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DECIPHERING THE TRANSCRIPTIONAL ACTIVITIES OF GENES CODING FOR ADIPOKINES AND THEIR RECEPTORS IN PORCINE *EX SITU*-PROTECTED MESENCHYMAL STEM CELLS UNDERGOING ADIPOGENIC DIFFERENTIATION*

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Abstract

The aim of this study was to investigate the impact of the source of mesenchymal stem cells (MSCs) and their adipogenic derivatives on the relative abundances (RAs) noticed for mRNA transcripts of the selected adipokines (adiponectin and leptin) and their receptors. MSCs were isolated from bone marrow (BM) and subcutaneous adipose tissue (AT) samples collected post mortem from a total of four gilts (each at the age of 6 months). The stemness of the MSCs was proven via recognizing their abilities to differentiate into adipocytes, osteoblasts, and chondrocytes. By using real-time PCR (RT-qPCR), the quantitative levels of transcriptional activity pinpointed for the adiponectin (*ADIPOQ*), adiponectin receptor 1 (*ADIPOR1)*, adiponectin receptor 2 (*ADIPOR2)*, leptin (*LEP*), and leptin receptor (*LEPR*) genes were comparatively analyzed between non-differentiated AT-MSCs and BM-MSCs and their cell counterparts undergoing differentiation into adipocytes. A significantly higher RA for *ADIPOR1* transcripts was identified in the AT-MSCs compared to the BM-MSCs and their adipogenic derivatives (P≤0.05). Moreover, the quantitative levels of *LEPR* transcripts were shown to increase significantly among adipocytes originating from differentiated BM-MSCs compared to undifferentiated BM-MSCs, AT-MSCs, and AT-MSC-derived adipocytes (P≤0.05). Nonetheless, there was no significant inter-group variability in the RAs of the *ADIPOQ*, *ADIPOR2*, or *LEP* mRNA transcripts (P>0.05). Cumulatively, the *in vitro* models focused on the identification and detailed exploration of transcriptomic signatures of undifferentiated pig BM- and AT-MSCs, and the molecular mechanisms underlying the adipogenic differentiation pathways of the above-indicated two distinct sources of stem cells were developed and optimized in the current investigation for the very first time. These *ex vivo* porcine models might confirm the expedited functional mobilization of *ex situ*-protected MSCs and their enhanced capacity to be transcriptionally reprogrammed into adipocytes due to physiopathological alterations in the expression profiles of adipokines and their receptors, which are prompted and progressed in obese and superobese women patients at the peri-pubertal period of ontogenesis. Finally, the models providing comprehensive molecular insights into *ex situ*-protected porcine BM- and AT-derived MSCs and their differentiated derivatives can be largely suitable for the biotechnologically assisted rescue and restitution of endangered representatives of rare native breeds of domestic pig.

Key words: porcine, AT-MSCs, BM-MSCs, adipogenic differentiation, mRNA, adipokines, receptors, *ADIPOQ*, *ADIPOR1*, *ADIPOR2*, *LEP*, *LEPR*

Mesenchymal stem cells (MSCs) represent a population of non-hematopoietic stem cells that display such phenotypic and functional traits as enhanced adherence to plastic substrata, a fibroblast-like appearance, and a relatively high capability to regenerate tissues due to their specific properties of self-renewal and differentiation into bone, adipose, and cartilage cells. MSCs can be isolated from a variety of tissues including bone marrow (BM-MSCs) or adipose tissue (AT-MSCs) (Sobhani et al., 2017). Cell culture engineering with the use of BM-MSCs offers (1) a low concentration of BM-MSCs (0.001% to 0.01%) of the cells in bone marrow concen-

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trate, and (2) high invasiveness of the surgical procedure of recovering red BM and the accompanying isolation of BM-derived biopsies constituting a bioreservoir of MSCs (Brozovich et al., 2021). Therefore, taking the above into account, an alternative source of MSCs (both in terms of the abundance of MSCs and the low invasiveness of the tissue biopsy isolation procedure itself) seems to be AT. Adipose tissue can be harvested using various methods, such as resection, conventional liposuction, and syringe aspiration. The viability of the isolation cells present in the harvested tissue may increase when using specific liposuction methodologies (Meyer et al., 2015). Thus far, MSCs have been used to treat around 90 diseases, including lymphoblastic leukemia, cardiomyopathy, COVID-19, Alzheimer's disease, Parkinson's disease, type 1 and type 2 diabetes, stroke, heart attack, multiple sclerosis, lupus, and cerebral palsy (Kouchakian et al., 2021).

MSCs have emerged as an important model for the study of adipogenesis and adipocyte development. MSCs could be a new preadipocyte reservoir and generate adipocytes through the process of adipogenesis (Burgess et al., 2012). Adipogenesis is driven by a signaling cascade that is regulated by the activity of several key transcription factors. One of the best-known transcription factors is peroxisome proliferator-activated receptor gamma (PPARγ). During the adipogenic differentiation of MSCs, the increased expression of PPARγ promotes the expression of adipogenic markers such as adipokines, whose the most recognizable representative is adiponectin (Ishikane et al., 2021). PPARγ agonists have been shown to upregulate plasma adiponectin levels and increase adipose mass via the production of small adipocytes (Burgess et al., 2012).

Adipokines are a group of cytokines synthesized and secreted by adipocytes of white adipose tissue. They are paracrine and endocrine factors that regulate energy expenditure, metabolic homeostasis, inflammatory and immune responses, cardiovascular function, and many other physiological processes (Pham et al., 2023). The relatively well-investigated adipokines are adiponectin, leptin, resistin, chemotactic protein 1 (MCP-1), tumor necrosis factor-α (TNF-α), interleukin 6 (IL-6), interleukin 1β (IL-1β), interleukin 10 (IL-10), and transforming growth factor-β (TGF-β) (Farkhondeh et al. 2020). One of the most important functional properties of adipocytes is their endocrine activity, reflected by the increased capacity to produce two key adipokines (leptin and adiponectin) under the conditions of induced adipogenic differentiation (Uckan et al., 2009).

Leptin is a 16-kDa polypeptide and is the first adipokine that has been discovered so far. Leptin is produced by adipocytes and regulates food intake and energy homeostasis by acting on leptin receptor (*LEPR*)-expressing neurons in the hypothalamus (Yue et al., 2016). Although leptin is primarily secreted by adipocytes, MSCs are also known to express it (Pham et al., 2023). Leptin has been suggested to affect the processes of osteogenesis and adipogenesis by acting within the bone marrow to regulate the differentiation of mesenchymal stromal cells (Yue et al., 2016). The reciprocal negative impacts of adipogenesis on osteogenesis have been extensively recognized, but the underlying molecular mechanisms are complex (Yue et al., 2016). Furthermore, leptin supports the osteogenesis of MSCs isolated from bone marrow. Leptin-overexpressing BM-MSCs have an increased ability to differentiate into osteoblasts compared to the wild-type cells. MSCs modified through this pathway may be used as a promising strategy to improve the therapeutic efficacy of MSCs in female patients afflicted with osteoporosis (Pham et al., 2023). Unless leptin promotes the osteogenic differentiation of MSCs under the pathological conditions of obesity, the long-term culture of MSCs at high concentrations of leptin leads to impaired osteoblast differentiation (Pham et al., 2023).

Leptin receptor (*LEPR*) is a bone-marrow-derived MSC marker and *LEPR*-positive MSCs have been shown to differentiate into osteoblasts/osteocytes, chondroblasts/chondrocytes, and adipocytes under *in vitro* culture and *in vivo* transplantation. Studies have revealed that leptin can act directly on *LEPR*-expressing MSCs in adult bone marrow to influence their lineage allocation *in vivo* by inhibiting bone formation and inducing marrow adipogenesis (Zhang et al., 2020). Fate mapping demonstrated that *LEPR*-positive cells are generated after birth and give rise to the formation of most of the bones and adipocytes present in adult bone marrow, including bone regenerated after fracture. *LEPR*-positive cells are not active, but become active after injury; therefore, these cells are the main source of bone and adipocytes in adult bone marrow (Zhou et al., 2014). *LEPR* mediates diet-induced fat accumulation in the bone marrow and inhibits osteogenesis (Yue et al., 2016). Because *LEPR*s promote adipogenic differentiation and inhibit the osteogenic differentiation of BM-MSCs, treatment with peripheral *LEPR* antagonists could be a potential strategy to treat osteoporosis (Yue et al., 2016). The paracrine action of leptin released from BM-derived adipocytes on *LEPR*-positive MSCs could possibly be involved in the homeostasis of MSCs and MSC-derived cells (Matsuzaki et al., 2014).

