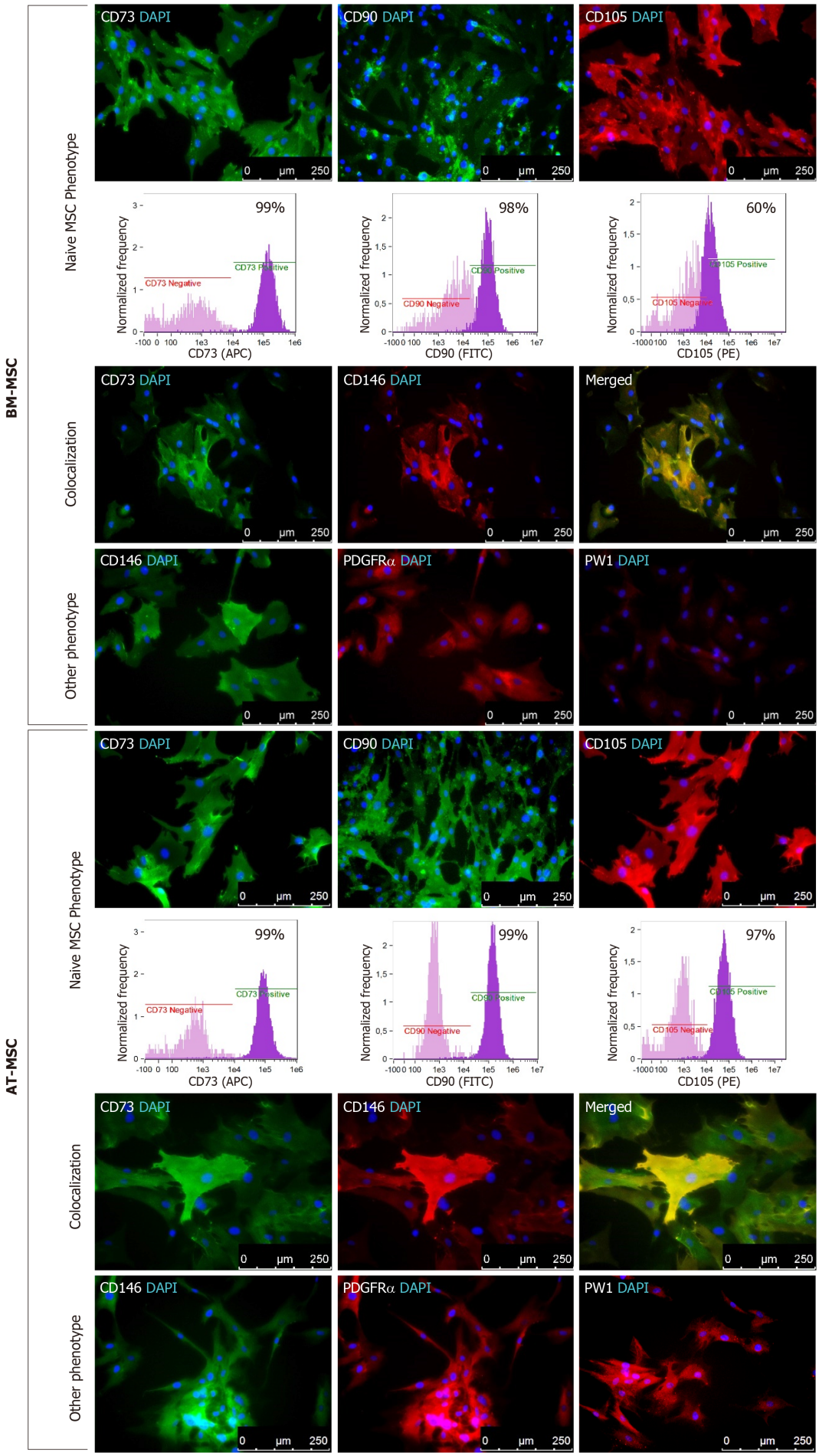
Figure 2 summarizes the changes in the percentage of MSCs during long-term culture in P1, P5, and P10.

### Assessment of antigen expression with fluorescence intensity

Changes in the dynamics of the fluorescence intensity of the examined antigens were strictly dependent on the source of the cells (Figure 3). MSCs isolated from bone marrow showed the most stable CD73 and CD90 expression during the subsequent passages. CD73 dynamics slightly increased in AT-MSCs in P6 ($1.99 \times 10^5$), whereas CD90 expression increased in P2 ($2.52 \times 10^5$), after which it gradually decreased in P10 ($6.23 \times 10^4$). The highest visible fluorescence of CD90 in SM-MSCs was observed in P5 ($4.49 \times 10^5$), whereas the expression of CD73 varied ($4.66 \times 10^5$ - $7.57 \times 10^4$). Interestingly, in SM-MSCs, the expression of all MSC antigens, CD73, CD90, and CD105, increased in P10. SK-MSCs showed the strongest fluorescence intensity of both CD73 and CD90 in P1 ($5.32 \times 10^5$ and $3.99 \times 10^5$, respectively) among all of the tested tissues; however, their expression was downregulated with the age of culture.

Fluorescence intensity of CD105 and CD146 showed the highest diversity. For BM-, AT-, and SK-MSCs, the highest fluorescence intensity peak of CD105 was observed between P1 and P2. In AT-MSCs and SK-MSCs, fluorescence intensity of CD105 gradually decreased in the subsequent passages up to P10. A different pattern of the fluorescence intensity of CD105 was observed in BM-MSCs and SM-MSCs, which displayed three peaks of elevated fluorescence signal during the follow-up period.

BM-MSCs showed the longest time of CD146 expression; at P10, about 33% of the original signal could still be captured. In AT-MSCs and SM-MSCs, fluorescence intensity of CD146 was measurable at the early passages P1 and P2, after which the signal gradually decreased, reaching a critical point of signal loss at around P6. This observation was in contrast to CD146 expression in SK-MSCs, which was the weakest among all MSC sources and undetectable from P0 ($3.50 \times 10^3$) to P5 (0.00), after which it increased up in P6, reaching its highest value of $5.50 \times 10^3$.


BM-MSC
AT-MSC
Naive MSC Phenotype
Colocalization
Other phenotype
CD73 DAPI
CD90 DAPI
CD105 DAPI
CD146 DAPI
PDGFRα DAPI
PW1 DAPI
Merged
99%
98%
60%
99%
99%
97%
CD73 Negative
CD73 Positive
CD90 Negative
CD90 Positive
CD105 Negative
CD105 Positive
Normalized frequency
CD73 (APC)
CD90 (FITC)
CD105 (PE)
0 μm 250

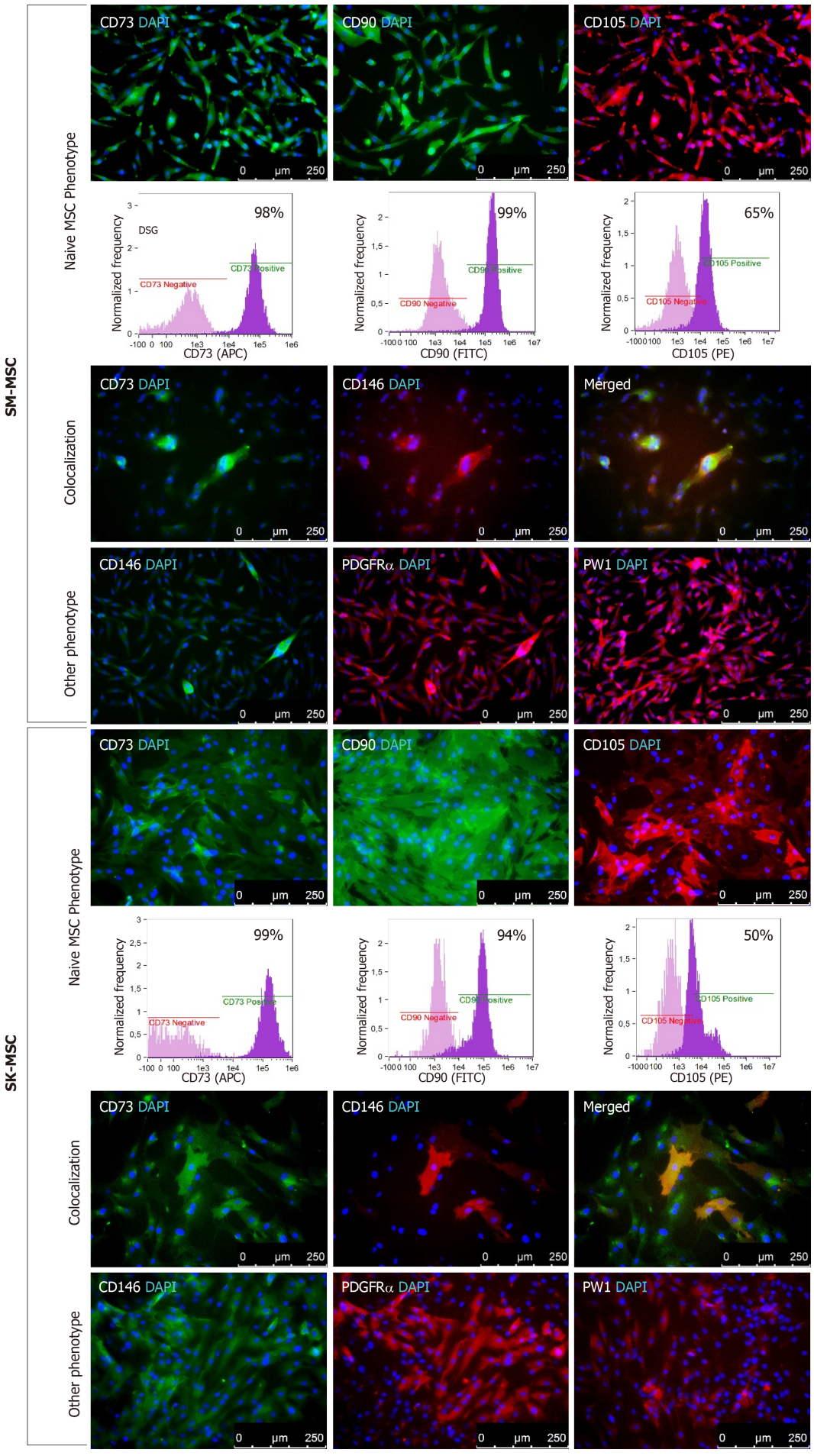

SM-MSC
SK-MSC
Naive MSC Phenotype
Colocalization
Other phenotype
CD73 DAPI
CD90 DAPI
CD105 DAPI
CD146 DAPI
PDGFRα DAPI
PW1 DAPI
Merged
98%
99%
65%
99%
94%
50%
CD73 Negative
CD73 Positive
CD90 Negative
CD90 Positive
CD105 Negative
CD105 Positive
Normalized frequency
CD73 (APC)
CD90 (FITC)
CD105 (PE)
0 µm 250

**Figure 1 Immunofluorescence and flow cytometry analysis of mesenchymal stem cells in P0, isolated from bone marrow, adipose tissue, skeletal muscle and dermis.** Isolated adherent cells express naïve MSC markers CD73, CD90, and CD105, proangiogenic markers CD146 and PDGFRα, and PW1 stem cell marker specific for MSCs derived from solid tissues. The subpopulation of MSCs co-expresses CD73/CD146 antigens. BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

Additionally, CD56 was evaluated in SM-MSCs in order to assess the spontaneous maturation of muscle progenitor cells. Expression of CD56, characteristic for muscle progenitor cells, increased between passages P3 ($2.90 \times 10^4$) and P5 ($2.17 \times 10^4$), after which it rapidly decreased in P6 ($6.00 \times 10^2$) (Figure 3). Interestingly, at the same time, the fluorescence intensity of basic MSC markers increased, showing a prospective role of naïve MSC markers (CD73, CD90, and CD105) in myogenesis.

