

Angiopoietin-like growth factor-derived peptides as biological activators of adipose-derived mesenchymal stromal cells

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ABSTRACT

Adipose-derived mesenchymal stromal cells (AD-MSCs) are an essential issue in modern medicine. Extensive preclinical and clinical studies have shown that mesenchymal stromal/stem cells, including AD-MSCs, have specific properties (ability to differentiate into other cells, recruitment to the site of injury) of particular importance in the regenerative process. Ongoing research aims to elucidate factors supporting AD-MSC culture and differentiation *in vitro*. Angiopoietin-like proteins (ANGPTLs), known for their pleiotropic effects in lipid and glucose metabolism, may play a significant role in this context. Regeneration is a complex and dynamic process controlled by many factors. ANGPTL6 (Angiopoietin-related growth factor, AGF), among many activities modulated the biological activity of stem cells. This study examined the influence of synthesized AGF-derived peptides, designated as AGF9 and AGF27, on AD-MSCs. AGF9 and AGF27 enhanced the viability and migration of AD-MSCs and acted as a chemotactic factor for these cells. AGF9 stimulated chondrogenesis and lipid synthesis during AD-MSCs differentiation, influenced AD-MSCs cytokine secretion and modulated transcriptome for such basic cell activities as migration, transport of molecules, and apoptosis. The ability of AGF9 to modulate the biological activity of AD-MSCs warrants the consideration of this peptide a noteworthy therapeutic agent that deserves further investigation for applications in regenerative medicine.

1. Introduction

1.1. AD-MSCs

In regenerative medicine adipose tissue is recognizable as a rich source of stem cells named Adipose-derived Mesenchymal Stromal Cells, AD-MSCs [1–5]. *In vitro* cultured AD-MSCs are slow-dividing cells with

doubling time between 2 and 4 days, lengthening over culture [6–9]. The ability to adhere allows assessment of their migratory and chemotactic properties in *in vitro* culture [10,11]. These cells produce a number of proteins, the secretion of which can be modified by different factors [12–15]. In the inflammatory phase of wound healing, the presence of AD-MSCs induces an influx of leukocytes and macrophages in the healing wound. In the growth phase, they stimulate the proliferation and

Abbreviations: AD-MSCs, adipose-derived mesenchymal stromal cells; MSCs, mesenchymal stem/stromal cells; AGF, angiopoietin-related growth factor; ANGPTLs, angiopoietin-like proteins; RGD, tripeptide with arginine, glycine and aspartic acid residues; GAG, glycosaminoglycans.

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migration of cells (keratinocytes, fibroblasts), which allow granulation of the wound [16,17]. *In vitro*, they have a wide range of abilities to differentiate into other cells [12,18–23]. However, according to the recommendations of the International Society for Cell & Gene Therapy (ISCT) Mesenchymal Stromal Cell (ISCT MSC), AD-MSCs should have the ability to differentiate into three cell lineages: adipocytes, osteocytes and chondrocytes in *in vitro* cultures [1]. When using appropriate inductive agents, AD-MSCs differentiate into adipocytes and lipid-filled vacuoles formation traced by triglyceride staining, e.g. with oily red is observed [24,25]. In media from differentiating cultures, the presence of many adipokines of a hormone nature is detected, e.g. resistin, leptin, adiponectin or factors regulating the immune system (TNF- α , TGF- β , IL-1 β , IL-6, IL-8, IL-10, MCP-1), proteins associated with the coagulation system (PAI-1, TF), factors stimulating angiogenesis (VEGF, NGF) [12, 26–28]. AD-MSCs also have the ability to differentiate into osteocytes leading to mineralization of the extracellular matrix, which is detectable through various methods such as alizarin red staining [24,29–32]. MSCs after 2–4 weeks of culture show increased secretion of alkaline phosphatase, osteocalcin, and calcium phosphates [24,29,33–36]. In the case of AD-MSCs differentiation towards chondrocytes, cells are cultured at a higher density, and the extracellular matrix (ECM) compounds such as glycosaminoglycans (GAG) and collagen increase are observed [13,37]. AD-MSCs are researched as a possible factor supporting the regenerative processes [5,12,38–42].

1.2. AGF

Angiopoietin-related growth factor (AGF, ANGPTL6, ARP3 or ARP5) belongs to the family of angiopoietin-like proteins (ANGPTLs). It is a group of secreted glycoproteins composed of eight members (ANGPTL 1–8) which share structural similarity with angiopoietins, but lack the necessary domains to bind with the classical angiopoietin receptors, Tie1 or Tie2. They all contain an amino-terminal, a linker region, and a carboxy-terminal fibrinogen-like domain. ANGPTLs are involved in multiple biological processes such as angiogenesis, lipid metabolism, inflammation and hematopoietic stem cell activity [43–45]. The AGF in the fibrinogen-like domain has a sequence of the RGD motif (tripeptide with arginine, glycine and aspartic acid residues; Arg-Gly-Asp), recognizable by integrins during a cell interaction with the external environment [46,47]. RGD plays a vital role in cell adhesion and migration. It has been observed that AGF promotes the migration of cells such as keratinocytes, fibroblasts, and endothelial cells and influences cell proliferation and differentiation [48–50]. Conversely, RGD conjugated with alginate hydrogel RGD in a 3D culture model increases the differentiation of AD-MSCs towards adipocytes [51]. The RGD-containing biomaterials have been reported in diverse regenerative applications, including spinal cord repair [52], increased bone formation [53] and accelerated healing of cardiovascular textiles [54]. It has been observed that in the transgenic mice with expression of the AGF expressed under control of keratin K14 promoter (K14-AGF mice), the number of skin vessels increased [47]. AGF participates in the restoration of the epidermis (epidermal phase), granulation and wound remodelling; stimulates keratinocytes to migrate and proliferate, and accelerates the granulation process; and rebuilds cartilage and vessels in the mouse auricle following punch wound [47,55]. AGF, like some others ANGPTLs, is presented in blood serum and exosomes [49]. Its mechanism of cell action through plasma membrane receptors is still an open question. Zang et al. noticed that AGF-containing RGD motif is an integrin ligand and, as such it works as a mitogenic growth factor for keratinocytes in cutaneous wound healing [50]. Wang et al. suggested that ANGPTL6 presented in the serum exosomes regulates endothelial proliferation and migration during the blood vessels regeneration by activating MAPK pathways by through what receptor it is unknown [49]. However, the detailed molecular mechanism of AGF action in the cell remains unknown.