Adiponectin (*ADIPOQ*) is a 28–30 kDa collagen-like protein containing 244 amino acids. It is a hormone secreted exclusively by adipocytes. Adiponectin activates the adenosine-monophosphate-activated protein kinase (AMPK)-dependent signaling pathway. Adiponectin inhibits the apoptosis of MSCs induced by ischemic conditions and promotes the survival of MSCs *in vitro* via the AMPK pathway. AMPK is involved in regulating the cellular energy status by modulating the phosphorylation state of its substrates. Adiponectin stimulates biogenesis and exosome release by binding to T-cadherin during MSC-based heart failure therapy in mice (Nakamura et al., 2020). *ADIPOQ* has emerged as a potential biomarker for predicting various diseases. Low levels of plasma-derived adiponectin are correlated with various metabolic (obesity, insulin resistance, and type 2 diabetes mellitus) and (cardio)vascular diseases. Supplementation

with exogenous adiponectin and/or the use of chemical compounds that promote the secretion of adiponectin alleviate these diseases (Hossain et al., 2017). Interestingly, adiponectin can protect MSCs from serum deprivation or hypoxia-induced apoptosis. Similarly, adiponectin enhances the viability of MSCs under flow shear stress that simulates a stressful condition encountered during *in vivo* transplantation. Current evidence also suggests that the activation of AMPK, a key regulator of cellular responses to stress, critically contributes to the modulation of MSC fate by this adipokine. Based on these interesting findings, it may be predicted that recipients with low levels of adiponectin will receive fewer benefits from MSC-mediated therapy (Pham et al., 2023). Similar to leptin, adiponectin signaling has also been extensively linked to the potential for the osteocyte-/adipocyte-specific differentiation of MSCs. Adiponectin plays an important role in maintaining fat and bone balance by inhibiting adipogenesis or promoting osteogenesis. The overexpression of adiponectin increases osteogenic differentiation in MSCs (Pham et al., 2023).

Adiponectin has three receptors: two with enzymatic activity, adiponectin receptor 1 (*ADIPOR1*) and adiponectin receptor 2 (*ADIPOR2*); and one that binds adiponectin, but has no intracellular signaling domain, T-cadherin. *ADIPOR1* and *ADIPOR2* have constitutive ceramidase activity, which increases upon the binding of adiponectin. *ADIPOR1*/R2 deficiency causes increased insulin resistance and glucose intolerance compared to adiponectin deficiency alone. This is probably due to the fact that the receptors themselves are active even in the absence of adiponectin (Meacham et al., 2022). This may also denote that a level of *ADIPOR1*/R2 may be required to regulate MSC functions that do not necessarily require adiponectin. Despite the high structural homology, there are considerable differences in the distribution and function of these two receptors. In particular, *ADIPOR1* is expressed ubiquitously and at higher levels in skeletal muscle, whereas the expression of *ADIPOR2* is mostly restricted to the liver. With regard to molecular actions, the adiponectin-dependent stimulation of *ADIPOR1* and *ADIPOR2* results in the activation of AMPK and PPARs, respectively, which together regulate inflammatory responses, as well as glucose and lipid metabolism. As mentioned earlier, the overexpression of adiponectin triggers the differentiation of MSCs into osteoblasts. This effect appears to be mediated by *ADIPOR1*, because *ADIPOR1*-deficient MSCs do not respond to osteogenic stimulation by adiponectin. Mechanistically, adiponectin/*ADIPOR1* signaling modulates osteoblast/adipocyte-specific differentiation through various signaling mechanisms, including the activation of APPL1/AMPK, ERK, and GSK-3β/β-catenin. Moreover, adiponectin also exhibits beneficial effects on the processes of bone tissue regeneration by regulating the mobilization and recruitment of BM-MSC to injury sites via the modulation of chemotaxis-related genes, including CXCL-1, CXCL-8, and SDF-1 (Pham et al., 2023).

Taking into account all of the above-indicated molecular interrelations between adipokines and their receptors or selective receptor agonists/antagonists, the current investigation sought to comprehensively determine the transcriptomic profiles pinpointed for adiponectin and leptin and their receptors in porcine adipocytes differentiated from MSCs, depending on their different histological provenance (bone marrow or adipose tissue). Moreover, it is noteworthy to highlight that no research has been carried out so far to recognize and thoroughly explore the inter-transcriptomic crosstalk between adiponectins and their receptors in the *ex-vivo*-expanded non-differentiated subpopulations of BM- and AT-derived MSCs and their adipogenic derivatives. Therefore, this study is a brand new research direction focused on tracking the molecular hallmarks between mRNA transcripts coding for adipokines and their receptors in *in vitro* MSC-based models. The latter have been elaborated and optimized in our laboratory for the first time to unravel the formerly mentioned transcriptional interplay, referring to the intracellular mechanisms, by which the various origins of MSCs (BM- or AT-specific) impact the *ADIPOQ*-, *LEP*-, *ADIPOR*-, and *LEPR*-mediated transcriptomic landscapes. Deciphering the molecular interplay taking place in the *ex situ*-expanded BM- and AT-derived MSCs and their adipogenically reprogrammed cell derivatives can also be highly reliable and feasible for future efforts to generate cryopreserved cell bioreservoirs. The latter create the possibility of genetically protecting critically threatened indigenous breeds of domestic pig (*Sus scrofa domesticus*) by using modern assisted reproductive technologies (ARTs) such as cloning via intra- and/or cross-breed somatic cell nuclear transfer (SCNT).

Material and methods

Recovery of bone marrow and subcutaneous adipose tissue explants and the subsequent establishment of mitotically stable MSC lines

BM and AT samples were collected post mortem from a total of four 6-month-old gilts, including two specimens representing the Polish Landrace (PL) breed and two specimens representing Duroc×PL interbred hybrids. It is worth noting that both BM- and AT-derived bioptates were collected from the same female individuals. The BM-derived MSCs were isolated according to the protocol reported by Opiela et al. (2013). In turn, AT-derived MSCs were retrieved according to the protocol of Garcia et al. (2022) with slight modifications. Briefly, following the inactivation of the collagenase-mediated enzymatic reaction and two-step centrifugation of cell pellets, the cells were gradient-centrifuged at 400 × g for 20 min over a layer of Ficoll-Paque Plus (Stem Cell Technologies; SCT, Canada). Interphase cell layers were subsequently plated onto a T-75 tissue culture flask. The cell suspensions were cultured in low-glucose-formulated DMEM supplemented with 10% fetal bovine serum

(FBS). After the single MSC colonies had formed, cell cultures were carried out to reach 80% confluence.

Immunocytochemical verification of MSC provenance through the detection of adequate CD-specific surface antigens

To immunophenotypically recognize the stemness of the established MSC lines, the presence of plasma membrane markers was identified using the flow-cytometry-based method. Either to quantitatively confirm or to exclude the expression of highly specific surface antigens in the subpopulations of undifferentiated MSCs, flow cytometric analysis was accomplished using a wide array of monoclonal antibodies against clusters of differentiation: CD105 (Biorbyt, Cambridge, UK; positive expression), CD44 (Thermo Fisher Scientific, Waltham, MA, USA; positive expression), CD90 (Thermo Fisher Scientific, Waltham, MA, USA; positive expression), macrophage marker CD14 (Thermo Fisher Scientific, Waltham, MA, USA; negative expression), and hematopoietic stem cell marker CD45 (Thermo Fisher Scientific, Waltham, MA, USA; negative expression).