### *Differentiation potential of MSCs of different tissue-origin*

A high capacity for osteogenesis was observed in MSCs from all sources (Figure 4). The most efficient osteogenic differentiation, however, was observed for passages P5 and above. In BMMSCs and AT-MSCs, adipogenesis was characterized to be the most powerful. However, its efficiency decreased with the age of culture at P10, contrary to osteo- and chondrogenesis, which became more advanced with the subsequent passages. A similar effect of differentiation potential was observed in the culture of SK-MSCs. Adipogenesis in SK-MSCs was limited and less efficient than in BM-MSCs and AT-MSCs. Cells isolated from skeletal muscles showed a lack of the ability to perform adipogenic differentiation under the study conditions, which suggests that skeletal muscles are a source of stem/progenitor cells with a limited differentiation capacity. Chondrogenesis in all of the examined MSCs improved with the subsequent passages, and AT-MSCs were able to differentiate the most efficiently among all MSC sources. A positive Alcian blue staining in SK-MSCs was observed after 21 d of differentiation; however, the morphology of the formed cartilage was different from the solid structures observed in P1 and P5.

### *mRNA expression for proteins involved in the biological activity of MSCs originating from different tissues*

The multipotential character of MSCs was confirmed through Oct4 and Sox2 expression; however, compared to the iPSCs control, the expression of Sox2 and Oct4 was significantly lower ($P < 0.0001$) in MSCs isolated from all examined sources (Figure 5). Nevertheless, mRNA for Oct4 and Sox2 was detectable in all examined tissue-resident MSCs at different levels. The highest expression of Sox2, compared to iPSCs, was observed in SK-MSCs at P1 (RQ = $1.77 \times 10^{-3}$) compared to BMMSCs at P1 (RQ = $2.79 \times 10^{-5}$), AT-MSCs at P1 ($5.74 \times 10^{-5}$), and SM-MSCs at P1 (RQ = $2.22 \times 10^{-4}$). However, the expression of Sox2 in SK-MSCs decreased significantly during the subsequent passages, as observed in P5 (RQ = 0.07) and P10 (RQ = 0.058) ($P < 0.0001$). In AT-MSCs and SM-MSCs, Sox2 expression between passages P1 and P10 remained at a similar level. The increase of Sox2 expression was observed in one examined sample of BM-MSCs. BM-MSCs from other samples expressed Sox2 at P1, but this expression was insufficient for assessment in passages P5 and P10.

Oct4 expression in the examined MSCs was also lower than in iPSCs (Figure 5). Their relative quantity at P1 was as follows: RQ = $7.16 \times 10^{-5}$ for BM-MSCs, RQ = $6.89 \times 10^{-5}$ for AT-MSCs, RQ = $1.33 \times 10^{-4}$ for SM-MSCs, and RQ = $1.48 \times 10^{-5}$ for SK-MSCs. Tissue-dependent variation was observed in Oct4 expression. In BM-MSCs, expression between passages P1 and P10 gradually increased, reaching RQ = 2.12 in P5 and RQ = 7.46 in P10. Case-dependent variation in Oct4 expression was also observed here (SD = 4.8). A less rapid upregulation was observed in ATMSCs, for which RQ in P5 and in P10 was similar: 1.16 and 1.56, respectively. There were no significant differences in the expression of Oct4 in SK-MSCs. However, in SM-MSCs, a significant ten-fold decrease ($P < 0.005$) in Oct4 expression was observed between P1 (RQ = 1) and P5 (RQ = 0.11). Subsequently, in P10, Oct4 expression in SM-MSCs increased slightly (RQ = 0.62).

The expression of mRNA for p53 expression in MSCs was lower than the expression observed in iPSCs and at P1, RQ = 0.21 for BM-MSCs, RQ = 0.32 for AT-MSCs, and RQ = 0.19 for SM-MSCs and SK-MSCs (Figure 5). The dynamics of p53 expression between the passages in BM-MSCs and AT-MSCs was very similar. No differences were observed between P1 and P10; however, at P10, considerable case dependent variation in p53 expression was observed (SD = 0.96–1.23). P53 expression decreased significantly ($P < 0.05$) between P1 (RQ = 1) and P5 (RQ = 0.34) in SM-MSCs, after which it increased again in P10 (RQ = 0.81). A decrease in p53 expression was observed in SK-MSCs at P10 (RQ = 0.65).

c-Myc expression, as compared to iPSCs, was the lowest in BM-MSCs (in P1, RQ =

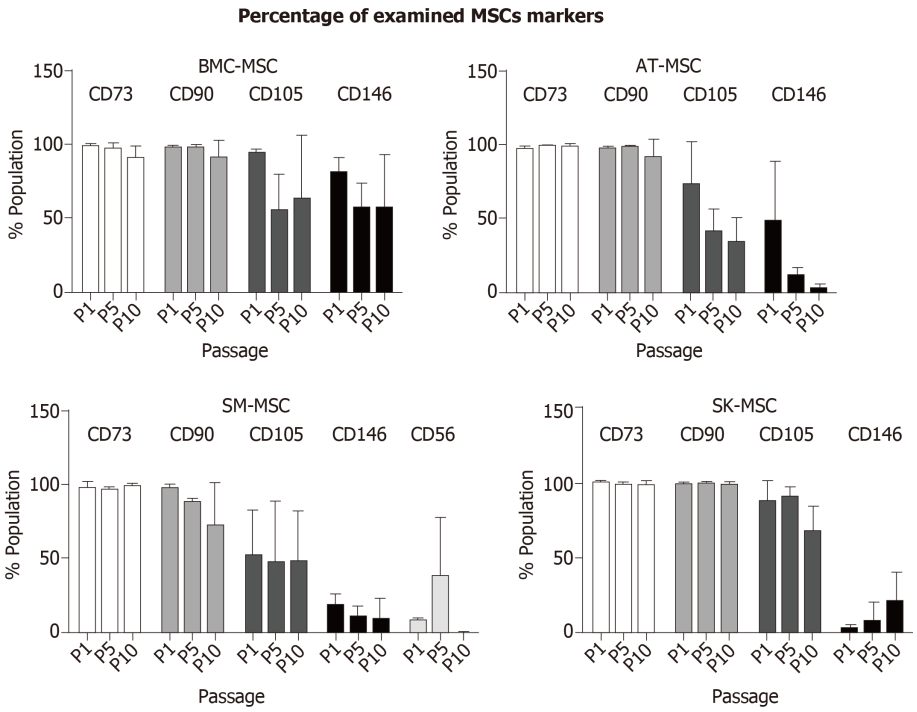



**Figure 2 Percentage of mesenchymal stem cells population positive for CD73, CD90, CD105, and CD146 in bone marrow - mesenchymal stem cells, adipose tissue - mesenchymal stem cells, and skin - mesenchymal stem cells, and CD56 only in skeletal muscle - mesenchymal stem cells.** Mesenchymal stem cells (MSCs) isolated from all examined tissues maintained the naïve MSC phenotype CD73, CD90, and CD105 during the follow-up period. A higher level of CD146 was observed in P1 in BM-MSCs, AT-MSCs, and SM-MSCs, but declined in the subsequent passages. In contrast, SK-MSC P1 was characterized with a low number of CD146+ cells; their level increased in P9. CD56 positive cells were present only in SM-MSCs, and the highest level was observed in P5. BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

0.47) and SM-MSCs (in P1, RQ = 0.35), and the highest in SK-MSCs (at P1, RQ = 0.82), and almost two times higher in AT-MSCs than in the iPSC control (at P1, RQ = 1.98). However, the expression of c-Myc in BM-MSCs was significantly upregulated ($P < 0.05$) in P5 (RQ = 1.87). The upregulated c-Myc expression in BM-MSCs continued until P10 (RQ = 1.44). In AT-MSCs, a significant downregulation ($P < 0.05$) of c-Myc was observed in P5 (RQ = 0.71). The expression was upregulated again in P10 (RQ = 0.91). SM-MSCs showed the most stable c-Myc expression between the passages. In SK-MSCs, a slight increase in c-Myc expression was observed in P5 (RQ = 1.27).

### *ELISA multiplex for cytokines and trophic factors secreted by MSCs*

Among the 27 cytokines and trophic factors covered by screening, it was observed that MSCs from all sources secreted a certain amount of MCP-1, IL-8, VEGF, IL-6, IL-5, IFNy, and MIP-1β. MCP-1 secretion was characteristic for MSCs isolated from all examined tissues, and the age of culture may have affected the cytokine concentration in post-culture media (Figure 6). MCP-1 concentration increased significantly between P1 (291.5 pg/mL) and P10 (697.1 pg/mL) ($P < 0.05$) in BM-MSCs. Similar results were observed in SM-MSCs, for which MCP-1 concentration also increased significantly between P1 (522.9 pg/mL) and P5 (1247.3 pg/mL) ($P < 0.05$).

The cytokines and growth factors involved in angiogenesis, IL-8 and VEGF, showed the highest concentration in the supernatant collected from the BM-MSC culture. The level of IL-8 in the supernatant from BM-MSCs at P5 increased compared to P1 (4234.6 pg/mL *vs* 1143.0 pg/mL, respectively) and was the highest compared to MSCs from other examined sources. In P10, the high level of IL-8 was maintained in the supernatant from BM-MSCs and significantly increased in the supernatant from SM-MSCs compared to P1 ($P < 0.05$). In contrast to IL-8 concentration, VEGF concentration was the highest in the supernatant from BM-MSCs at P1 (4234.6 pg/mL); however, it gradually decreased during the subsequent passages, and at P10, it was assessed at 776.8 pg/mL (Figure 6).