1.3. Aim of the study

In this study, we evaluate the effects of AGF-derived peptides on the biological properties of AD-MSCs due to their potential for applications in regenerative therapies.

2. Material and methods

2.1. Synthesis of AGF9 and AGF27 peptides

The main AGF peptide sequence, 338–346 fragment of AGF (ANGPTL6), named peptide AGF9 (T³³⁸SRGDHELL³⁴⁶-amide), was claimed as part of the patent (PL236332 B1,28 Dec 2020). AGF27 peptide is an elongated version of AGF9 with prolonged flanking sequences at N- and C-termini (Table 1). Two AGF-derived peptides were synthesized by the solid phase peptide synthesis method with the use of an automated peptide synthesizer (Quartet, Protein Technologies Inc) as C-terminal amides on TentaGel S RAM amide resin (loading 0.25 mM NH₂ group/g) using Fmoc chemistry and TBTU as a coupling reagent. The peptide was cleaved from the peptidyl-resin by treatment with a mixture of TFA/phenol/water/triisopropylsilane (TIPS) (88/5/5/2) at room temperature (RT) for 3 h under argon bubbles. The crude peptides were precipitated and washed with ice-cold diethyl ether. After lyophilization, the peptides were purified by a preparative RP-HPLC system (SpotPrep, Armen) using a Reprisil 100–18 column (Dr. Maisch GmbH, 40 × 250 mm, 10 μ m particle size). A linear gradient methods of 0–60 % (for AGF9) and 30–85 % (for AGF27) realized in 40 min of acetonitrile (ACN) with 0.08 % TFA with a flow rate of 25 ml/min was used throughout the purification. Fractions of the highest purity (> 95 %) were analyzed by analytical RP-HPLC system (Prominence, Shimadzu) using a Phenomenex Kinetex XB-C18 column with the dimensions of 150 mm × 4.6 mm, particles the size of 5 μ m with a linear gradient of 0–100 % of ACN with 0.08 % TFA (solvent B) in 20 min and a Phenomenex Aeris PEPTIDE column with the dimensions of 150 mm × 4.6 mm, particles size of 3,6 μ m (for AGF27) with the same type of gradient and a flow rate of 1 ml/min. Solvent A was composed of water with 0.08 % TFA. After purification and lyophilization, the peptides were dissolved in 0.01 M AcOH for trifluoroacetate to acetate ion exchange and lyophilized. Afterwards, the peptide homogeneity was characterized by analytical RP-HPLC (Prominence, Shimadzu) and ESI mass spectrometry (TripleTOF 5600+, ABSciex). The physicochemical properties of the peptides are summarized in Table 1. Analytical RP HPLC chromatograms and ESI MS spectra are shown in Supplementary Material (Fig. 1S, Fig. 2S).

2.2. Isolation and culture of AD-MSCs

Human subcutaneous adipose tissue was sampled from patients at the Plastic Surgery Clinic of the Medical University of Gdansk. The procedure was approved by the Independent Bioethics Committee for Research of the Medical University of Gdansk (NKBBN/387/2014; NKBBN/298/2021) in accordance with the Helsinki Declaration of the World Medical Association (Ethical Principles for Medical Research Involving Human Subjects, 2008). Each donor signed an informed written consent form for collecting and using cutaneous adipose tissue. The isolation of adipose-derived mesenchymal stromal cells (AD-MSCs) was based on a standard protocol described before [56]. Briefly, following enzymatic digestion and erythrocyte lysis, the cells were suspended in a culture medium Dulbecco's modified Eagle medium (DMEM low glucose, 1000 mg) supplemented with 10 % foetal bovine serum FBS (Sigma-Aldrich, Saint Louis, Missouri, U.S.A). After 24 h culture in standard conditions (37 °C, 5 % CO₂) non-adherent cells were removed. Adherent cells were seeded on Primaria flasks (Corning, U.S. A) at a density of 375,000 cells/75 cm² flask, and every week passages were performed by trypsinization (0.25 % trypsin/EDTA solution; Sigma-Aldrich, Saint Louis, Missouri, U.S.A). The multipotency of the

Table 1

Physicochemical properties of AGF-derived peptides. The sequence common in both peptides is bolded.