The MSC samples were rinsed with $CaCl_2$- and $MgCl_2$-free Dulbecco's modified phosphate-buffered saline (DPBS; Sigma-Aldrich, Merck, Poznań, Poland) and detached from the monolayer with 0.25% trypsin–ethylenediamine tetra-acetic acid (trypsin/EDTA) (Sigma-Aldrich, Merck, Poznań, Poland). The dissociated cells were centrifuged and washed with DPBS supplemented with 2% BSA, and incubated for 30 minutes at 24ºC in the dark with fluorescein isothiocyanate (FITC)-conjugated antigen-specific antibodies that were diluted to the adequate concentrations according to the manufacturer's protocols. Considering these protocols, the volume ratios noticed for anti-CD14 antibody (0.1 mg/mL; # MA1-82074, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), anti-CD44 antibody (# MA1-10228; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), anti-CD45 antibody (0.1 mg/mL; # MA5-28383, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), anti-CD90 antibody (# MA1-10228, Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and anti-CD105 antibody (orb44186; Biorbyt, Cambridge, UK) were as follows: 10 µL per 1×10^6 cells, 20 µL per 1×10^6 cells, 10 µL per 1×10^6 cells, 4 µL per 1×10^6 cells, and 20 µL per 1×10^6 cells, respectively. Afterwards, the cells were washed thrice in DPBS and resuspended in 500 mL DPBS. All of the fluorescence signals of the antibody-tagged MSC samples were measured using a CytoFlex flow cytometer (Beckman Coulter, California, USA). An excitation wavelength at 488 nm was supplied by an argon laser at 15 mW. An emission wavelength at the green fluorescence spectrum ranging from 480 to 530 nm was quantified in the FHL1 channel.

Positive and negative MSC populations were determined in relation to the unstained sample. The images of the stained and unstained samples were superimposed on a dot plot (FITC-derived green fluorescence; side scatter – SSC), and then the cell pool occupied by the unstained sample was subtracted, resulting in cytometrically selecting the positive MSC population. The cells were acquired using forward scatter and side scatter and gated to exclude debris. The analysis was performed using CytExpert 2.4 software (Beckman Coulter), where population gates were defined (%Gated) and mean fluorescence intensity values were generated by the software based on the fluorescence intensity of the gated cell populations.

Prompting the multi-lineage differentiation pathways in the *ex-vivo*-expanded MSCs

To confirm the stemness- and multipotency-related attributes of BM- and AT-derived MSCs, their *ex vivo* capabilities to osteogenically, chondrogenically, and adipogenically differentiate were evaluated.

Assessment of differentiability of MSCs towards osteoblasts/osteocytes

After the stable MSC lines had been established, the multipotent properties of their random representatives were verified by the induction of cytodifferentiation pathways into osteoblasts/osteocytes. The osteogenic differentiation took place in low-glucose-formulated DMEM (Sigma-Aldrich, Merck, Poznań, Poland) enriched with 15% FBS (Sigma-Aldrich, Merck, Poznań, Poland), 1× Glutamax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 1% antibiotic–antimycotic solution (AAS; Sigma-Aldrich, Merck, Poznań, Poland), and the cocktail of supplements triggering cytodifferentiation as elaborated by Rentsch et al. (2010). This cocktail was composed of 10 nM dexamethasone (Sigma-Aldrich, Merck, Poznań, Poland), 20 mM β-glycerophosphate (Sigma-Aldrich, Merck, Poznań, Poland), and 50 µM *L*-ascorbic acid 2-phosphate (Sigma-Aldrich, Merck, Poznań, Poland). The cells were cultured under osteogenic-differentiation-specific conditions for 21 days and the replenishment of the medium was performed every 2 days. After the osteogenic differentiation of the MSCs had been completed, the osteoblasts/osteocytes, which were generated from functionally converted MSCs, were specifically dyed with Alizarin Red S.

Assessment of differentiability of MSCs towards chondroblasts/chondrocytes

After the stable MSC lines had been propagated and multiplied, the multipotency-related attributes of their random representatives were identified by the ectopic onset of chondrogenic differentiability. The processes of the functional reprogramming of MSCs into chondroblasts/chondrocytes took place in low-glucose-formulated DMEM enriched with 15% FBS (Sigma-Aldrich, Merck, Poznań, Poland), 1× Glutamax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 1% antibiotic–antimycotic solution (AAS; Sigma-Aldrich, Merck, Poznań, Poland), and the cocktail of supplements triggering cytodifferentiation as elaborated by Branly et al. (2017). This cocktail comprised 100 nM dexametha-

sone (Sigma-Aldrich, Merck, Poznań, Poland), 50 μg/mL *L*-ascorbic acid 2-phosphate (Sigma-Aldrich, Merck, Poznań, Poland), 40 μg/mL *L*-proline (Sigma-Aldrich, Merck, Poznań, Poland), 1 nM sodium pyruvate (Sigma-Aldrich, Merck, Poznań, Poland), 1% insulin–transferrin–sodium selenite (ITS) solution (Sigma-Aldrich, Merck, Poznań, Poland), and 10 ng/mL transforming growth factor-β1 (TGF-β1; Sigma-Aldrich, Merck, Poznań, Poland). The cells were cultured under chondrogenic-differentiation-specific conditions for 21 days and the replenishment of the medium was performed every 2 days. After the chondrogenic differentiation of the MSCs had ceased, the chondroblasts/chondrocytes, which originated from functionally reprogrammed MSCs, were specifically tagged with Alcian Blue.

Assessment of differentiability of MSCs towards pre-adipocytes/adipocytes

After the mitotically stable MSC lines had been generated and *ex-vivo*-expanded, the multipotency-specific cytophysiological traits of their random representatives were recognized by extrinsically prompting the capabilities of MSCs to differentiate into pre-adipocytes and adipocytes. The stem cells were subjected to adipogenic differentiation in low-glucose-formulated DMEM enriched with 15% FBS (Sigma-Aldrich, Merck, Poznań, Poland), 1× Glutamax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), 1% antibiotic–antimycotic solution (AAS; Sigma-Aldrich, Merck, Poznań, Poland), and the cocktail of supplements triggering cytodifferentiation as elaborated by Gawrońska-Kozak (2014). The procedure of the adipogenic differentiation of MSCs was two-step and encompassed their culture in the first adipogenic induction medium to initiate, progress, and finalize the functional reprogramming of MSCs into pre-adipocytes. This medium comprised 100 mL DMEM/F12 (Sigma-Aldrich, Merck, Poznań, Poland) supplemented with a highly specific cocktail composed of 10% FBS, 1.7 mM insulin (Sigma-Aldrich, Merck, Poznań, Poland), 1.0 mM dexamethasone (Sigma-Aldrich, Merck, Poznań, Poland), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, Merck, Poznań, Poland). In turn, the second adipogenic induction medium, which was intended for the functional reprogramming of the MSC-derived pre-adipocytes into terminally differentiated adipocytes, comprised 100 mL DMEM/F12 supplemented with a highly specific cocktail composed of 10% FBS, 17 nM insulin (Sigma-Aldrich, Merck, Poznań, Poland), and 2 mM 2,4-thiazolidinedione (TZD; Sigma-Aldrich, Merck, Poznań, Poland). After reaching 100% confluency, the MSCs were cultured for 2 to 3 days in the first adipogenic induction medium followed by sequential cytodifferentiation by the exposure of the cells to the second adipogenic induction medium for a further 7 days. The second adipogenic induction medium was replenished every 2 days. After the termination of two-step adipogenic differentiation, the functionally reprogrammed cells were stained with Oil Red O.

Qualitative identification of differentiated status of MSC derivatives by highly specific staining followed by light microscopy

A variety of pathways of differentiation, which had been triggered using ectopic inducers of osteo-, chondro-, and adipogenic reprogramming, were cytochemically confirmed by applying a triad of specific dyes (Alizarin Red S, Oil Red O, and Alcian Blue). Prior to staining, all three types of differentiated MSCs and the negative control MSCs were fixed in 4% paraformaldehyde (PFA; Sigma-Aldrich, Merck, Poznań, Poland) for 1 h and then washed in DPBS.

The differentiation of MSCs towards osteoblasts/osteocytes was positively verified by tagging the fixed cells with 2% Alizarin Red S (Sigma-Aldrich, Merck, Poznań, Poland) for 30 min. The overabundance of dye molecules that had not undergone incorporation into bone cells was rinsed off with the aid of distilled water. The osteogenic provenance and characterization of the partially and terminally differentiated cell derivatives of MSCs were recognized by the detection of intracellular Alizarin-Red-S-mediated conjugates, which display a high affinity to various forms of inorganic and organic calcium salts (Sottile et al., 2002).