AT-MSCs showed the strongest ability to secrete IL-6 among the MSCs derived from all tested sources, assessed at 360.4 pg/mL in P1. A lower concentration of IL-6 was observed in the supernatant from the BM-MSC culture (99.7 pg/mL in P5). In

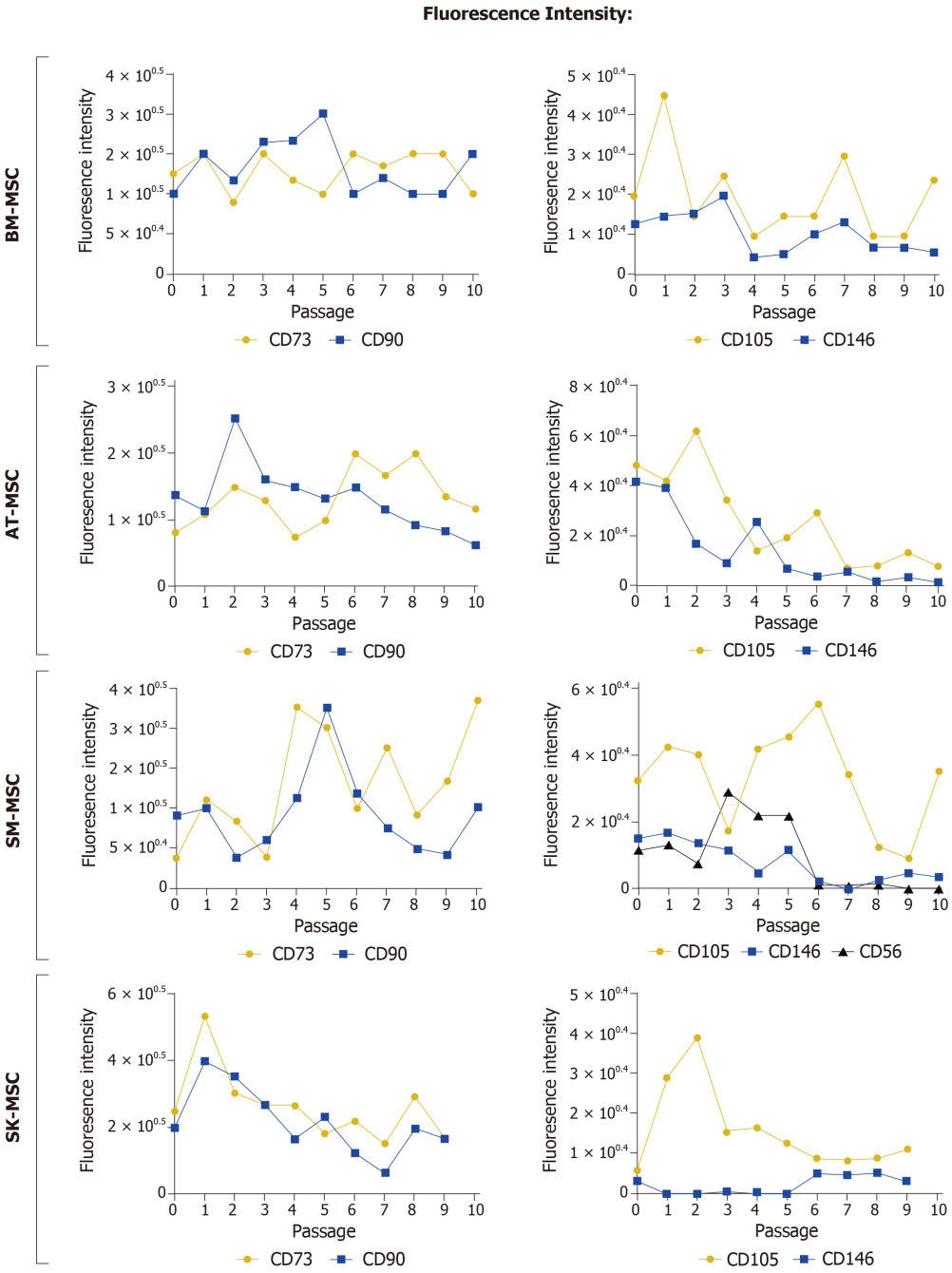
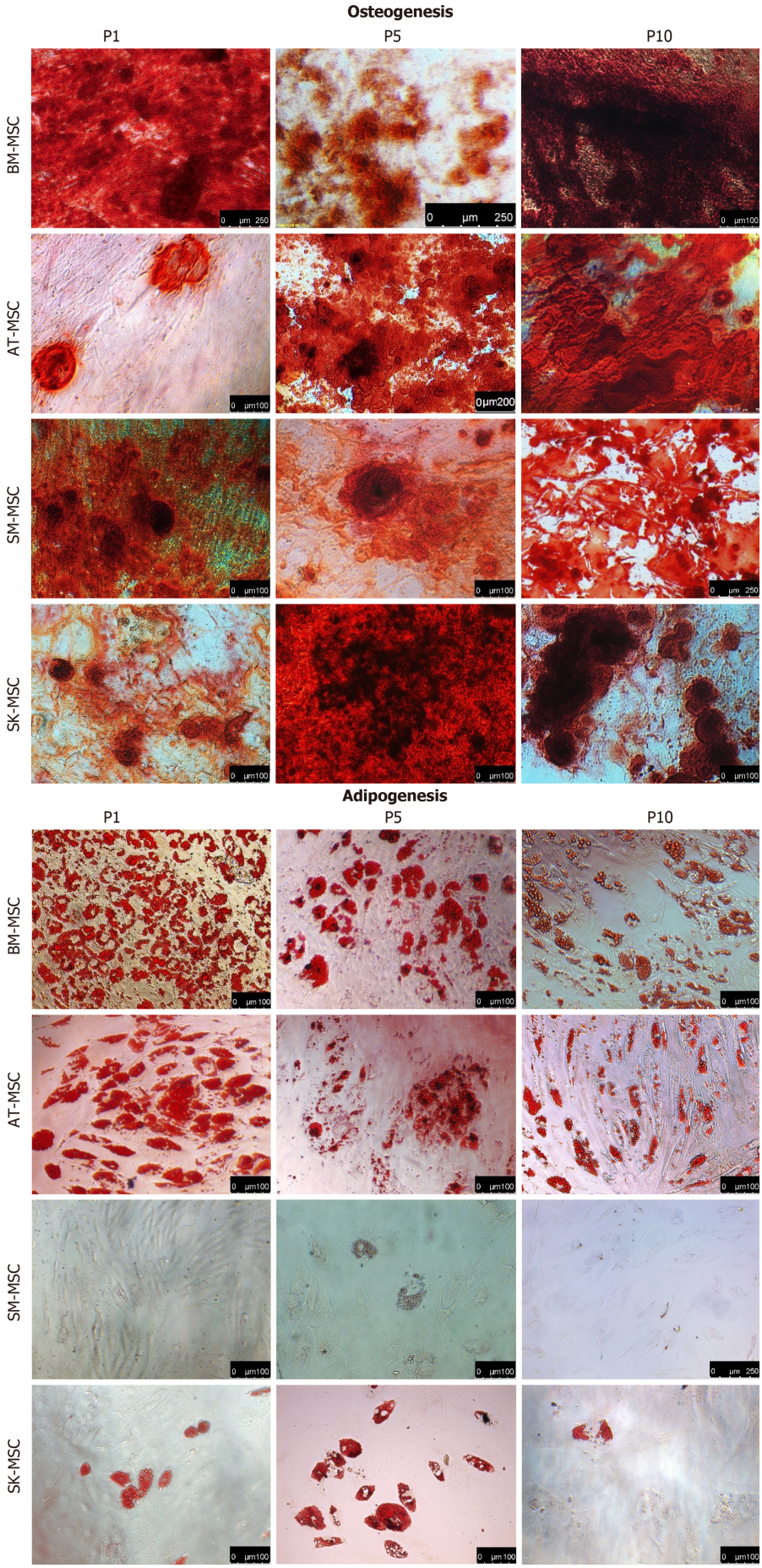



**Figure 3 Fluorescence intensity positive for MSC markers CD73 (APC), CD90 (FITC), CD105 (PE), and CD146 (PE) in bone marrow - mesenchymal stem cells, adipose tissue - mesenchymal stem cells, and skin - mesenchymal stem cells, and CD56 (PE) only in skeletal muscle - mesenchymal stem cells.** Heterogeneous population isolated from skeletal muscles showed the strongest instability of naïve MSC markers. Expression of CD73 and CD90 was preserved among the MSCs isolated from other tissues (BM, AT, and SK); however, fluorescence intensity decreased in AT-MSCs and SK-MSCs in the later passages. Expression of the CD105 marker was the most varied during the follow-up period. Fluorescence intensity of the proangiogenic marker CD146 was the strongest in BM-MSCs and AT-MSCs in the early passages. BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

MSCs from both of these tissues, significant changes in IL-6 expression were observed between the passages. In BM-MSCs, IL-6 concentration increased from 45.9 pg/mL in P1 to 79.01 pg/mL in P10 ($P < 0.05$), and for AT-MSCs, the concentration between the same passages decreased from 360.4 pg/mL in P1 to 111 pg/mL in P10 ($P < 0.05$). Cells from BM-MSCs, SM-MSCs, and SK-MSCs were able to secrete IL-6, IFN-γ, and MIP-1β at a very low level (< 110 pg/mL). Interestingly, SK-MSCs showed the lowest cytokine secretion ability compared to the evaluated MSCs originating from other sources (Figure 6).