Peptide label	Sequence	HPLC t_R [min]	M.W. (calculated) [g/mol]	ESI MS (determined) m/z
AGF9	T ³³⁸ SRGDHELL ³⁴⁶ -amide	8,86	1025,5	1026,4 [M+H] ⁺ 1048,4 [M+Na] ⁺
AGF27	G ³³⁰ L ³³¹ P ³³² V ³³³ Y ³³⁴ Q ³³⁵ L ³³⁶ T ³³⁷ SRGDHELL ³⁴⁶ V ³⁴⁷ L ³⁴⁸ L ³⁴⁹ D ³⁵⁰ W ³⁵¹ G ³⁵² G ³⁵³ R ³⁵⁴ G ³⁵⁵ -amide	12,72	3009,3	1505,1 [M+2 H] ²⁺ 1003,7 [M+3 H] ³⁺

obtained AD-MSCs has been confirmed by the presence of the surface molecules characteristic for AD-MSC such as CD73, CD105, and CD90 by flow cytometry method and the ability to differentiate into other cells as adipocytes, osteocytes and chondrocytes as has been described previously [56]. Shortly, for adipogenic and osteogenic differentiation, cells were cultured in differentiating media for two weeks (StemPro Adipogenesis Differentiation kit A100070–01, StemPro Osteogenesis Differentiation kit A100072–01, Gibco by Life Technology). Cells cultured in MesenPRO RS medium (Cat. 12746012, Gibco by Life Technology) were used as a control. After two weeks, fixed cells were stained with Oil Red O (Sigma, Cat. O0625) and Alizarin Red S (Sigma, Cat. A5533) to confirm adipocytes and osteocytes differentiation, respectively. Adipocytes were identified as cells with red-stained lipid vesicles. Osteocyte activity was identified as red-staining calcium deposits in the medium [57,58]. Chondrogenesis differentiation was assessed by micropellet formation during a two-weeks culture in a 96-well plate in a differentiating medium (Stem Pro Chondrogenesis Differentiation kit, Gibco by Life Technology). Cells were stained with 1 % Alcian Blue pH 2.5 (Sigma-Aldrich, Saint Louis, Missouri, U.S.A) in 3 % acetic acid and blue staining indicated the synthesis of proteoglycans by chondrocytes [59, 60].

Over 95 % of cultured cells have surface molecules characteristic for AD-MSC cells, such as CD73, CD105, and CD90 and differentiated into adipocytes, osteocytes and chondrocytes (data not shown).

2.3. Semi-quantitative assessment of adipogenesis, osteogenesis and chondrogenesis

During the semi-quantitative assays, AD-MSCs were cultured with AGF9 at a concentration 1 µg/ml for two weeks (adipogenesis, osteogenesis) or six weeks (chondrogenesis).

2.3.1. Adipogenesis (lipids content in cells)

For evaluation of lipids, the stained by Oil Red O cells were rinsed in distilled water, 100 µl isopropyl alcohol (Isopropanol IPA I-MAX 99.9 %, Cat. 1000845, I.P.A., Poland) was added and shaken at 1000 rpm for 10 min to remove stained lipids from cells. After this time, the solution was transferred to a new 96-well plate to measure absorbance at a wavelength of 490 nm (Victor 4 spectrophotometer, Perkin Elmer, Germany).

2.3.2. Osteogenesis (secretion of alkaline phosphatase to environment)

The process of osteogenesis was evaluated by the alkaline phosphate activity in culture supernatant according to the manufacturer's procedure (Biomaxima, Poland). For this purpose 10 µl of culture medium was collected and added to 100 µl of the mixture used for the determination of the enzyme alkaline phosphatase (Cat. 5020.2, Biomaxima, Poland) level. The intensity of the colour has been measured spectrophotometrically at a wavelength of 405 nm. (Victor 4 Perkin Elmer spectrophotometer, Germany).

2.3.3. Chondrogenesis (glycosaminoglycans, GAG, presence in extracellular matrix)

The pellet of AD-MSCs was cultured in 2 ml of chondrocyte differentiating medium without (control) or with AGF (1 µg/ml) for six weeks.

AD-MSCs formed a clump (pellet) that has been histologically prepared. The pellet was fixed with 4 % formaldehyde for 24 h at RT, dehydrated by applying the ethanol in increasing concentration and embedded in paraffin. Five-micron thick tissue histological sections were prepared using a microtome. The paraffin-embedded tissues were deparaffinized, hydrated and stained with 1 % Alcian Blue pH 2.5 (Sigma-Aldrich, Saint Louis, Missouri, U.S.A) in 3 % acetic acid for 30 min at RT. After staining, the slides were counterstained with Harris's hematoxylin (Sigma-Aldrich, Saint Louis, Missouri, U.S.A) for 1 min at RT. Blue staining indicated the synthesis of glycosaminoglycans (GAG) by chondrocytes. The GAG presence was assessed in a greyscale on the photos of pellet histological slides using the ImageJ v. 1.52 program (U.S.A National Institutes of Health; <http://rsb.info.nih.gov/ij/>).

2.4. Viability and cytotoxicity assays

AD-MSCs were plated at a density 4000 in 100 µl per well on the 96-well plates in DMEM supplemented with 10 % FBS. After 24 h, the medium was changed to serum-free medium and the AGF peptides were added at a concentration from 0.001 to 150 µg/ml for 48 and/or 72 h. All solutions used in the experiments were prepared with water under sterile conditions. After incubation time the XTT test and LDH test were done. The XTT cell viability assay was performed according to the manufacturer's instructions (Cat.No 11465015001 Roche Diagnostic). AD-MSCs were incubated with AGF9 and AGF27 for 48 and 72 h, and then XTT reagent was added, and incubated for 4 h. The absorbance was measured at 490 nm using a spectrophotometer plate reader (Victor 4 spectrophotometer; Perkin Elmer, Germany). Cell viability was normalized with respect to the viability of AD-MSCs incubated without AGFs (control, 100 %). Cytotoxicity of AGFs was quantified by measuring lactate dehydrogenase (LDH) activity in cell supernatants (Cat. No. MK401; Takara, Japan). After 48 h of AD-MSCs incubation with AGF9 and AGF27, the supernatants were collected for LDH content analysis according to the manufacturer's procedure (Takara, Japan). Cell death was normalized with respect to the level of cell death in a non-AGFs-treated control (0 %). Triton X-100 detergent (1 %) was used as a positive control for maximum LDH release (100 % cytotoxicity).