The terminal differentiation of MSCs into adipocytes was positively identified by labeling the fixed cells with Oil Red O solution. In the first step, the fixed cells were washed with 60% isopropyl alcohol (Sigma-Aldrich, Merck, Poznań, Poland) and then exposed to 0.5% Oil Red O (Sigma-Aldrich, Merck, Poznań, Poland) for 1 h. The surplus of dye molecules that had not been incorporated into fat cells was rinsed again with 60% isopropyl alcohol and, finally, with distilled water. The adipogenic origin and phenotypic landscape of the terminally differentiated cell derivatives of the MSCs were proven by the presence of red-tinted lipid droplets in a cytoplasm of fat cells (Sottile et al., 2002; Rentsch et al., 2010).

To perform the cytochemical analysis that revealed the structuro-functional attributes of the positive differentiation of MSCs into chondroblasts/chondrocytes, the fixed subpopulations of presumptive MSC-derived cartilage cells were incubated for 30 min in the presence of a 1% benzoporphyrin congener designated as Alcian Blue (copper complex compound of phthalocyanine; Sigma-Aldrich, Merck, Poznań, Poland) diluted in 3% acetic acid (Sigma-Aldrich, Merck, Poznań, Poland). The diagnosed cells were subsequently rinsed with an electrolytic solution of 0.1 N hydrochloric acid (Sigma-Aldrich, Merck, Poznań, Poland) and evaluated under a light microscope. The chondrogenic provenance and cartilage-related cytological traits of the terminally differentiated cell derivatives of MSCs were demonstrated by the identification of Alcian-Blue-conjugated intra- and intercellular negatively charged clusters of acidic mucopolysaccharides, proteoglycans, and neutral, sulfonated, and phosphorylated glycosaminoglycans (GAGs) combined with copper-coordinated cationic dye molecules, whose occurrence is highly specific for cytosolic compartments,

organelles, cytoskeletons, and membrane skeletons, as well as an extracellular matrix of chondroblasts/chondrocytes (Rentsch et al., 2010).

RNA isolation, cDNA synthesis, and real-time PCR (q-PCR)

Approximately 1 × 10^6 BM-derived or AT-derived MSCs were collected before and after adipogenic differentiation. Briefly, after reaching 90% confluence, the cells were harvested using the trypsin/EDTA treatment methods. The cells were subsequently washed 4 times with DPBS and centrifuged to eliminate any residual trypsin from the cell suspensions. Afterwards, the cells were transferred into 1.5 mL tubes followed by centrifugation, resulting in the removal of the debris of supernatants, and then frozen in liquid nitrogen and maintained at –80°C until RNA isolation. To determine the mRNA levels of adiponectin and leptin and its receptors, the TaqMan Gene Expression Cells-to-CT Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for RNA isolation and cDNA synthesis following the manufacturer's protocols. The quantities of RNA and cDNA were determined by measuring the absorbance at 260 nm and 280 nm via spectrophotometry. Subsequently, TaqMan Gene Expression Master Mix and specific primers (Thermo Fisher Scientific, Waltham, MA, USA) for adiponectin (ADIPOQ, no. Ss03384375_u1), ADIPOR1 (no. Ss03378803_u1), ADIPOR2 (no. Ss03391825_g1), leptin (LEP, no. Ss03392404_m1), and LEPR (no. Ss03379257_u1) were used and amplifications were performed following the manufacturer's instructions and as we previously described (Rak et al., 2017) using the StepOnePlus system (Applied Biosystems, Carlsbad, CA, USA). As a reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used (Kurowska et al., 2020). Briefly, 100 ng cDNA, 1 µL TaqMan Gene Expression primers, and 10 µL TaqMan PCR Master Mix (Applied Biosystems, Waltham, MA, USA) were used in the reaction. The thermal cycling conditions to determine the cycle threshold number (Ct) for quantitative measurement were as follows: 2 min at 50°C; 10 min at 95°C; and 40 cycles of 15 s at 95°C and 1 min at 60°C. The relative mRNA expression levels of the studied genes were determined using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittegen, 2001). To minimize the error associated with the differences in the quantity of applied template, the analyses were run in quadruplicate (four biological replicates and three technical replicates within each biological replicate), and the results were averaged.

Statistical analysis

The occurrence of inter-group variability in the quantitative levels of expression pinpointed for the selected mRNA transcripts encoding adipokines and their receptors between undifferentiated BM- and AT-derived MSCs and their functionally reprogrammed adipogenic cell derivatives was assessed using one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test (GraphPad Prism version 8). Additionally, a statistical analysis was performed using the unpaired Student's *t* test (GraphPad Prism version 8) between the differentiated and undifferentiated cell derivatives of BM-MSCs or AT-MSCs. Differences with a probability (p) equal to or less than 0.05 were considered to be statistically significant.

Results

Identification of MSC-specific clusters of differentiation

Flow-cytometry-mediated analyses aimed at immunophenotyping the BM-derived MSCs revealed that approximately 87% of them exhibited the presence of the CD44 surface marker, approximately 96% expressed CD90, approximately 20% expressed CD105, nearly 12% expressed CD14, and approximately 14% expressed CD45 (as thoroughly detailed in Table 1).

In turn, flow-cytometry-based analyses focused on immunophenotyping the AT-derived MSCs revealed that approximately 78% of them displayed the presence of the CD44 antigen, over 99% expressed CD90, almost 68% expressed CD105, more than 20% expressed CD14, and nearly 30% expressed CD45 (as comprehensively specified in Table 1).

A significantly higher percentage of CD105-positive cells was identified in AT-MSCs than in BM-MSCs.

Determination of MSC-specific capabilities to *ex vivo* differentiate via induced pathways of osteo-, chondro-, and adipogenic reprogramming

The cytochemical diagnostics of porcine AT- and BM-derived MSCs have proven that both types of *ex vivo*-expanded stem cells were characterized by a high differentiability into osteoblasts/osteocytes, chondroblasts/chondrocytes, and pre-adipocytes/adipocytes under the conditions prompting their functional reprogramming. The occurrence of terminally differentiated osteogenic cell derivatives was confirmed as a result of the 21-day ectopic reprogramming of MSCs of each provenance (BM and AT), as verified via Alizarin-Red-S-mediated staining (Figure 1 A, B). On Day 21 of the differentiating culture, a comparable (oscillating at about 80%) percentage of osteogenically reprogrammed cell derivatives originating from BM-MSCs and AT-MSCs was identified. As a consequence of the 21-day culture in the medium intended for chondrogenic reprogramming, the multiple subpopulations of chondroblasts/chondrocytes were found to colonize *in vitro* and to multiply in the form of the typical micropellets of compacted ellipsoidal (disk-shaped) cells exhibiting a multi-layered, extracellular-matrix-rich morphology, which is highly specific for cartilage cells. Due to the Alcian-Blue-based dyeing of chondrogenic cell derivatives of MSCs, the intensified biosynthesis, externalization, and bioaccumulation of extracellular-matrix-related mucopolysaccharides, pro-

teoglycans, and neutral, sulfonated, and phosphorylated GAGs were strongly and cumulatively recognized (Figure 1 C, D). A total of 90% chondrogenically converted BM- and AT-derived MSCs was observed on Day 21 of the differentiating culture. Following a total of 9 to 10 days of induced adipogenic differentiation of each type of MSC, a high incidence of terminally reprogrammed adipocytes was shown on the basis of Oil-Red-O-mediated tagging (Figure 1 E, F). After 10 days of culture in the differentiation medium, the percentage of adipogenically reprogrammed cells ranged from 80% for BM-MSCs to 85% for AT-MSCs.

Table 1. Structure of quantitative distribution in the immunophenotypic profiles of expression noticed for CD14, CD44, CD45, CD90, and CD105 surface antigens estimated by cytometrically assisted immunofluorescent staining of porcine BM-MSCs and AT-MSCs

	CD14 (%)	CD44 (%)	CD45 (%)	CD90 (%)	CD105 (%)
BM-MSCs	11.96±3.38	86.68±3.37	14.19±4.54	96.31±1.56	20.18±6.82 a
AT-MSCs	20.47±0.8	77.59±5.17	29.91±3.21	99.40±0.17	67.71±1.79 b

BM-MSCs: bone-marrow-derived MSCs, AT-MSCs: adipose-tissue-derived MSCs. Quantitative distribution in the immunophenotypic profiles presented as a percentage (%) of positive cells ± standard error of the mean (SEM). The positive expression of surface markers (clusters of differentiation) depicted in the form of gray boxes. Various small letters (a or b) indicate statistically significant differences ($P \leq 0.05$; unpaired Student's *t* test). Number of biological replicates = 3. Number of technical replicates = 3 (within each biological replicate).