Another screened cytokine, IL-1RA, was observed to be secreted in a very small amount only by BM-MSCs and SM-MSCs. The highest levels of IL-1RA measured in the supernatant after *in vitro* MSC culture were detected in P1 (41.3 pg/mL) for BM-


Osteogenesis
P1
P5
P10
BM-MSC
AT-MSC
SM-MSC
SK-MSC
Adipogenesis
P1
P5
P10
BM-MSC
AT-MSC
SM-MSC
SK-MSC

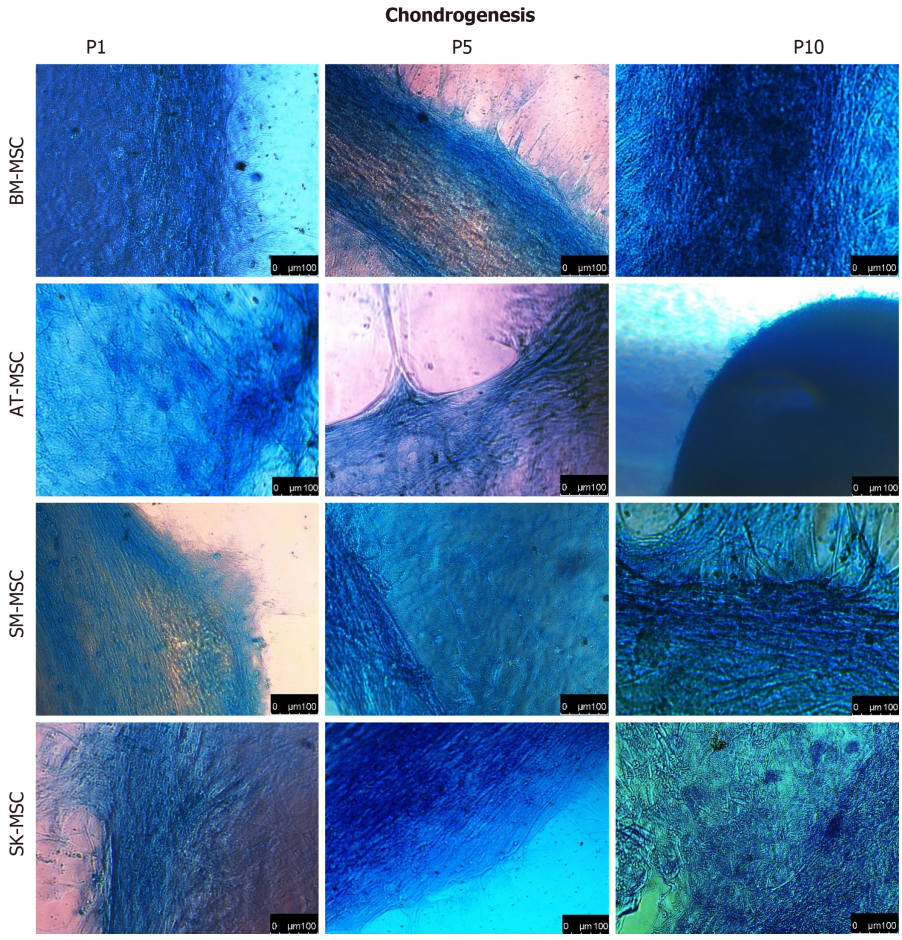
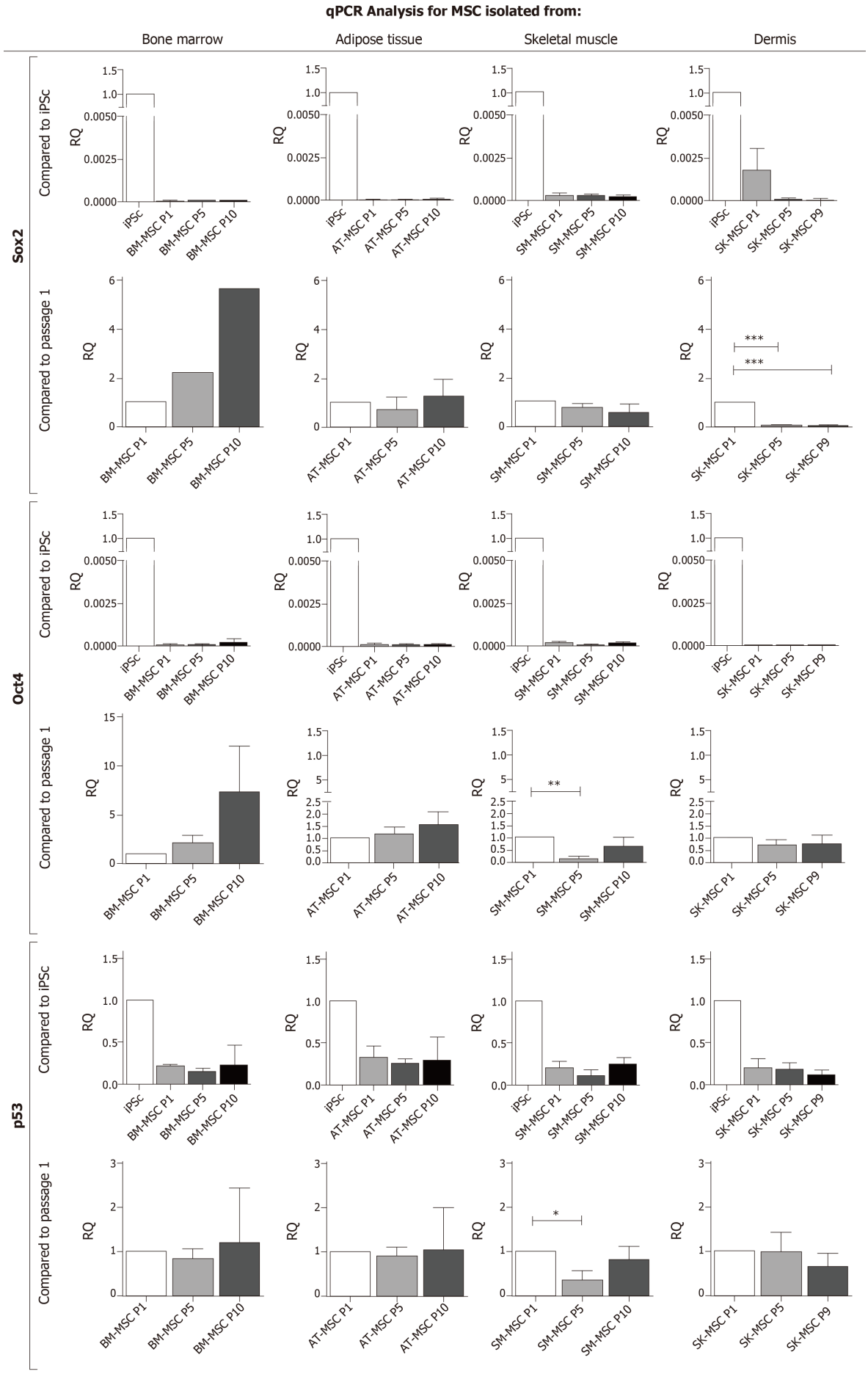



**Figure 4 Osteogenic, adipogenic, and chondrogenic potential of bone marrow - mesenchymal stem cells, adipose tissue - mesenchymal stem cells, skeletal muscle - mesenchymal stem cells and skin - mesenchymal stem cells, examined from P1 to P9–10.** Improvement of the osteogenic potential and downregulation of adipogenic abilities with the number of passages was observed in BM-, AT- and SK-MSCs. Only SM-MSCs were unable to differentiate into adipocytes. BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

MSCs and in P10 (6 pg/mL) for SM-MSCs. BM-MSCs, AT-MSCs, SK-MSCs, and SM-MSCs also secreted G-CSF. For BM-MSCs and AT-MSCs, the highest level of G-CSF detected in the supernatant was 19.4 pg/mL for BM-MSCs at P5 and 31.3 pg/mL for AT-MSCs at P10. The highest concentration of G-CSF from the SM-MSC culture amounted to 209.9 pg/mL at P10, whereas in the supernatant from the SK-MSC culture, the highest concentration amounted to 52.1 pg/mL in P5. BM- (P5), AT- (P10), and SM- (P10), but not SK-MSCs, secreted IP-10 at a concentration of 30 pg/mL, 43.5 pg/mL, and 39.9 pg/mL, respectively. BM-MSCs in P1 and AT-MSCs in P10 secreted low amounts of RANTES (15.6 pg/mL and 5.3 pg/mL, respectively). SM-MSCs secreted RANTES only in passage P10 at a concentration of 23.2 pg/mL. Other pro-inflammatory cytokines that were detected only in a late passage (P10) after SM-MSC culture were TNF-α (14.7 pg/mL), MIP-1α (0.3 pg/mL), IL-9 (1.6 pg/mL), IL-2 (2.4 pg/mL), and IL-17 (5.6 pg/mL), suggesting that the level of differentiation of myogenic precursor cells or changes occurring due to long-term culture affected the secretion of pro-inflammatory cytokines. Low amounts of TNF-α were also detected in P1, P5, and P10 in BM- and AT-MSC cultures. The highest level of TNF-α for BM-MSCs was observed in P5 (13.9 pg/mL), and for AT-MSCs, in P10 (13.4 pg/mL). A very low concentration of IL-4 was detected in the BM-MSC culture (with the highest concentration in P5, 1.3 pg/mL), AT-MSC culture (P10, 1.5 pg/mL) and SM–MSC culture (P10, 1.4 pg/mL). Only AT-MSCs and SK-MSCs were able to secrete Eotaxin into the supernatant. The highest concentration of Eotaxin in the supernatant was observed in the AT-MSC culture in P10 (8.9 pg/mL) and SK-MSC culture in P1 (0.9 pg/mL). BM-MSC was the only culture able to secrete low amounts of IL-7 (6.3 pg/mL in P5). The presence of cytokines, such as IL-1β, IL-12, IL-13, IL-15, GM-CSF, PDGF-bb, or IL-10, was not observed in the supernatant after the culture of any of the


qPCR Analysis for MSC isolated from:
Bone marrow
Adipose tissue
Skeletal muscle
Dermis
Sox2
Oct4
p53
Compared to iPSc
Compared to passage 1
RQ
iPSc
BM-MSC P1
BM-MSC P5
BM-MSC P10
AT-MSC P1
AT-MSC P5
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SM-MSC P1
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SM-MSC P10
SK-MSC P1
SK-MSC P5
SK-MSC P9
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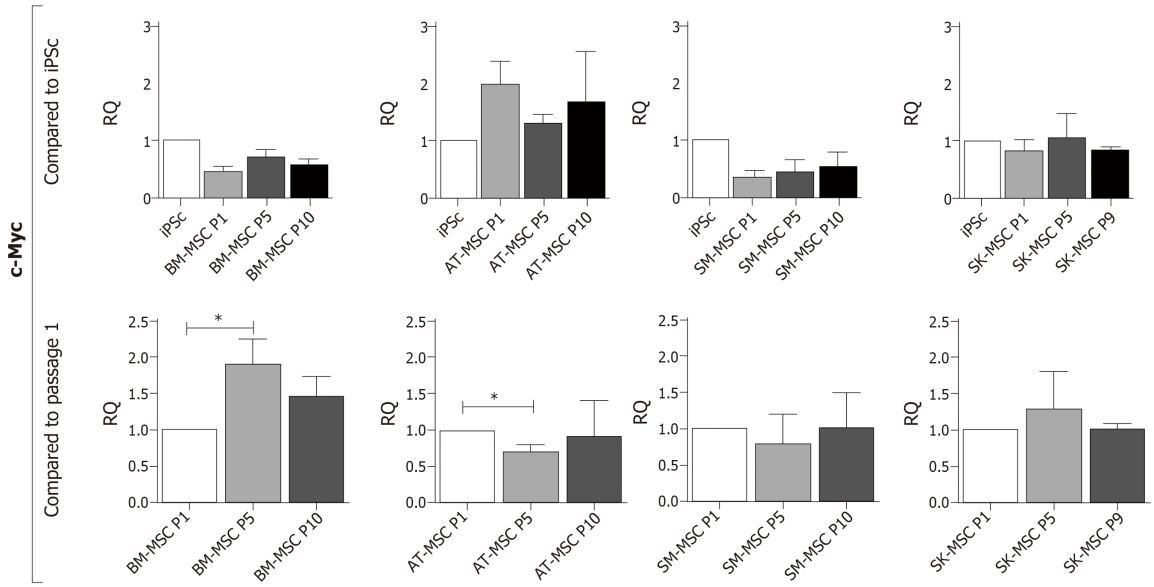



**Figure 5 qPCR analysis for pluripotency and the genetic stability of mesenchymal stem cell.** mRNA for Sox2 and Oct4 characterizes the pluripotency of MSCs, tumor suppressor gene p53, and proto-oncogenes c-Myc expression for the genetic stability of BM-MSCs, AT-MSCs, SM-derived stem/progenitor cells (SM-MSCs) and SK-MSCs as compared to iPSC control and to an early passage, P1. ($^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$). BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

cell types included in this study.