2.5. Migration and chemotaxis assays

The effect of AGFs on AD-MSCs cell migration after 24 h was determined using culture-insert 2 well with a defined cell-free gap (Cat. No. 81176; Ibidi, GmbH, Germany). 5000 cells in culture medium (DMEM, 10 % FBS, antibiotics) were sown into each chamber of the insert and incubated for 24 h. After removing the insert attached cells create a cell-free gap of approximately 500 µm. Then, a serum-free medium with mitomycin C (1 µg/ml) was added for 30 min to block cell proliferation (Cat. M0503, Sigma-Aldrich, U.S.A). Next, the medium was changed, and the cells were stimulated with the appropriate concentration of AGF9 at 0.1 and 1 µg/ml. After 24 h, the cells were fixed with 3.7 % paraformaldehyde for 30 min, and stained with 0.05 % crystal violet (Cat. 1092180500, Merck, Germany) for 20 min, and rinsed with water. Migrating cells were assessed under the microscope (Aixovert 100, Carl Zeiss, Germany) at 50x magnification and documented by taking pictures (four photos from different parts). The surface

was analyzed by using Imaging Software NISElements Basic Research. The effect of AGFs on AD-MSCs chemotaxis was assessed with ThinCert cell culture inserts (Cat. 662638, 8.0 µm; Greiner Bio-One, Germany BD, U.S.A). Cells were seeded on inserts placed in a 24-well plate at a density of 100,000 cells per well in a serum-free medium. Culture media with AGF9 and AGF27 at a concentration 1 µg/ml were added to the plate well (lower compartment). After 24 h, the cells were stained with 8.0 µM Calcein-AM (cell viability dye; Cat no.C3099, Life Technologies, U.S.A), migratory cells were detached with trypsin-EDTA, and their fluorescence was measured with a plate reader at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

2.6. Analysis of selected cytokines secretion by AD-MSCs

To evaluate the effect of the AGF9 peptide on the paracrine properties of AD-MSCs, the level of selected cytokines was estimated using Luminex xMAP® technology. Supernatants from AD-MSCs cultured in MesenPro medium in the presence of AGF9 at a final concentration of 1 µg/ml for 14 days were collected and prepared according to the manufacturer's instruction (Cat. HADCYMag-61 K, Merck Millipore, U.S.A; Panel is an 11-plex kit for the simultaneous quantification of the following cytokines: Adiponectin, HGF, IL-β, IL-6, IL-8, Leptin, MCP-1, NGF, PAI-1 (total), Resistin, TNFα) and read with a Luminex MAG-PIX® analyzer. Data were analyzed in the xPONENT 4.2 program.

2.7. Transcriptome analysis

AD-MSCs were seeded without and with AGF9 at a final concentration of 0.1 µg/ml for 48 h. Cells were then collected, followed by RNA isolation was performed using the kit miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with modifications [61]. RNA quantity and quality (RNA Integrity Number [RIN]) were assessed using the Bioanalyzer 2100 Instrument and RNA 6000 Nano Kit (Agilent, Waldbronn, Germany). Total RNA with RIN >8.0 was used for sequencing was library preparation. Ribosome reduction kit (Arraystar Inc, Rockville, U.S.A) and SureSelect Strand Specific mRNA library kit (Agilent, Santa Clara, U.S.A) were utilized according to standard procedures and the cDNA libraries were quantitated using qPCR in a Roche LightCycler 480 with the Kapa Library Quantification Kit for Illumina (Kapa Biosystems, Woburn, U.S.A). Paired-end 50 bp RNA sequencing were run on the Illumina HiSeq 2500 (Illumina Inc., San Diego, U.S.A) and the data was converted to the FASTQ Sanger format using the bcl2fastq converter. Trim Galore was used to remove adapter sequences from the reads (<https://www.bioinformatics.babraham.ac.uk>). The reads were mapped to a reference human genome (hg19 edition) using TopHat software [62,63]. Differential expression analyses comparing the AGF9-treated and control cells were performed with the Cufflinks package using the complete workflow for version 2.2.0 (and higher) as described by the authors (<http://cole-trapnell-lab.github.io/cufflinks/manual>). The results of the comparative analyses were used to build differential gene expression matrices that were further processed by Ingenuity Pathway Analysis (IPA Spring Release 2017, Redwood City, U.S.A).

2.8. Statistical analysis

The non-parametric Mann-Whitney U test and Wilcoxon matched paired test were used to analyze the significance of differences between the studied groups, at a significance level of $p < 0.05$. Statistical analysis was performed using the software Statistica v13.3 (Statsoft, Warsaw, Poland) and for the graphical results presentation GraphPad Prism 5 was used (GraphPad Software, La Jolla, U.S.A).