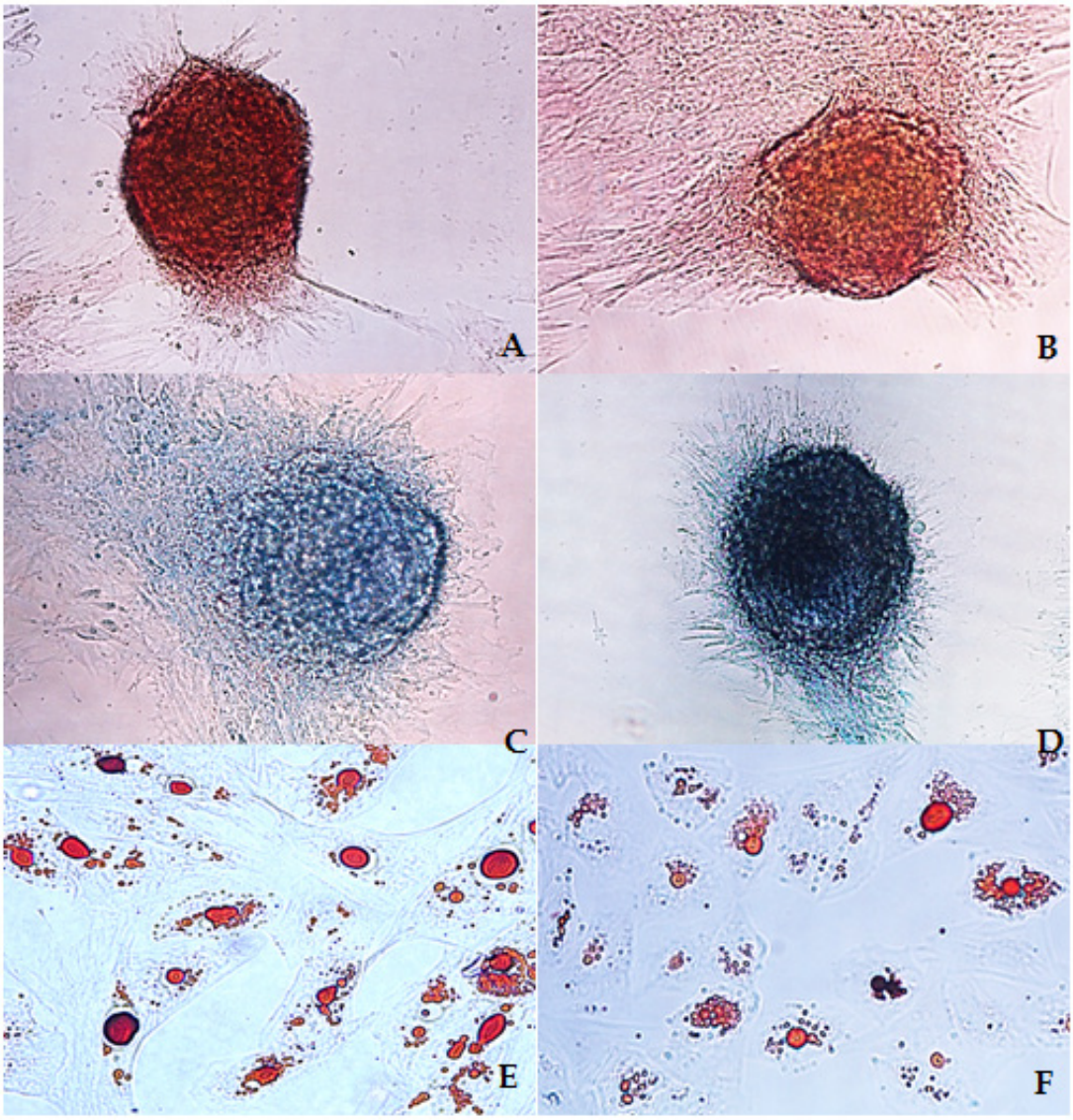

Figure 1. Differentiation of adipose-tissue (AT)- and bone-marrow (BM)-derived mesenchymal stem cells (MSCs) into osteoblasts/osteocytes (confirmation of osteogenic reprogramming by Alizarin-Red-S-mediated staining; A: AT-MSCs, B: BM-MSCs); chondroblasts/chondrocytes (confirmation of chondrogenic reprogramming by Alcian-Blue-mediated staining; C: AT-MSCs, D: BM-MSCs); and adipocytes (confirmation of adipogenic reprogramming by Oil-Red-O-mediated staining; E: AT-MSCs, F: BM-MSCs). Images were taken at a magnification of 200×

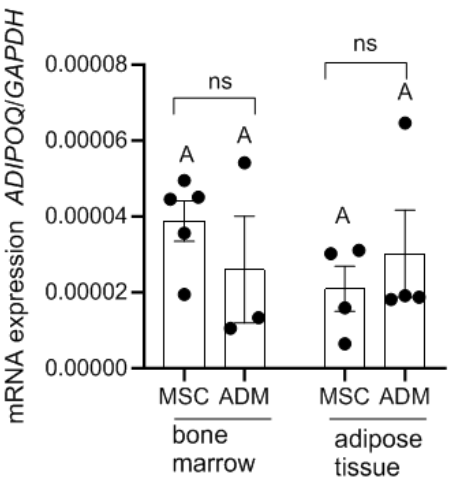

Figure 2. Relative mRNA expression profiles (mean ± SEM) for adiponectin (*ADIPOQ*) gene in bone-marrow-derived MSCs, adipose-tissue-derived MSCs, adipocytes originating from differentiated bone-marrow-derived MSCs (ADM), and adipocytes originating from differentiated adipose-tissue-derived MSCs (ADM). Different capital letters (A or B) indicate statistically significant differences (P≤0.05); one-way ANOVA followed by Tukey's *post hoc* test and unpaired Student's *t* test. Ns: no significant differences

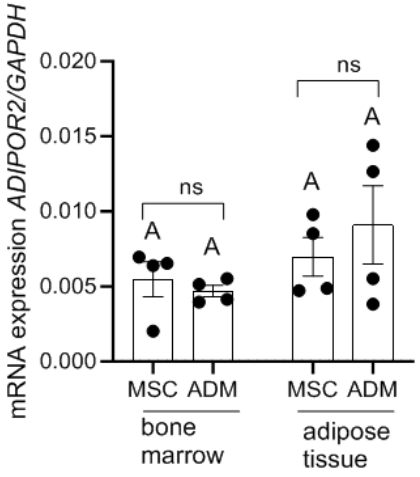

Figure 4. Relative mRNA expression profiles (mean ± SEM) for adiponectin receptor 2 (*ADIPR2*) gene in bone-marrow-derived MSCs, adipose-tissue-derived MSCs, adipocytes originating from differentiated bone-marrow-derived MSCs (ADM), and adipocytes originating from differentiated adipose-tissue-derived MSCs (ADM). Different capital letters (A or B) indicate statistically significant differences (P≤0.05); one-way ANOVA followed by Tukey's *post hoc* test and unpaired Student's *t* test. Ns: no significant differences

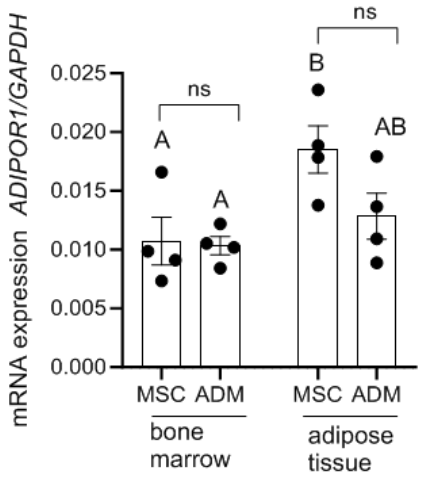

Figure 3. Relative mRNA expression profiles (mean ± SEM) for adiponectin receptor 1 (*ADIPOR1*) gene in bone-marrow-derived MSCs, adipose-tissue-derived MSCs, adipocytes originating from differentiated bone-marrow-derived MSCs (ADM), and adipocytes originating from differentiated adipose-tissue-derived MSCs (ADM). Different capital letters (A or B) indicate statistically significant differences (P≤0.05); one-way ANOVA followed by Tukey's *post hoc* test and unpaired Student's *t* test. Ns: no significant differences