Similarities and differences between biological activities of examined MSCs are summarized in the Table 2.

### *Co-cultures of MSCs isolated from different tissues*

Co-cultures of MSCs of different tissue origin were created in two arrangements of isolated MSCs and were observed for seven days. BM-MSCs and SM-MSCs dyed with PKH26 (red) and PKH67 (green), respectively, were able to form the first spontaneous fusions after 24 h of co-culture (Figure 7). The first observation was that BM-MSCs (red) surrounded SM-MSCs (green), and some of the cells gained a yellow color, which suggested green and red dye immersion (fusion) (Figure 7A, white arrows). Fused cells were present during the follow-up period, and after 120 h of coculture, showed the formation of a yellow structure that resembled myotube and consisted of both BM-MSCs and SMMSCs. A very similar state was observed after 24 h of AT-MSC (PKH 67, green) and SM-MSC (PKH 26, red) co-culture. After one day of mixed culture, spontaneous fusion between AT-MSCs and SM-MSCs was observed. After 120 h of coculture, fused cells resembled a mostly fusiform, but not myotube-like, shape (Figure 7B).

To confirm the spontaneous fusion between the co-cultured MSCs of different tissue origin, mixed cultures were detached from the culture plate after the images were taken, and single cells were analyzed using flow cytometry to assess the presence of cells that displayed merged fluorescence signals. In Figure 7, measurements performed with a cytometer documented the presence of a population of cells with fluorescence emission within the 480-560 nm range of the spectrum (Channel 2), characteristic for PKH67, the 595-642 nm range (Channel 4), characteristic for PKH26, and the 560-695 nm range (Channel 3), which confirmed the immersion of two dyes with each other. Furthermore, 15.4% of the BM- and SM- population and 11% of the AT-MSC population revealed fluorescence emission in Channel 3. Images of cells showing a specific morphology and the strongest fluorescence in the three channels indicated that double or enlarged single-cell nuclei were a characteristic feature of some of these cells (Figure 7).

BM-MSCs also revealed the ability to perform spontaneous fusion with SK-MSCs (6%) and AT-MSCs (7%), and the first cells sharing the red and green dye appeared after 24 h and 48 h, respectively. Fusion between the AT-MSCs and SK-MSCs reached only 2%, and the first fusions were observed after 48 h. SK-MSC and SM-MSC fusion was also ineffective: about 3.5% of the cells emitted a signal in Channel 3. The first fusions were observed after 96 h of incubation (data not shown).

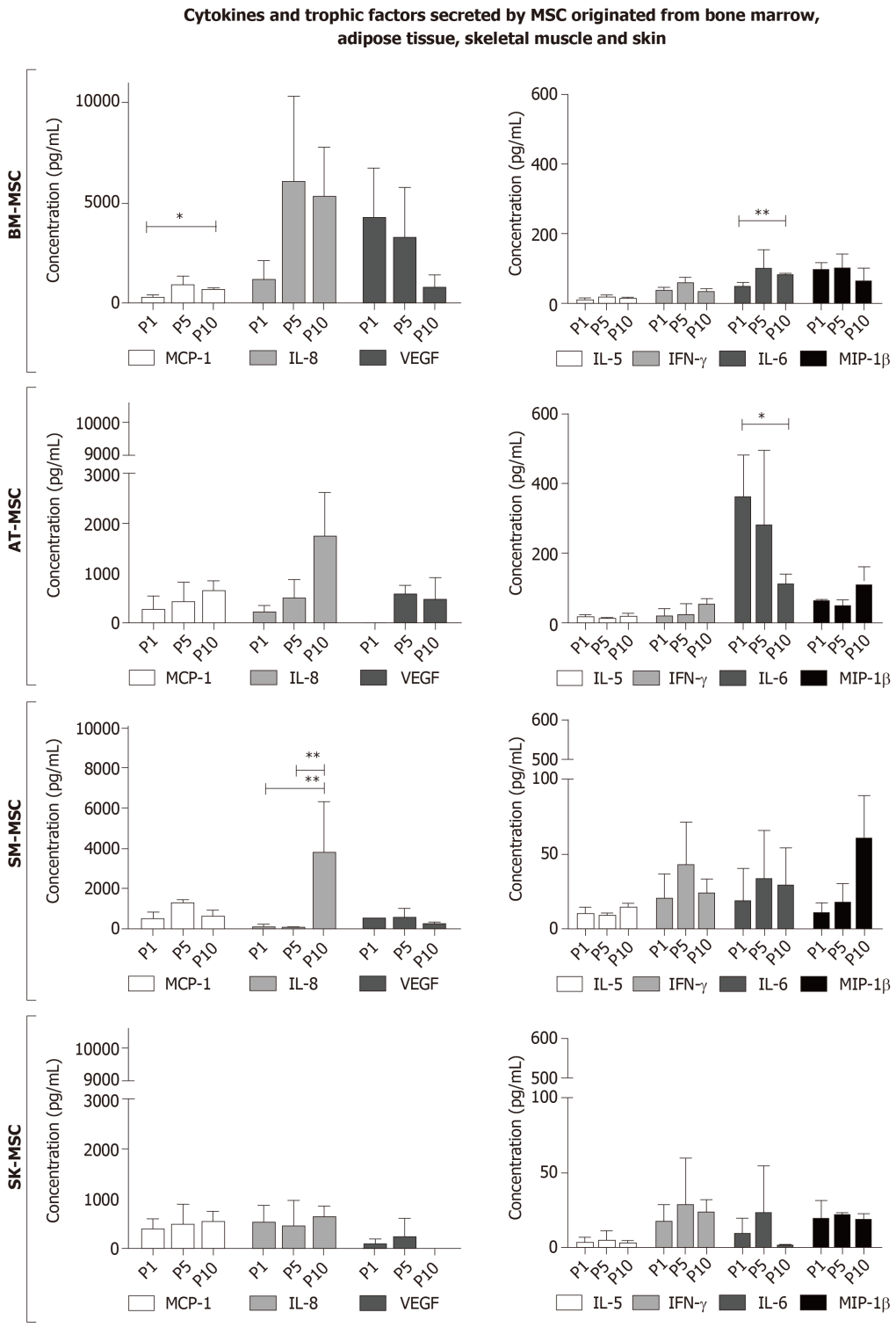



**Figure 6 Cytokine and trophic factor expression through Multiplex ELISA analysis in supernatants after cell culture of bone marrow - mesenchymal stem cells, adipose tissue - mesenchymal stem cells, skeletal muscle - mesenchymal stem cells and skin - mesenchymal stem cells.** MCP-1 was detected in all supernatants; however, statistical significance was only proven in BM-MSCs and SM-MSCs. The highest concentration of IL-6 was observed in supernatants after AT-MSC culture. In the supernatants of all examined MSCs, a low concentration of IL-5 and IFN-γ was observed. The highest concentrations of VEGF and IL-8 were observed in supernatants from the BM-MSC culture; however, smaller amounts of VEGF and IL-8 were also detected in the later passages in AT-MSCs and SM-MSCs. ($^{*}P < 0.05$, $^{**}P < 0.001$). BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

## DISCUSSION

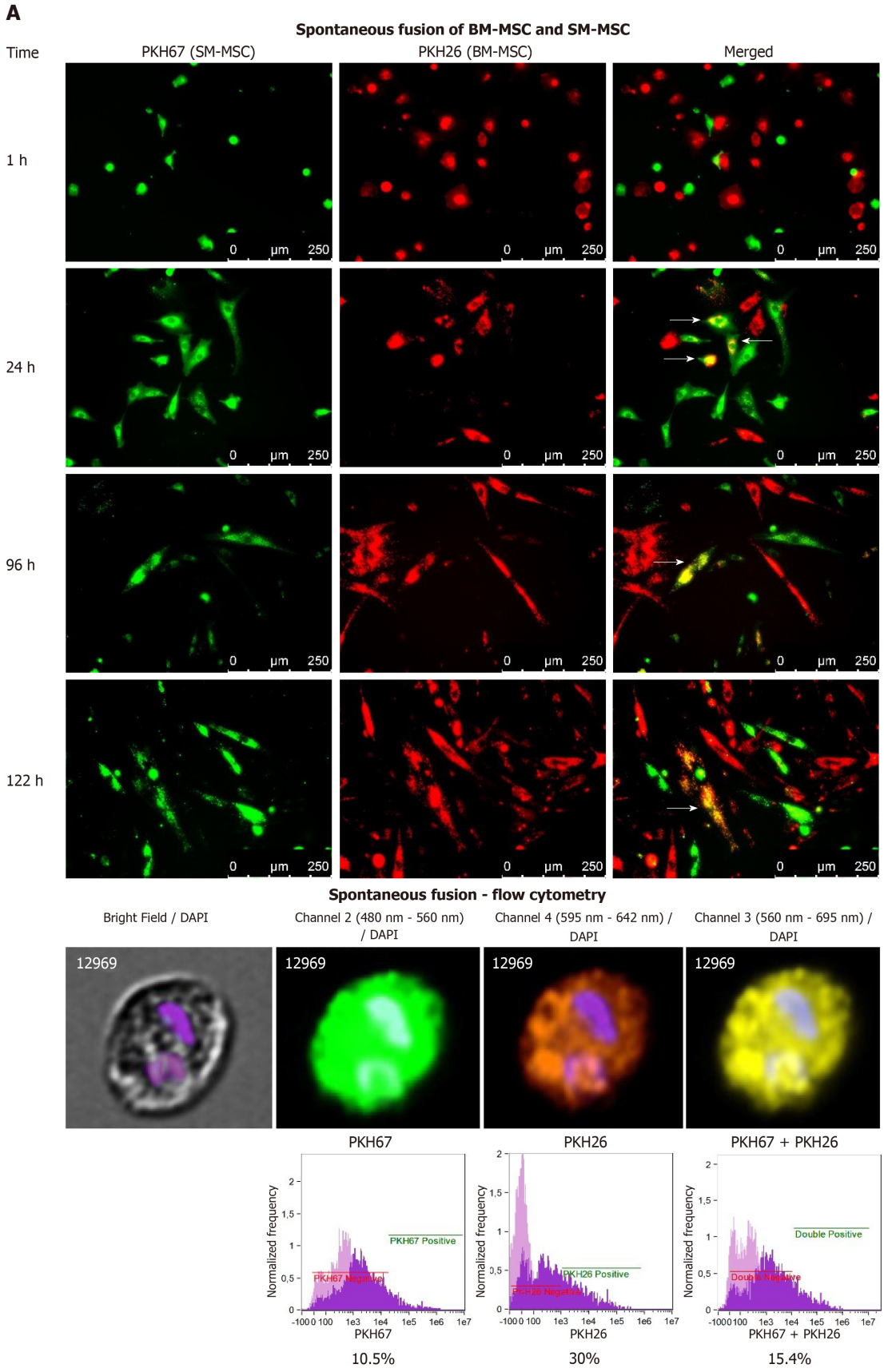
Mesenchymal stem/stromal cells are present in many human tissues and organs, which mean that their biological properties may differ depending on their environment. In this study, we demonstrated that MSCs obtained from adult bone