3. Results

3.1. Design of AGF-derived peptides

Literature data show that the pleiotropic protein AGF is involved in cell proliferation and differentiation. These processes are closely linked to cell adhesion, in which the integrin-binding RGD motif, present in many extracellular matrix proteins, is involved. In the sequence of the human AGF protein, the RGD motif corresponds to amino acid residues 340–342 (R³⁴⁰GD³⁴²) (Fig. 1; The multiple sequence alignment of mammalian AGF proteins was carried out by MUSCLE 3.8; [64]). It has been reported that this motif may play an important role in repair and cartilage tissue engineering processes [65]. Since the binding specificity of the RGD motif is determined by the peptide sequences flanking the N- and C-terminus of this motif [66], we designed a peptide that is a fragment of the human AGF protein designated as AGF9 (T³³⁸SRGDHELL³⁴⁶-amide) to investigate the effect of the RGD motif in the sequence context unique for AGF as confirmed by blast analysis (Supplementary Fig. 3 S) on ASCs biology, in particular on chondrogenesis. The selected sequence is conserved in AGF homologues in different mammalian species, including the human and the mouse variants of the AGF protein (Fig. 1). The second peptide designated as AGF27 (G³³⁰LEPVYQLT³³⁸SRGDHELL³⁴⁶VLEDWGGRG³⁵⁶-amide) contains elongated at both the N- and C-terminal flanking sequences of the RGD motif (Fig. 1). The spatial structure of the human AGF protein suggests that it should adopt a stable β-sheet structure in this region [UNIPROT ID Q8NI99; <https://www.uniprot.org/uniprotkb/Q8NI99/>]. Both peptides were synthesized by the solid support peptide synthesis (SPPS) method (in the form of C-terminal amides), and, after purification (>95 % purity) and replacement of the trifluoroacetate counterion with an acetate one, were used for cellular studies.

3.2. AGF-derived peptides enhance AD-MSCs viability and are not cytotoxic to AD-MSCs

Tests of lactate dehydrogenase (LDH) release showed that neither AGF9 nor AGF27 were cytotoxic to AD-MSCs during 48-h incubation (Fig. 2 C,D). The peptide AGF9 compared to the control group significantly enhanced the viability of AD-MSCs at all tested concentrations from 0.001 to 150 µg/ml, both after 48 and after 72 h (Fig. 2A; $p < 0.05$). This compound at a concentration of 0.01 µg/ml after 48 h caused an increase in cell viability by 25 %. AGF27 did not change AD-MSCs viability (Fig. 2B).

3.3. AGFs effect on AD-MSCs migration and chemotaxis

During the AD-MSCs migration test, AGF9 significantly reduced the scratch surface area already at a dose 0.1 µg/ml, while AGF27 got this level at 1 µg/ml (Fig. 3A). Additionally, both compounds showed a pro-chemotactic effect on AD-MSCs (Fig. 3B; $p < 0.05$).

Considering the higher activity of AGF9 observed compared to AGF27 on AD-MSCs cultures, further analyses concerning AD-MSCs' differentiation and secretory activities were concentrated on the former peptide. The difference in activity was particularly striking in cell viability tests, even taking into account that the molar concentrations of the RGD active motif are approximately three times lower for the equivalent mass concentrations in AGF9 relative to AGF27.

3.4. AGF9 affects adipogenesis and osteogenesis

AD-MSCs cultured in adipocyte differentiating medium (Fig4. A "C Adipo") reached the highest level of lipids content and as such were treated it as a positive control (100 %). It was observed that addition of AGF9 did not change this level of lipids (Fig. 4A "AGFAdipo"). However, when AD-MSCs were in a non-differentiation medium MesenPro with the addition of AGF9 (AGF9Mesenpro) the lipid synthesis increased by



Fig. 1. Multiple sequence alignment of mammalian AGF proteins [Mouse vs. Human 73 % identity]. AGF9 sequence is marked with a red, AGF27 with a blue one. Consensus sequence positions are marked with a star. The multiple sequence alignment was carried out by MUSCLE (3.8).