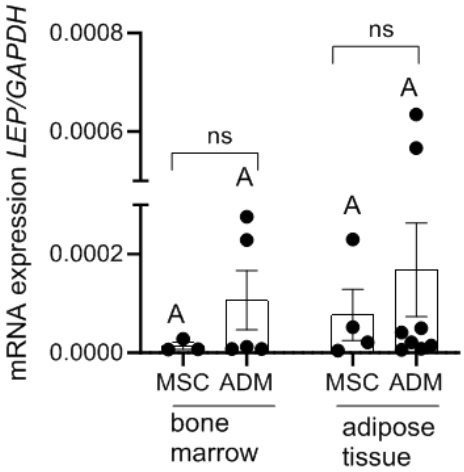

Figure 5. Relative mRNA expression profiles (mean ± SEM) for leptin (*LEP*) gene in bone-marrow-derived MSCs, adipose-tissue-derived MSCs, adipocytes originating from differentiated bone-marrow-derived MSCs (ADM), and adipocytes originating from differentiated adipose-tissue-derived MSCs (ADM). Different capital letters (A or B) indicate statistically significant differences (P≤0.05); one-way ANOVA followed by Tukey's *post hoc* test and unpaired Student's *t* test. Ns: no significant differences

Total quantitative profiles of expression for DNA transcripts encoding adipokines and their receptors in undifferentiated AT- and BM-derived MSCs and their counterparts undergoing adipogenic differentiation

The statistical variabilities in the levels of transcripts for *ADIPOQ*, *ADIPOR1*, *ADIPOR2*, *LEP*, and *LEPR* were estimated between the undifferentiated and differentiated AT-MSCs and BM-MSCs and are presented in Figures 2 to 5.

Additionally, a significantly higher RA for *ADIPOR1* transcripts was identified in the AT-MSCs compared to the BM-MSCs and their adipogenic derivatives at the quantitative level of 0.01852±0.00201 compared to the levels of 0.01073±0.00202, 0.01034±0.00077, and 0.01285±0.00195 in the BM-MSC, BM-MSC/ADM, and AT-MSC/ADM groups, respectively (Figure 3; P≤0.05). Moreover, the RAs of *LEPR* mRNA were shown to increase significantly among AT-MSCs and

AT-MSC-derived adipocytes at the quantitative level of 0.001366±0.000242 compared to 0.000247±0.000210, 0.000012±0.000003, and 0.000367±0.000176, respectively (Figure 6; P≤0.05). The results of the analyses performed using the unpaired Student's *t* test also confirmed a statistically significant increase in the *LEPR* mRNA expression of adipocytes originating from the differentiated BM-MSCs compared to the undifferentiated BM-MSCs (Figure 6; P≤0.05). Nonetheless, there was no significant inter-group variability in the RAs of the *ADIPOQ* (Figure 2), *ADIPOR2* (Figure 4), or *LEP* (Figure 5) mRNA transcripts (P≤0.05).

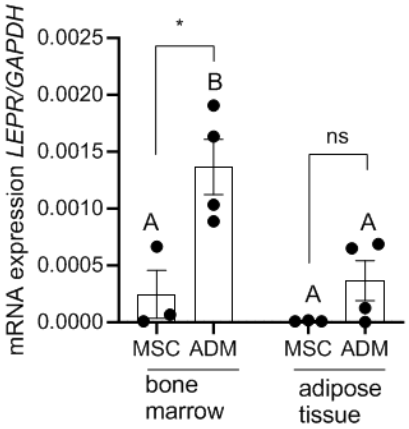

Figure 6. Relative mRNA expression profiles (mean ± SEM) for leptin receptor (*LEPR*) gene in bone-marrow-derived MSCs, adipose-tissue-derived MSCs, adipocytes originating from differentiated bone-marrow-derived MSCs (ADM), and adipocytes originating from differentiated adipose-tissue-derived MSCs (ADM). Different capital letters (A or B) indicate statistically significant differences (P≤0.05); one-way ANOVA followed by Tukey's *post hoc* test and unpaired Student's *t* test. Ns: no significant differences

Discussion

The results of the present study clearly confirmed that the histological provenance of MSCs and their adipogenic derivatives affects the quantitative levels noticed for the transcriptional activity of the selected adipokines (adiponectin and leptin) and their receptors.

The reasons for the differences in the differentiability of myeloid and adipogenic MSCs are not fully recognized, yet frequently arise from the occurrence of inter-specimen physiological variation. Therefore, the RT-qPCR-mediated determination of the relative abundances noticed for the selected adipokines and their receptors was accomplished by simultaneously using explants recovered from two sources (BM and AT) of autologous origin. Put differently, the individual donors of BM- and AT-derived bioptates were represented by the same female specimens (gilts). This denotes that the specimen-dependent impacts of diversification resulting from dissimilar cytohistological and anatomo-topographical provenance were minimized as much as possible.

The expression profile of surface markers of myeloid and adipogenic MSCs determines a broad spectrum of immunophenotypic attributes related to molecular background and cytobiochemical landscape, but it is not identical in both subpopulations of these stem cells. Adipogenic MSCs display the expression of the plasma-membrane-specific antigen CD49d and the simultaneous lack of the expression of the CD106 antigen, while myeloid MSCs are characterized by the expression of the surface biomarker CD106 and the lack of the expression of the CD49d biomarker (Ranera et al., 2011). In our present investigation, the positive expression profile of mesenchymal stemness-related surface markers was similar in BM- and AT-derived MSCs; however, the cells originating from AT exhibited a higher quantitative level of CD90 (oscillating at nearly 100%), but a lower level of CD44 expression. Taking into consideration the negative immunophenotypic background for CD antigens, the stem cells recovered from BM were characterized by remarkably diminished quantitative levels of cytobiochemical signatures. It is widely known that considerable variability in the extent of CD-mediated immunophenotypic profiles between the MSCs collected from different species and histological sources occurs. The surface marker CD105, which is considered to be the positive marker correlated with mesenchymal stemness in humans, is not always expressed in other mammalian species. The results of the study by Paebst et al. (2014) proved that the CD105-based expression profile in equine AT-MSCs reaches the level of approximately 40%, whereas the incidence of this cytobiochemical trait has not been shown in BM-MSCs. Similar results have been confirmed by research focused on rabbit and ovine AT-MSCs (Martinez-Lorenzo et al., 2009) and ovine BM-MSCs (Adamzyk et al., 2013). On the other hand, the absence of such CD-related expression and wide divergence in the immunophenotypic profiling may be due to the lack or negligible reactivity and affinity of the used antibodies to the clusters of differentiation antigens undergoing the expression in the plasma membranes of porcine MSCs.

BM-MSCs serve as the source of stem cells that seems to be most frequently used for the therapeutic treatment of spinal cord injury (SCI), with more than 20 clinical trials registered on the ClinicalTrials.gov database. The retrieval of MSCs from AT, due to the low invasiveness of the procedures utilized for their *in vivo* recovery and a more abundant population of adult stem cells compared to BM, appears to be an ideal alternative (Liau et al., 2020). The results of the research by Hur et al. (2016) confirmed a desirable impact of the intrathecal transplantation of autologous AT-MSCs on the regenerative and reconstructive anatomo-histological parameters of clinical therapeutic modalities in a total of 14 patients afflicted with SCI. These modalities resulted in the relatively strong improvements in neurological function, totally without the incidence of the enlargement of the anatomo-topographical extent of the lesion. However, no clinical studies have yet compared the regenerative and reconstructive susceptibility of MSCs derived from dif-