**Table 2 Summary of biological properties of MSCs isolated from different tissues**

| Summary | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Phenotype** | **Marker** | **BM-MSC** | | | **AT-MSC** | | | **SM-MSC** | | | **SK-MSC** | | |
| | | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** |
| | CD73 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | CD90 | +++ | +++ | +++ | +++ | +++ | ++ | +++ | +++ | ++ | +++ | +++ | +++ |
| | CD105 | +++ | ++ | ++ | +++ | ++ | + | ++ | ++ | ++ | +++ | +++ | ++ |
| | CD146 | +++ | ++ | ++ | +++ | + | +/- | + | + | +/- | + | + | ++ |
| | PDGFRα | ++ | ++ | +/- | +++ | + | +/- | ++ | + | + | + | + | + |
| | PW1 | - | +/- | +/- | ++ | +/- | +/- | +++ | +/- | +/- | ++ | +/- | +/- |
| **Differentiation** | **Tissue** | **BM-MSC** | | | **AT-MSC** | | | **SM-MSC** | | | **SK-MSC** | | |
| | | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** |
| | Osteo- | ++ | ++ | +++ | + | ++ | +++ | ++ | ++ | ++ | + | ++ | +++ |
| | Adipo- | +++ | ++ | + | +++ | ++ | ++ | - | - | - | + | ++ | +/- |
| | Chondro- | ++ | ++ | +++ | ++ | ++ | +++ | + | ++ | ++ | + | ++ | ++ |
| **mRNA ecpression** | **Gene** | **BM-MSC** | | | **AT-MSC** | | | **SM-MSC** | | | **SK-MSC** | | |
| | | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** |
| | Sox2 | + | ++ | +++ | + | + | + | + | + | + | +++ | + | + |
| | Oct4 | + | ++ | +++ | + | + | + | ++ | +/- | + | + | + | + |
| | p53 | + | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ | ++ | ++ | + |
| | c-myc | + | +++ | ++ | +++ | ++ | +++ | ++ | ++ | ++ | ++ | ++ | ++ |
| **Cytokines and chemokines** | **Cytokine** | **BM-MSC** | | | **AT-MSC** | | | **SM-MSC** | | | **SK-MSC** | | |
| | | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** | **P1** | **P5** | **P10** |
| | MCP-1 | + | ++ | ++ | +/- | + | + | + | ++ | + | + | + | + |
| | IL-8 | + | +++ | +++ | +/- | + | ++ | - | - | ++ | + | + | + |
| | VEGF | +++ | +++ | + | - | + | +/- | + | + | +/- | - | +/- | - |
| | IL-5 | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | +/- | - | - | - |
| | IFN-γ | +/- | + | +/- | +/- | +/- | + | +/- | + | +/- | +/- | +/- | +/- |
| | IL-6 | +/- | + | + | +++ | +++ | ++ | +/- | + | + | - | +/- | - |
| | MIP-1β | + | + | + | + | + | + | - | +/- | + | +/- | +/- | +/- |

The gradation reflects the changes in the value of the examined parameters compared to passage 1: (-) negative, (+/-) weak, (+) moderate positive, (++) positive, (+++) strong positive.

marrow, adipose tissue, skeletal muscles, and dermis showed certain differences, despite meeting the minimal criteria of MSC characterization[15]. Based on the results obtained in this study, we also considered alternative, more accessible sources of MSCs for potential clinical application. However, clinical application requires biologically active MSCs, which are difficult to obtain in a sufficient amount during the first step of isolation at P0. To obtain a sufficient number of MSCs for therapeutic use, subsequent passages are usually needed, which may change the biological properties of the MSCs. One of the criteria of MSCs is their surface phenotype, and in this study, we compared the stability of basic MSC phenotypes during the follow-up period up to P10. The dynamics of CD73, CD90, CD105, and CD146 marker expression, presented here as fluorescence intensity, confirmed that MSCs from all sources showed an expression of basal MSC markers (CD73, CD90, and CD105). The purity of the population of cells that expressed CD73 and CD90 in MSC cultures from all four studied sources was above 95%. Heterogeneity of MSCs isolated from different tissues was confirmed through a diverse expression of CD105 in P0, which varied between the tissues of origin and was equal to 78% for SM-derived stem/progenitor cells, 92% for SK-MSCs, and up to 98% for AT-MSCs and BM-MSCs. Expression of CD105 marker increased in the *in vitro* culture in passage P1 for BM-MSCs and SM-MSCs and in passage P2 for SK-MSCs and AT-MSCs. The fluorescence intensity of other MSC markers, CD73 and CD90, varied the most in cells isolated from skeletal muscles, and decreased slightly with age in the culture isolated from the dermis. In BM-MSCs and AT-MSCs, CD73 and CD90 expression was mostly stable, as confirmed by fluorescence intensity. Despite some variance in fluorescence intensity, the overall population of CD73+ and CD90+ cells in MSCs from all examined tissues reached 95%–99% during the follow-up period. This observation confirmed that long-


A
Spontaneous fusion of BM-MSC and SM-MSC
Time
PKH67 (SM-MSC)
PKH26 (BM-MSC)
Merged
1 h
24 h
96 h
122 h
0 µm 250
Spontaneous fusion - flow cytometry
Bright Field / DAPI
Channel 2 (480 nm - 560 nm) / DAPI
Channel 4 (595 nm - 642 nm) / DAPI
Channel 3 (560 nm - 695 nm) / DAPI
12969
PKH67
PKH26
PKH67 + PKH26
Normalized frequency
PKH67 Positive
PKH67 Negative
PKH26 Positive
PKH26 Negative
Double Positive
Double Negative
10.5%
30%
15.4%