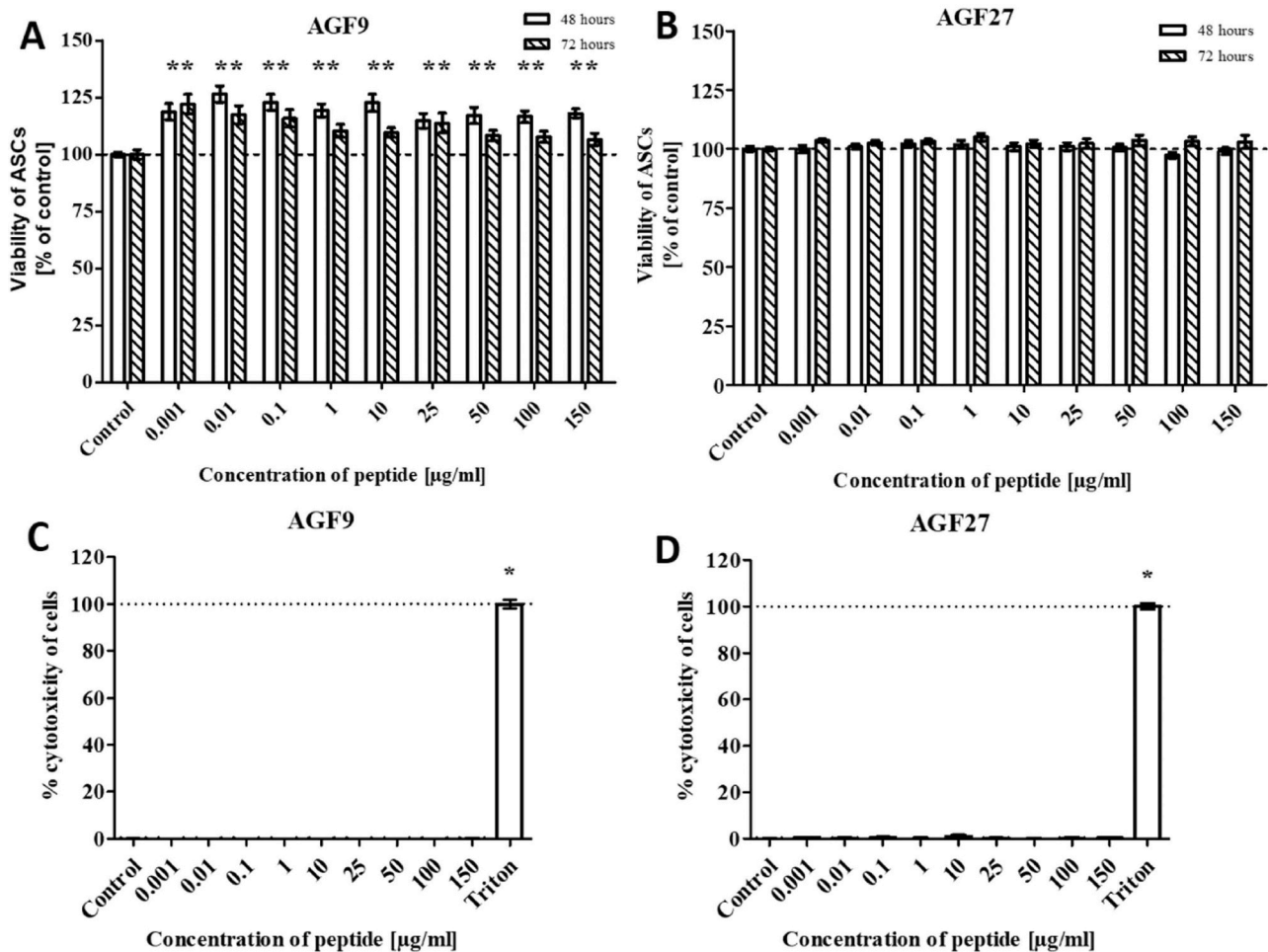


Fig. 2. AGF9 (A, C) and AGF27 (B, D) effect on AD-MSCs viability (A, B) and cytotoxicity after 48 h (C, D). The bars represent the means ± SEM from 4 independent experiments (each in 4 repetitions). TritonX100-treated cells were the positive control of cytotoxicity (100 %). * statistically significant differences in comparison to control $p < 0.05$, Mann-Whitney U test.

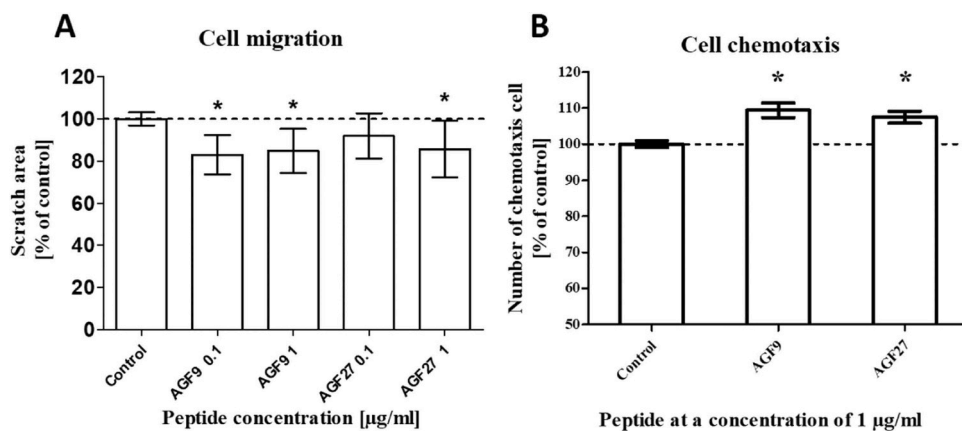


Fig. 3. Effect of AGF9 and AGF27 on AD-MSCs migration (A) and chemotaxis (B) after 24-h incubation. The bars show the means ± SEM from 4 independent experiments (each in 4 repetitions). * statistically significant differences $p < 0.05$, Mann-Whitney U test.

13 % ($p < 0.05$; Fig. 4A). The addition of this peptide to the medium MesenPro did not change the alkaline phosphatase presence (marker of osteogenesis), but as a result of AGF adding to osteogenic medium "Osteo" (AGF9Osteo), a statistically significant ($p < 0.05$) decrease in alkaline phosphatase secretion by 7 % was observed (Fig. 4B).

3.5. AGF9 stimulates chondrogenesis

The histological slides prepared from AD-MSC pellets and stained with Alcian blue to visualize the presence of glycosaminoglycans (GAG) were analyzed. The GAG content in the extracellular matrix was assessed based on grey intensity determined following photo processing (Fig. 5).

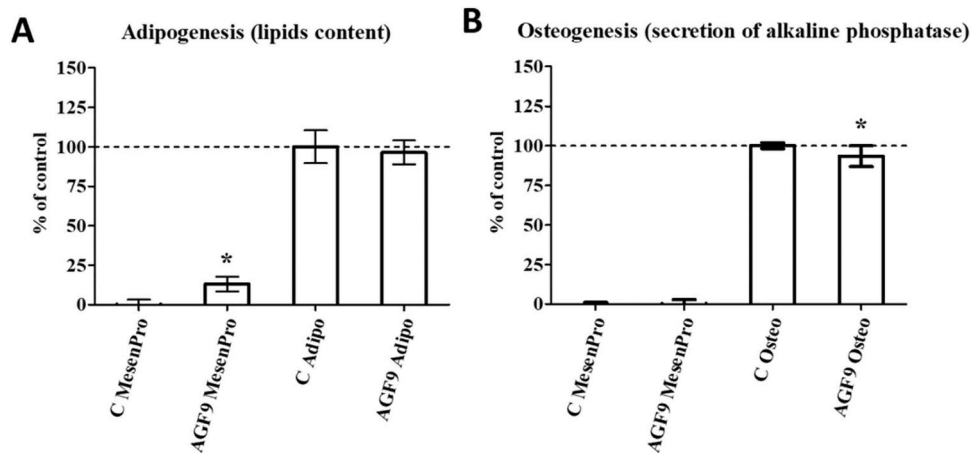


Fig. 4. Effect of AGF9 on lipids content (A) and alkaline phosphatase presence (B) after 14 days in the medium non-differentiating ("MesenPro") or adipocyte ("Adipo") and osteocyte ("Osteo") differentiating media. AGF at a concentration of 1 µg/ml was used. Bars represent the means ± SEM of 4 independent experiments (4 replicates each). * statistically significant increase of lipidogenesis $p < 0.05$; * statistically significant decrease of alkaline phosphatase secretion $p < 0.05$; Mann-Whitney U test.