ferent tissue sources. Thus far, Takahashi et al. (2018) verified the efficacy of AT-MSCs and BM-MSCs in treating SCI with the aid of a mouse model. The results of their research confirmed that, compared to BM-MSCs, AT-MSCs display a higher *in vitro* resilience and subsequent adaptability to the conditions of micro-environmental deficiencies such as hypoxia, trophic deprivation (i.e., serum depletion), and the attenuation of cytophysiological defense/capacity/protection against oxidative stress, or, in other words, diminishments in the enzymatic or non-enzymatic scavengers of reactive oxygen species (ROS). Moreover, after transplantation into sites of severe SCI, AT-MSCs have exhibited *in vivo* survival rates that are considerably higher than those noticed for BM-MSCs. Moreover, the investigations carried out by Takahashi et al. (2018) demonstrated that the regenerative capacity pinpointed for each type of stem cell is equivalent or comparable (independently of their AT- or BM-specific provenance), particularly taking into account the remarkable amelioration of a broad post-clinical spectrum of physiological, senso-motoric, and kineto-dynamic functions. This suggests that AT-MSCs seem to be a tremendously attractive stem-cell-based therapeutic option for the tissue-engineered treatment of severe SCI due to their enhanced resistance to ROS-induced oxidative stress and stemness-related secretion profile. Such a finding indicates that this variety of cytophysiological properties should be considered when selecting a particular source of MSCs for the purposes of disorder-dependent preclinical or clinical therapeutic modalities or other research applicability in the context of their histologically dependent and stemness-related advantages or disadvantages. Therefore, further comprehensive and comparative analyses of the stem-cell-specific differences between AT-MSCs and BM-MSCs are indispensable. Taking into account the BM- or AT-specific origin and multipotent extent of MSCs, the molecular pathways determining their adipogenic differentiation have been characterized in the most thorough and multi-faceted manner (Kozlowska et al., 2019). Variability in the capabilities to *in vitro* differentiation has been found between myeloid and adipogenic MSCs. As reported by Kozlowska et al. (2019), the proliferative activity of AT-derived MSCs is higher than that for BM-derived MSCs. Moreover, both types of MSCs exhibit the relatively abundant secretome comprising such endocrine-active polypeptides as basic fibroblast growth factor and insulin-like growth factor-1 and immunomodulatory proteins, e.g., interferon-γ. It is also noteworthy that BM-derived MSCs are signified by increased osteogenic and chondrogenic differentiability compared to AT-derived MSCs. Additionally, the results of the study by Li et al. (2015) targeted at the exploration of stemness-related secretomic proteome confirmed that each type of MSC does not fail to secrete stem-cell-derived factor-1 and hepatocyte growth factor. Research conducted by Niemeyer et al. (2010) confirmed that, as a result of the re-implantation of AT-derived MSCs into the medullary cavity of the tibia of domestic sheep, the AT-MSCs exhibited a diminished potential for their cytodifferentiation into bone-forming cells (osteoblasts) and their terminal derivatives (osteocytes). This may be due to the fact that undifferentiated MSCs obtained from adipose tissue may have a higher predisposition to differentiate into terminal cells of fat tissue (adipocytes) (Molnar et al., 2022).

The *ADIPOQ* gene can serve as an excellent marker to distinguish matured adipocytes from other cell types (Straub and Scherer, 2019). In the current investigations, the RAs identified for *ADIPOQ* mRNA transcripts in undifferentiated forms of porcine AT- and BM-derived MSCs have been shown to be comparable, which denotes that the results of our research are analogous to those estimated in the study by Dicker et al. (2005) for human MSCs of both AT- and BM-specific origin. Moreover, due to the *ex vivo* differentiation of MSCs into adipocytes, the enhancements in the transcriptional profiles of the *ADIPOQ* gene were recognized both for adipogenically converted MSCs isolated from BM and their cell counterparts derived from AT. However, the augmented quantitative profiles of *ADIPOQ* mRNAs have been found to persist at higher levels in BM-MSCs, which could primarily indicate that this type of stem cell is characterized by a much more increased capacity to be differentiated towards pre-adipocytes and adipocytes. In our present investigation, the RAs pinpointed for *ADIPOQ* mRNA transcripts were proven to be retained at similar levels in almost all types of non-differentiated MSCs and their adipogenically reprogrammed cell counterparts, irrespective of their histological origin (BM or AT). The quantitative profiles observed for mRNAs transcribed from the *ADIPOQ* gene increased remarkably after the cytodifferentiation of BM-MSCs into adipocytes. The recognized enhancements in the levels of transcriptional activity pinpointed for the *ADIPOQ* gene coincide with the research by Martella et al. (2014) conducted on adipogenically induced human-BM-derived MSCs. Interestingly, intensifying the *in vitro* secretive activity of adipo-induced human BM-MSCs related to the endocrine-active biosynthesis of adiponectin was recognized and comparatively assessed, depending on the ontogenetic variability between the donor specimens of BM, out of which the MSC lines had been previously established. While the presence of secreted adiponectin in the culture media conditioned by the endocrine activity of adipogenically reprogrammed stem cells was detectable after 10 days of the adipogenic differentiation of human BM-MSCs at a median concentration of 5.13 ng/mL, the extent of the stem-cell-mediated secretion of adiponectin varied significantly in a time- and donor-specimen-dependent manner. The degree of adiponectin-related secretive activity usually increased considerably with the progression of adipo-induction (from Day 3 up to Day 10) between a variety of adipogenically cytodifferentiated BM-derived MSC lines derived from a total of eight patient donors (Martella et al., 2014).

Furthermore, the results of our study confirmed that undifferentiated AT-MSCs are characterized by higher expression levels identified for *ADIPOR1* and *ADIPOR2* mRNA transcripts than for BM-MSCs. Moreover, the transcriptional profiles noticed for the previously mentioned receptors did not change considerably during the induced reprogramming of both types of MSCs towards adipocytes. The research by Fasshauer et al. (2004) demonstrated, for the first time, that *ADIPOR1* and *ADIPOR2* genes are transcriptionally active in murine 3T3-L1 preadipocytes and their expression profiles undergo gradual upregulation during the differentiation process of these preadipocytes (Fasshauer et al., 2004). In turn, Bauer et al. (2010) proved that the mRNA expression of both receptors was induced in terminally differentiated forms of mature adipocytes, while the proteomic profiles pinpointed for *ADIPOR1* and *ADIPOR2* receptors were not altered during the adipogenic differentiation of murine 3T3-L1 cells. The assumption of this finding resulting from the investigations carried out by Bauer et al. (2010) is that mRNA expression is not suitable for predicting the proteomic levels of adiponectin receptors. In the study by Rasmussen et al. (2006), *ADIPOR1* mRNA transcripts were found to be highly expressed in human AT, indicating that adiponectin may have biological impacts on fat cells via autocrine and paracrine pathways. The diminishments in the transcriptional activity of the *ADIPOR1* gene took place in the subcutaneous and omental AT of obese patients and, in contrast, the increase in the expression profiles noticed for *ADIPOR1* mRNA transcripts occurred after weight loss (Rasmussen et al., 2006).

The explants of visceral adipose tissue derived from obese mice synthesize and secrete the increased levels of leptin, which are proportional to the fat tissue content. It is well known that the quantitative profiles of leptin mRNA noticed for subcutaneous adipose tissue were maintained at levels shown to be approximately twofold higher than those identified for omental adipose tissue, and this difference has been found consistently in both lean and obese mice (de Assis-Ferreira et al., 2021). Leptin, which is expressed by primary pre-adipocytes and terminally differentiated (i.e., mature) adipocytes, modulates inflammatory responses. The results of the research by Bruno et al. (2021) proved that, in both pre-adipocytes and mature adipocytes, leptin and isoform 1 of transforming growth factor-β (TGF-β1) significantly decreased the expression of a homeodomain transcription factor designated as pre-B-cell leukemia homeobox (Pbx)-regulating protein-1 (PREP1), alone and following concurrent Toll-like receptor 4 (TLR4) activation. Moreover, in mature adipocytes, but not in pre-adipocytes, leptin increased TLR4 and interleukin-33 (IL-33) expression. However, the effects of leptin and TGF-β1 on immune receptors and cytokines are limited to mature adipocytes, suggesting that modulating the immune responses depends on the differentiation status of AT-MSCs (Bruno et al., 2021). Furthermore, leptin has been shown to promote apoptotic cell death in human BM-MSCs via the MEK/ERK/cPLA2/cytochrome c and JAK/STAT1 pathways (Kim et al., 2003). In turn, Laschober et al. (2009) reported that the number of colonies formed by leptin-treated MSCs remained unchanged. The relative abundances for mRNA molecules encoding molecular stemness- and plutipotency-related markers (*DNA-binding transcription factors*) such as Nanog (homeobox-containing transcription factor, whose name stems from the Celtic/Irish mythical word Tír na nÓg, i.e., Tir Na Nog; The Land of the Ever-Young) and Rex1/Zfp-42 (reduced expression gene 1 encoding reduced expression protein 1 or zinc finger protein 42 homolog) have been also found to be unaffected (Laschober et al., 2009). Despite the fact that there was no significant inter-group variability in the RAs of *LEP* mRNA transcripts, a higher concentration of leptin was observed in AT-derived MSCs. This result is analogous to that obtained in the investigation by Dicker et al. (2005), in which the quantitative profiles pinpointed for *LEP* mRNA transcripts in AT-MSCs have been proven to be retained at two-to-fourfold higher levels than those for BM-MSCs. These differences appear to be MSC-source-dependent. It is postulated that the serum leptin concentration is positively correlated with the mass/content of adipose tissue in humans. The augmentation of serum leptin concentration meticulously entails both an increase in the number of adipose cells and the enhancement of the transcriptional activity of genes coding for leptin per single fat cell (Jéquier, 2006). Adequately, Monteiro et al. (2019) demonstrated the elevated expression RAs noticed for leptin transcripts that were triggered after the adipogenic differentiation pathway of AT-derived MSCs had been prompted. However, the RAs noticed for adipogenically reprogrammed AT-MSCs were almost twofold higher than those identified for their undifferentiated stem cell counterparts. As confirmed by Monteiro et al. (2019), leptin may be predestined to serve as a factor of paramount importance not only for the induction of adipogenesis processes in precursor fat cells (pre-adipocytes), but also for the onset and progression of lipolysis in mature adipocytes.