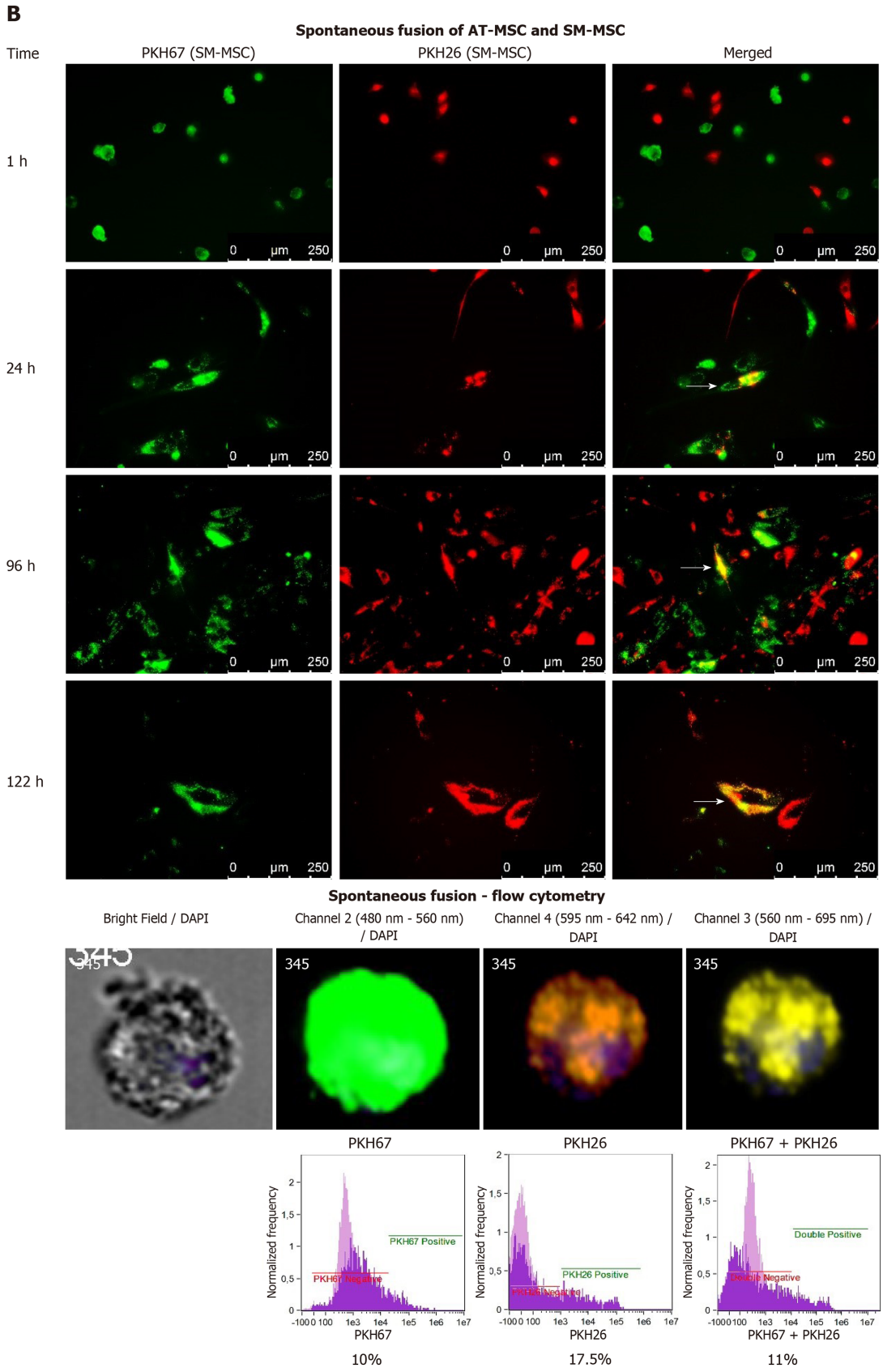



**Figure 7 Co-culture and spontaneous fusion of skeletal muscle - mesenchymal stems with bone marrow - mesenchymal stems or adipose tissue - mesenchymal stems.** Panel A. Co-culture of BM-MSCs (PKH26 red) and SM-MSCs (PKH67 green) revealed spontaneous fusion (white arrows) as early as 24 h after the mixed culture was started. During the follow-up period, fused cells created structures resembling myotubes; 120 h after the observation, MSCs were detached and single cells were analyzed using flow cytometry for fluorescence in the 560–695 nm range, which confirmed the immersion of red (PKH26) and green (PKH67) dyes. Panel B Fusion between SM-MSCs (PKH26 red) and AT-MSCs (PKH67 green). Flow cytometry analysis confirmed the presence of fused cells in the culture; however, after 120 h of co-culture, the fused cells assumed a mostly fusiform, but not myotube-like, shape. Flow cytometry for fluorescence in the 560-695 nm range confirmed the presence of fused cells. BM-MSC: Bone marrow - mesenchymal stem cell; AT-MSC: Adipose tissue - mesenchymal stem cell; SK-MSC: Skin - mesenchymal stem cell; SM-MSC: Skeletal muscle - mesenchymal stem cell.

term culture preserves the basic phenotype of MSCs, regardless of the tissue from which they were obtained.

The proangiogenic properties of MSCs were analyzed through CD146 expression. A fraction of CD146-positive MSCs was detected in MSCs isolated from all examined tissues at different levels. AT-MSCs showed the highest expression of CD146-positive cells, whereas SK-MSCs produced the lowest signal, as confirmed through fluorescence intensity. This is an important observation in terms of selecting a tissue for MSC isolation in order to stimulate angiogenesis for therapeutic use. However, long-term culture of MSCs is unfavorable for maintaining the proangiogenic function of MSCs. Expression of the CD146 marker decreased in the AT-MSC and SM-MSC populations and disappeared nearly entirely in P6. Interestingly, cells isolated from the dermis showed a weak CD146 expression in passage P6, despite the lack of CD146-positive cells in P1. In BM-MSCs, fluorescence intensity of the CD146 marker also decreased; however, it maintained a level of about 50% of the original signal, indicating that this source of MSCs was the most stable for CD146 expression. This observation confirmed that the tissue-origin niche of MSCs is important for a specific MSC phenotype and the resulting biological function. Therefore, for the purposes of clinical application, it is important to consider the source of MSCs with the preferred biological activity in order to achieve the desired effect. For instance, if MSCs are needed to treat a systemic disease (*e.g.*, graft-versus-host disease), the proangiogenic activity of MSCs is less important than their anti-inflammatory ability, whereas in order to regenerate injured tissue (*e.g.*, in muscular dystrophy or myocardial infarction), MSCs with an anti-inflammatory activity and proangiogenic potential should be considered.

The proangiogenic properties of MSCs were also confirmed through PDGFRα expression in P0 in the population of cells obtained from all sources. However, around passage P5, expression of PDGFRα decreased in MSCs from all examined sources, as confirmed by immunofluorescence staining. This observation suggests that the biological properties of the studied MSCs may play a role in the angiogenesis and regeneration of impaired blood vessels, and considering the proangiogenic activity of MSCs in therapeutic application, cells from early passages should be used[44,45]. However, subject literature also states that the PDGFRα receptor plays a role in connective tissue remodeling through cross-talk with the extracellular matrix[46]. Another surface marker examined in this study was the PW1. A recent study based on the mouse model demonstrated that a population of PW1-positive endothelial cells showed an increased ability to proliferate and regenerate and arrange blood vessels[29]; moreover, PW1 expression was also associated with the differentiation of hair follicle cells *in vivo*[47]. PW1 with the co-expression of PDGFRα was characteristic for myogenic precursor cells induced for adipogenic differentiation[27]. However, according to the results presented in this study, a large population of MSCs originating from skeletal muscles expressed PW1, but in the study conditions, the cells were unable to differentiate into adipocytes. The presence of PW1 in AT-, SK- and SM-derived myogenic stem/progenitor cells, but not BM-MSCs, suggests that PW1 expression may be associated with tissue specificity. Research on the effect of PW1 on MSC differentiation potential confirmed that cardiac PW1$^+$ cells may affect mesenchymal differentiation and were able to give rise to multiple cardiovascular and mesenchymal lineages[48]. We assume that PW1 plays a supportive role in tissue homeostasis and regeneration. However, their role in tissue regeneration is variable and depends on the activity of stem/progenitor cells residing in a given tissue.

Sox2, in cooperation with Oct4 and Nanog, is one of three key transcription regulators of pluripotent stem cells. Sox2 and Oct4 play an important role in mesodermal and ectodermal differentiation during embryonic development[49]. Their role is not limited to embryogenesis; it is also required for the proper proliferation and differentiation of adult stem cells, such as MSCs. In the context of MSCs, Sox2 is often referred to as a cell fate factor because it constitutes an up- or downregulation trigger into the adipogenic or osteogenic differentiation stage for cells[50,51]. In this study on the MSCs of different tissue origin, Sox2 expression was significantly lower in the examined MSCs than in iPSCs. However, when compared between the subsequent passages, the expression of Sox2 in BM-MSCs and AT-MSCs increased with culture age. Unfortunately, in case of BM-MSCs, the expression of Sox2 was difficult to determine in some patients in the later passages. Interestingly, BM-MSCs and AT-MSCs differentiated the most efficiently into osteoblasts and chondrocytes in P10, when Sox2 expression was the highest. The adipogenic ability of AT-MSCs and BM-MSCs in the later passages decreased, being the most effective in P1, when Sox2 expression was lower than in P10. These results are in contrast to the observations made by Park *et al*[34] on the MSCs of umbilical cord-origin with inhibited Sox2 expression, in which the authors revealed abnormalities in adipogenic differentiation in favor of osteogenesis. However, the authors explained that human umbilical cord

MSCs exhibited a stronger expression of Sox2 compared to human BM-MSCs and AT-MSCs, which may explain the differences in our results. In our study, the level of Sox2 in the examined MSCs was associated with their ability to differentiate into osteoblasts and increased with the age of cell culture, as observed in BM- and AT-MSCs. Long-term culture of BM-MSCs and AT-MSCs also resulted in an increased expression of Oct4. The greatest difference was again seen in BM-MSCs, for which Oct4 expression in P10 was over seven times higher than in P1. Oct4 is one of the key regulators of pluripotency and has the ability to bind and form a complex with Sox2 and regulate the transcription of many target genes[52]. Piccinato *et al*[53] observed that a high expression of Oct4 in human BM-MSCs was related to a longer lifespan of BM-MSCs cultured *in vitro*.

In the case of the SM-MSC and SK-MSC populations, the number of passages was unfavorable for Sox2 expression, and a significant downregulation was observed. SK-MSCs revealed the highest expression of Sox2 in P1, but during the subsequent passages, the expression decreased fourteen times in P5 and P10. However, the decreased level of Sox2 impaired the quality of chondrogenesis; this observation suggests that the differentiation potential of MSCs is related to their niche of origin. MSCs isolated from adult dermis also revealed a limited ability to differentiate into adipocytes in the study conditions. The dermis is a reservoir of Sox2-positive cells that are mesenchymal progenitors involved in hair follicle formation[54]. The observation of a relatively higher expression of Sox2 in P1 for SKMSCs matches the positive PW1+ results after isolation. In previous studies on skin, the expression of PW1/Peg3 in adult dermis was associated with hair follicle formation[47]. This finding suggests that the dermis-derived stem/progenitor cells, which meet the minimal criteria for MSC characterization, may possibly display a higher potential to form hair follicles *in vitro*. This observation may be important for tissue engineering and should be further studied.

For SM-derived stem/progenitor cells, not only did the expression of specific markers, such as CD56, show variability, but there were also changes in the relative quantity of Oct4 and p53. Compared to P1, the expression of these two markers decreased in P5 by over a half, to increase again in P10. Changes in the expression of Oct4 and p53 do not affect the capacity for chondrogenesis and osteogenesis, and may be associated with the upregulation of CD56 expression, which in turn is related to the myogenic differentiation of satellite cells[55,56]. p53 is known as a tumor suppressor protein, and it also plays a role in the regulation of the proliferation and differentiation of stem cells[57]. Research also suggests that p53 regulates the balance between differentiation and the return to the quiescence of the satellite cell pool in the skeletal muscle niche[58]. Under specific conditions, p53 was observed to inhibit myogenin expression[59]. PW1/Peg3 is a p53 binding proteins. Research has documented that PW1-deficient mice revealed a decreased number of myogenic progenitors in the quiescent stage[60]. The decrease in the expression of PW1 in P5 could be related to the spontaneous differentiation of SM-derived stem/progenitor cells *in vitro* observed during the cell culture period, which was also confirmed by a decrease in CD56 expression. Moreover, the loss of expression of PW1 in P5 in the SM-MSC population was permanent and did not appear again in P10 (data not shown). The decrease in p53 expression in P5 could be related to the lack of one of its binding factors.

c-Myc, a transcriptional factor implicated in a wide range of cellular functions, including cell growth, proliferation, differentiation, metabolism, and apoptosis, is also an important regulator of adipogenesis, as shown in a study by Deisenroth *et al*[61]. The lack of the adipogenic differentiation of SM-MSCs in our study was likely associated with a decreased activity of Sox2, Oct 4, and c-Myc in the later passages. The greatest differences between an early (P1) and a late passage (P10) in the expression of the c-Myc protooncogenic factor were observed in BM-MSCs. This observation may be related to the upregulation of Sox2 and Oct4 in the older passages. The expression of c-Myc did not change in the case of MSCs derived from other sources; however, compared to iPSCs, AT-MSCs revealed a surprisingly high expression of c-Myc beginning with P1. A high level of c-Myc expression is associated with increased proliferation and differentiation capacity, and is controlled by Sox2[34]. For AT-MSCs, the high level of c-Myc expression cannot be ignored, and the risk of oncogenesis should be studied further.

It is worth mentioning here that some MSCs in passage P10 showed a noticeably high SD (standard deviation). This observation suggests that gene expression in the late passages varies between patients, which lead to the conclusion that a comprehensive assessment of the gene expression background is required before cells from late passages can be transplanted, even if the graft is autologous. There are many factors that can influence the biological behavior of cells in patients, including individual genetic differences.