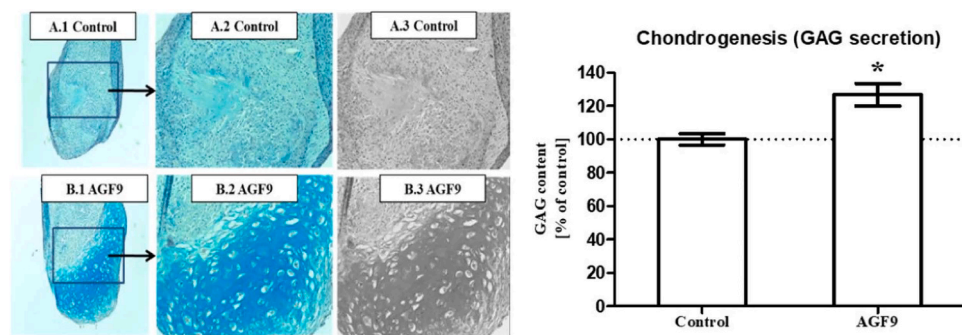


Fig. 5. A representative computer analysis of glycosaminoglycans (GAG) abundance as a marker of ongoing chondrogenesis. AD-MSC pellets were cultured for six weeks in a chondrocyte differentiation medium without (panel A control) or with AGF9 (panel B; AGF9 concentration 1 µg/ml) and the histological slides were done. A1 and B1 magnification 50x; A2 and B2 magnification 200x; A3 and B3 computer analysis of grey intensity by ImageJ v. 1.52 The right panel presents the results of the GAG content examination in the AD-MSC pellets from 4 experiments as means ± SEM. * statistically significant increase in GAG secretion by cells cultured with the addition of AGF9; $p < 0.05$; Mann-Whitney U test.

The obtained results indicated a statistically significant increase of GAG content in the presence of AGF9 ($p < 0.05$, Fig. 5).

3.6. AGF9 affects cytokine secretion by AD-MSCs

AD-MSC secretory activity could be modulated by AGF9. The influence of AGFs was investigated after stimulation with AGF9 peptide at a final concentration of 1 µg/ml in a non-differentiation culture. Of the 11

detectable cytokines selected with a set of antibodies, six were found to get a higher level in the presence of AGF9: NGF, IL-6, leptin, IL-8, HGF, MCP-1. The obtained results are presented in Fig. 6.

The observed differences were statistically insignificant. MCP-1 (Monocyte Chemoattractant protein-1), also known as Chemokine (CC-motif) ligand 2 (CCL2), displayed the strongest response following AD-MSCs exposure on AGF9.

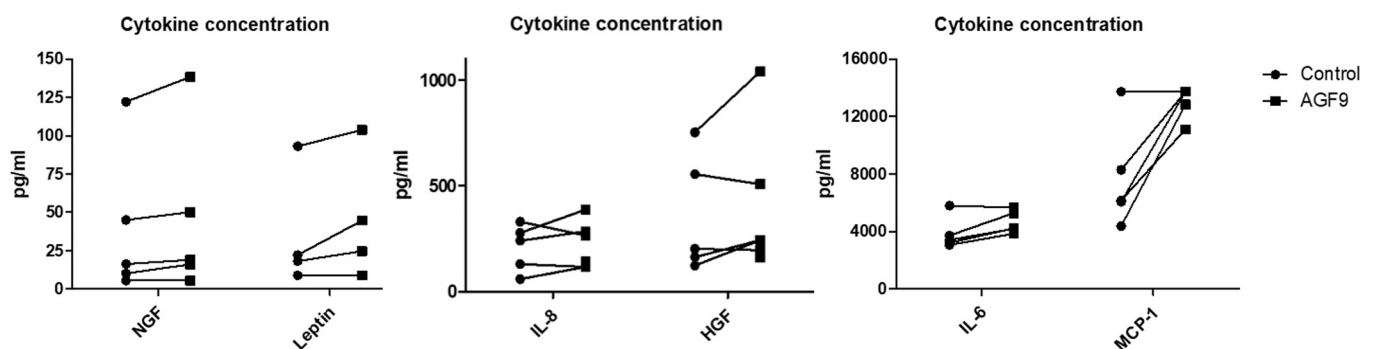


Fig. 6. Cytokine concentrations in supernatants after 14 days of AD-MSCs cultured without (control, black dots; 5 donors) and with AGF9 at a final concentration of 1 µg/ml (AGF9, black squares; 5 donors). Wilcoxon matched paired test.

3.7. AD-MSC transcriptomic response to AGF9

The analysis of transcriptome changes after AD-MSCs 48-h incubation with AGF9 peptide performed in Ingenuity Pathway Analysis is presented in Table 2. While no conclusive results regarding the regulatory system were revealed, activation of several related biological processes was observed, especially cell migration and transport of molecules. Interestingly, statistically significant inhibition of the pathways involved in apoptosis was also observed.