According to the study conducted by Zhou et al. (2014), which was additionally subjected to comprehensive review in the scientific comment paper from Matsuzaki et al. (2014), leptin receptor (*LEPR*) seems to be a pivotal candidate as an especially important marker of undifferentiated BM-MSCs in mice, whereas *LEPR*-positive BM-derived stem cells have been shown to exhibit a relatively high potential to functionally convert into partially or terminally differentiated bone cells (osteoblasts/osteocytes), cartilage cells (chondroblasts/chondrocytes), and fat cells (pre-adipocytes/adipocytes both under *in vitro* culture conditions and upon intracorporeal transplantation). The paracrine impact of leptin released from BM-derived adipocytes on *LEPR*-positive MSCs might be involved in the homeostasis of MSCs and their differentiated cell derivatives (Matsuzaki et al., 2014). In contrast, an investigation by Zhang et al. (2020) demonstrated that exogenous leptin potentiated the BMP9-

prompted osteogenic reprogramming of murine BM-derived MSCs under both *ex vivo* and *in vivo* conditions, simultaneously suppressing BMP9-triggered adipogenic cytodifferentiation. BMP9 has been proven to evoke the enhancements in the transcriptional and translational activities of *LEP* and *LEPR* genes/mRNA transcripts in BM-derived MSCs, whereas ectopic *LEP* contributed to ameliorated BMP9 expression in partially, but not terminally, differentiated BM-MSCs. On the other hand, the competitive inhibition of JAK-based signal transduction strongly alleviated leptin-mediated osteogenic differentiation that had been initiated by BMP9. All of these findings resulting from the study carried out by Zhang et al. (2020) indicate that leptin appears to be a potent stimulatory adipokine, which elicits BMP9-promoted osteogenic cytodifferentiation via the onset and progression of inter-transcriptomic and inter-proteomic crosstalk and molecular hallmarks between BMP9-related and JAK/STAT-based pathways of signal transduction occurring in BM-derived MSCs. These findings also provide new scientific insights into the discovery of the above-mentioned molecular hallmarks. The latter might be extrapolated and implemented to modern biomedical and surgical treatments focused on novel therapeutic modalities. In these modalities, focused on regenerative and reconstructive medicine of the human osteoskeletal system, simultaneous BMP9- and leptin-mediated approaches can play a superior role in promoting not only the attenuation of biological events related to adipogenic reprogramming in BM-MSCs, but also the intensified incidence of their osteogenic cytodifferentiation.

Nonetheless, considering the research of Zhou et al. (2014), the subpopulations abundant in *LEPR*-positive cells may serve as the main source of adipocytes in adult bone marrow. Furthermore, in compliance with the investigation carried out by Zhou et al. (2014), the establishment of *LEPR*-positive-cell-based compartments takes place during the postnatal phase of ontogenesis and gives rise both to the formation of the vast majority of bone types and to the cytodifferentiation into adipocytes present in adult bone marrow. On the other hand, *LEPR*-positive cell subpopulations are maintained in the quiescence status, and following the generation of osteogenic injuries, they undergo rapid enhancements of proliferative activity, resulting in the regeneration of bones subjected to irradiation or fracture (Zhou et al., 2014). In turn, according to the study carried out by Yue et al. (2016), *LEPR* mediates diet-induced fat accumulation in bone marrow and inhibits osteogenesis. Since *LEPR* promotes adipogenic conversion and suppresses osteogenic reprogramming among BM-MSCs, the treatment modalities targeted at the competitive inhibition of peripheral *LEPR* isoforms may be a potential therapeutic strategy in women patients afflicted with osteoporosis (Yue et al., 2016). For all of these reasons, modifying the leptin/*LEPR* expression ratio leads to the up- or downregulation of adipo- and osteogenesis in BM-derived MSCs in response to a high-fat diet and adiposity (Yue et al., 2016). The quantitative levels of *LEPR* transcripts have been shown to increase significantly among adipocytes originating from differentiated BM-MSCs compared to undifferentiated BM-MSCs, AT-MSCs, and AT-MSC-derived adipocytes.

Leptin triggers inflammation-associated programmed cell death in AT-derived MSCs. In turn, according to the review article by Pham et al. (2023), the intensified expression of *LEPR* has been proven to be directly correlated with the number of cell divisions during the long-term culture of MSCs. Finally, although the molecular mechanisms underlying both intra-/inter-cellular hallmarks and inter-transcriptomic/inter-proteomic communication, which is accompanied by the upregulation of a wide array of *LEPR* isoforms, have not been completely recognized, leptin receptors have been increasingly postulated as a biomarker of paramount importance for the multifaceted exploration of the processes of the obesity-induced senescence of cells and decrease in the incidence of cellular aging by the regenerative and reconstructive processes arising from the relatively high adipogenic differentiability of BM- and AT-derived MSCs (Pham et al., 2023).

Interestingly, no study has yet indicated an interaction between any adipokines, including leptin–adiponectin interplay. However, the literature data clearly show that adipokines directly affect receptor expression, which may partly explain the changes in the *ADIPOR1* or *LEPR* levels between the studied cell types. For example, elevated serum leptin decreases the transcriptional activity of *LEPR* gene in mouse liver (Hegyi et al., 2004). Similarly, another member of the adipokine family, designated as vaspin, also contributes to the decrease in the expression of its 78-kDa receptor, termed glucose-regulated protein 78 (GRP78), in porcine ovarian follicles (Kurowska et al., 2020). Spexin acts differently depending on the receptor type, i.e., it leads to either an increase in the expression of galanin receptor 2, or a decrease in the expression of galanin receptor 3 in human granulosa cells (Kurowska et al., 2023).

Conclusions and future targets

To sum up, in the current investigation, *in vitro* models focusing on the differentiation of porcine BM- and AT-MSCs into adipocytes were developed and optimized. These models were found to be suitable for exploring, for the very first time, the pathways by which the source of MSCs affects the RAs noticed for the selected mRNA transcripts encoding adipokines and their receptors. While the AT-MSCs were characterized by a higher overall expression of AdipoR1 transcripts, the transcriptional activity of *LEPR* was shown to increase in the BM-derived MSCs. Furthermore, the adipogenic derivatives of BM-MSCs displayed enhancements in the total quantitative profiles indicated for *LEPR* proteins.

Finally, the *ex vivo* porcine models, which were elaborated in the present study, might confirm the accelerated and specimen-specific recruitment of MSCs and

their intensified transcriptional reprogrammability into adipocytes under the intracorporeal conditions of the metabolic dysregulation of adipokines and their receptors arising from obesity and morbid obesity in peri-pubertal female humans. Furthermore, thoroughly exploring the inter-transcriptomic hallmarks occurring in the *ex vivo*-protected BM- and AT-derived MSCs and their adipogenically differentiated cell counterparts seems to play a pivotal role in the prospective attempts focused on the creation of MSC-based biorepositories. The latter can be indispensable, to a large extent, to initiate the realization of efficient ART-mediated strategies applied to the *ex situ* conservation of drastically vanishing subpopulations of autochthonous pig breeds and their subsequent re-introduction into the local biotopes of agricultural ecosystems. These strategies can be achieved using the innovative methods of embryonic genome engineering, which encompass intra- and inter-breed somatic cell cloning.

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