Immunoregulation is a crucial capability of MSCs, making them very special cells

that affect not only immune cells, but also the cell niche during regeneration. A screening of 27 human cytokines, chemokines, and growth factors showed that the MSCs, derived from all of the examined sources, had the ability to release a relatively high level of the MCP-1 protein into the culture supernatant. MCP-1 is a chemoattractant for monocytes and macrophages, and it also affects the stimulation of T lymphocytes into the secretion of IL-4. The migration of immune cells to the regeneration site has two aspects: it may be helpful during an invasion of pathogens into the damaged tissue and whenever it is necessary to remove the necrotic tissue from the niche. Another reason why monocytes and macrophages are attracted to the site of damage is the fact that, when appropriately stimulated, they take a proregenerative form (M2) and support regeneration by creating a favorable microenvironment[62,63]. MSCs secreted MCP-1 in a similar concentration, regardless of their origin; however, the highest concentration of MCP-1 in the culture supernatant was observed in SM-MSCs in P5. The P5 passage was unique in relation to the SM-MSC cells for several other reasons: it showed the highest (compared to the other MSCs) fluorescence intensity of CD73 markers, CD90 (characteristic peak of expression), and CD105, and a strong decrease in Oct4 and p53 gene expression. The maintenance of the MSC phenotype suggests that SM-MSCs represent muscle-resident non-satellite cells, which can act as immunoregulatory cells by releasing promyogenic cytokines that stimulate muscle progenitors to regenerate muscle fibers. Moreover, a very interesting observation related to SM-MSCs was their ability to secrete relatively high levels of IL-8 in P10, which may be related to the fact that the cells started to display the characteristics of differentiated cells, as evidenced by the expression of desmin and dystrophin (not shown).

However, the screening of cytokines showed that BM-MSCs had the highest ability to secrete proangiogenic factors, such as IL-8 or VEGF. IL-8 is a cytokine that is a chemoattractant for neutrophils and monocytes, but is also known as a cytokine with proangiogenic properties[64]. An increased production of IL-8 and VEGF by AT-MSCs has been reported in recent studies on the angiogenic activity of MSCs[65]. The secretion of proangiogenic factors may be a very desirable feature, especially in the context of tissue engineering and attempting to artificially create a living tissue, such as skin, bone, or a skeletal muscle. The engineered tissue must be supplied with blood vessels for proper tissue nutrition. However, the lack of an increase in the secretion of the proangiogenic factor VEGF in the later passages is intriguing; this is likely compensated by an increased IL-8 secretion in all examined MSCs, except those isolated from the dermis.

A characteristic feature of AT-MSCs is the ability to secrete the highest levels of the pleiotropic cytokine IL-6. During infection, IL-6 is able to engage cells involved in both the innate and adaptive immune response[66]. However, from the perspective of MSCs, the ability to secrete IL-6 is associated with the undifferentiating stage of MSCs, which has a positive effect on their immune privilege[67]. Research on ischemic brain damage in a rat model suggests a protective, antiapoptotic effect of IL-6 secreted by MSCs on astrocytes under hypoxic conditions[68]. In our study, the highest concentration of IL-6 was observed in the supernatant from AT-MSCs and BM-MSCs, and the lowest was observed in SK-MSCs and SM-MSCs. These observations are in line with a study performed by Priciola *et al*[67] and by Li *et al*[69], which suggests that IL-6 is more efficiently produced by undifferentiated cells.

MSCs are known for their ability to modulate the local environment. In this study, cells from different sources were seeded together in order to assess their influence on each other. Although all combinations of co-culture were examined (data not shown), the most interesting results were obtained when co-culturing BM- or AT-MSCs with SM-derived stem/progenitor cells. When cultured together, BM-MSCs and SM-MSCs as well as AT-MSCs and SM-MSCs were able to fuse. The myogenic abilities of BM-MSCs were introduced in the experimental model of Duchenne muscular dystrophy (DMD)[70]. Moreover, both BM-MSCs and SM-MSCs have a proangiogenic potential and may support the dystrophic niche for vascular regeneration, which is crucial for proper muscle function[71]. Co-culture of BM-MSCs with myoblasts in the presence of trophic factors resulted in myogenic differentiation in a 3D matrix[72].

This observation opens new possibilities to treat muscular dystrophies, including DMD, a genetic disease associated with a mutation in the dystrophin gene leading to a progressive deficiency of dystrophin. Cellular therapies have been the focus of clinical application for DMD patients for over 25 years. However, MSCs of bone marrow origin or stem/progenitor cells of skeletal muscle origin transplanted individually resulted in a limited therapeutic effect due to the complexity of DMD and the biological properties of the transplanted cells[73]. BM-MSCs are able to participate in myogenesis and have a proangiogenic potential that supports vascular regeneration, which is critical for muscle function. SM-MSCs are able to differentiate into myoblasts, as confirmed by CD56 expression, and are characterized by the

strongest PW1 expression among all examined MSCs, specific for muscle-resident stem cell population involved in adult muscle regeneration[74]. We suggest that a combined therapy involving BM-MSCs and skeletal muscle stem/progenitor cells can improve muscle function in DMD patients through fusion with the damaged muscles and the immunomodulatory properties of the transplanted cells[75]. The proposal to use skeletal muscle progenitors from healthy donors under the immunomodulatory cover of BM-MSCs, which is an original proposal of the senior author of this paper, is currently being verified in an experimental clinical procedure. Moreover, similar observations of the fusion of the SM-MSC fraction with the BM-MSC or AT-MSC fraction provide hope for an alternative source of MSCs for this purpose. This observation is supported by a previous *in vitro* study on the participation of AT-MSCs in myotube formation when cocultured with differentiating myoblasts from DMD patients[76].

The lack of fusion between SK-MSCs and SM-MSCs may be related to the fact that the cells were fused in P2, during which the SK-MSCs cells showed a low expression of the CD146 antigen. This observation also confirmed the role of cells with the proangiogenic function for the regenerative potential of MSC.

## CONCLUSION

All examined MSCs maintained the basic phenotype of naïve MSCs up to P10. The differentiation capacity of MSCs isolated from bone marrow and adipose tissue was maintained during the follow-up period up to P10 and increased in terms of osteogenesis and chondrogenesis. In contrast, MSCs from the skin had a limited capacity to differentiate into adipocytes, and MSCs from skeletal muscles were unable to form adipocytes, and were instead considered to constitute progenitor cells with a bipotential capacity. The most stable biological activity for BM-MSCs and AT-MSCs was observed for up to 5 passages. However, proangiogenic markers, such as CD146 or PDGFRα, decreased in AT-MSCs, but were compensated by an increased activity of IL-8 and VEGF.

Our study showed that MSCs from bone marrow and adipose tissue expressed stemness markers Sox2 and Oct4 in long-term culture up to P10, whereas MSCs from skeletal muscles and from the skin revealed a decreased ability to express Sox2 and Oct4. The expression of stemness markers varied among MSCs derived from different tissues.

The phenotypic similarity between BM-MSCs and AT-MSCs, their ability to differentiate, secretion of bioactive factors, and the fact that AT-MSCs fused with SM-MSCs as effectively as BM-MSCs indicate a high biological similarity between these two MSC sources, and AT-MSCs may serve as an alternative source for BM-MSCs.

In sum, the obtained results document differences in the biological activity of MSCs obtained from various tissues during long-term culture, which may be significant from the point of view of application in regenerative medicine. The choice of MSCs with specific biological properties creates the possibility for using targeted therapies, in which the source of MSCs and the duration of the culture will be an important consideration for their selection for their regenerative potential and genetic stability.

## ARTICLE HIGHLIGHTS

### *Research background*

Mesenchymal stromal/stem cells (MSCs) are applied in experimental clinical procedures as a promising tool in regenerative medicine. Cells with basic MSCs characteristics can be isolated from different human tissues. However, their biological properties are still not fully characterized. Although MSCs from different tissues exhibit many common characteristics, some markers and biological properties are different and depend on their tissue of origin.

### *Research motivation*

The biological diversity of MSCs, depending on their original tissue location, methodology of isolation, and culture conditions encouraged us to explore the biological properties of MSCs of different tissue-origin in long-term *in vitro* culture. Recognizing the activity of factors that underlie MSC biology should constitute important points for consideration before clinical MSC application.

### *Research objectives*

In this study, we characterize the biological properties of MSCs during longterm culture isolated from: bone marrow (BM-MSCs), adipose tissue (AT-MSCs), skeletal muscles (SM-MSCs), and skin (SK-MSCs).

### Research methods
MSCs were isolated from the examined tissues and cultured up to 10 passages. MSCs were assessed for: phenotype with immunofluorescence and flow cytometry, multipotency with differentiation capacity for osteo-, chondro-, and adipogenesis, stemness markers with qPCR for mRNA for Sox2 and Oct4, and genetic stability for p53 and c-Myc. Furthermore, 27 bioactive factors were screened with the multiplex ELISA array, and spontaneous fusion involving a co-culture of SM-MSCs with BM-MSCs or AT-MSCs stained with PKH26 (red) or PKH67 (green) was carried out.

### Research results
All examined MSCs showed the basic MSC phenotype CD73, CD90, CD105 stable up to P10. However, their expression decreased with the age of culture, as confirmed by fluorescence intensity. The proangiogenic properties of MSCs were confirmed by CD146 expression, however, long-term culture is unfavorable for maintaining the proangiogenic function of examined MSCs, but not for BM-MSCs. All examined MSCs, except BM-MSCs, expressed PW1, a marker associated with differentiation capacity and apoptosis. BMMSCs and ATMSCs expressed the stemness markers Sox2 and Oct4 in long-term culture. All examined MSCs were stable in terms of p53 and c-Myc expression. The differentiation capacity of BM-MSCs and AT-MSCs was maintained during the follow-up period. In contrast, SK-MSCs and SM-MSCs had a limited ability to differentiate into adipocytes. BM-MSCs and AT-MSCs revealed similarities in phenotype maintenance, the ability to undergo multilineage differentiation, and secretion of bioactive factors. The fact that AT-MSCs fused with SM-MSCs as effectively as BM-MSCs indicates that AT-MSCs may serve as an alternative source for BM-MSCs.

### Research conclusions
Long-term culture affects the biological activity of MSCs obtained from various tissues. The source of MSCs with specific biological properties and the duration of the culture will be an important consideration for their selection for regenerative medicine.

### Research perspectives
Knowledge of MSC biology is developing, but remains incomplete, and there is still much room for exploration in basic *in vitro* and *in vivo* research before MSCs can be used in therapy.

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