3.8. Conclusion from the results

Our findings indicate that AGF9 augmented AD-MSCs viability, migration and acts as a chemotactic factor for these cells. AGF9 demonstrates a potential in stimulating the chondrogenesis process during AD-MSCs differentiation, thus it is predicted for further examination as a noteworthy factor for potential applications in regenerative medicine.

4. Discussion

In addition to thermoregulatory function and hormone secretion, adipose tissue is an important source of stem cells [2,67,68]. AD-MSCs are an alternative source compared to bone marrow-derived mesenchymal stromal cells [13,69]. These cells show a high potential for differentiation into other cells, documented in many preclinical and clinical studies [70–73]. Transgenically delivered AGF was demonstrated to induce tissue regeneration in an ear pinna model and promote angiogenesis in mice [47,55]. As shown in a transgenic mouse model, AGF antagonizes obesity and insulin resistance [74], which may have

implications for diabetic wound healing. High expression of the gene encoding the AGF protein was found in liver cells, and as a circulating protein secreted by the liver, it promotes wound healing [55,74,75]. AGF also stimulates the proliferation and migration of keratinocytes, fibroblasts, and endothelial cells [50,76]. Changing the gene expression for AGF affects the metabolic pathways associated with lipid circulation and their deposition in adipose tissue [47,55,74,76–79]. Considering the important role of targeted migration of AD-MSCs to locally damaged tissue, the pro-migratory and pro-chemotactic effect of AGF9 seems to be interesting in the context of the recruitment and influx of mesenchymal cells to the damaged place [10,11]. So far, the literature has focused on the AGF protein as a whole, or has studied transgenic mice with increased expression of the gene encoding ANGPTL6. In K14-AGF mice, with AGF expression under the control of the keratinocyte-specific promoter, an increase in angiogenesis, epithelialization and granulation was observed [47,55,80]. The AGF present in the extracellular matrix, via the RGD fragment, mediates the binding of integrins and influences the processes of adhesion, proliferation, migration and differentiation of skin cells [50]. The mechanism of cell signalling under the influence of AGF is unknown. The available data is very fragmentary and mainly concerns non-AD-MSC cells [50,76,79,81]. AGF9 enhanced AD-MSCs viability and interacted with differentiation into other cells. The induction of the adipogenesis demonstrated in the present study corroborates with others' observations that there is a link between AGF and lipid metabolism [82–86]. The AGF gene expression is related to cholesterol and fatty acids serum levels [74]. A strong relationship between the redistribution of adipose tissue and the AGF (ANGPTL6) gene expression was demonstrated by Oike et al. in several studies [47,55,74]. It has been proven that AD-MSCs exhibit a

Table 2

Biological processes of AD-MSCs affected by AGF9 peptide. The ontological terms with $p < 0.05$ are considered statistically significant. The data comes from the analysis of material from 3 donors. IPA, Ingenuity Pathway Analysis.

Biological process	p-value	Direction of change	Number of transcripts involved in	Genes
Cell movements	0.0000155	Activation	29	A2M,AGT,ALB,APOA1,BCAM,CDH1,CX3CL1,EFNA1,ELF3,ERBB3, EREG,F12,F7,FGA, FOSB,GRB7,ID1,IGF2,KRT19,KRT8,LIN28B,NR1H4,NR4A1,ORM1, PROC,SERPINA1, SERPINA5,ST6GAL1,VTN
Blood vessel formation (vasculogenesis)	0.0000110	Activation	13	AGT,CDH1,EGR3,F12,FGFR3,ID1,IGF2,KLK1,NR4A1,ORM1,PROC, ST6GAL1,VTN
Cell migration	0.0000306	Activation	26	A2M,AGT,ALB,APOA1,BCAM,CDH1,CX3CL1,ERBB3,EREG,F12,F7, FGA,GRB7,ID1,IGF2, KRT19,KRT8,LIN28B,NR1H4,NR4A1,ORM1, PROC,SERPINA1,SERPINA5,ST6GAL1,VTN
Transport of molecules	0.0000154	Activation	22	A2M,ABCC2,AGT,ALB,APOA1,APOB,APOC2,APOC3,ATP2B2, CENPF,ERBB3,FGA,FGF, FGG,HBB,HSD17B2,SERPINA7,SLC38A3, SLC7A3,TF,TFR2,TTR
Cell movements embryonic lines	0.000153	Activation	6	AGT,APOA1,FGA,IGF2,NR4A1,VTN
Transport of amino acids	0.00000274	Activation	6	ABCC2,ALB,SERPINA7,SLC38A3,SLC7A3, TTR
Eicosanoid metabolism	0.00553	Activation	5	AGT,ALB,CYP1A1,IGF2,VTN
Fatty acid metabolism	0.000484	Activation	10	ABCC2,AGT,ALB,APOA1,APOB,APOC2, APOC3,CYP1A1,IGF2,VTN
Growth of epithelial cells	0.00599	Activation	8	EGR3,EREG,F12,FGFR3,HPN,ID1,NR4A1, PROC
Cancer cell movements	0.0000196	Activation	22	A2M,AGT,BCAM,CDH1,CX3CL1,EFNA1, ERBB3,EREG,F7,FGA,FOSB,GRB7,ID1,IGF2,KRT19,KRT8,LIN28B, NR1H4,SERPINA1, SERPINA5,ST6GAL1,VTN
Synthesis of reactive oxygen form	0.00414	Activation	7	AGT,ALB,CRYAA/CRYAA2,CYP1A1,F7,HBB,SERPINA1
Protein secretion	0.00186	Activation	4	AGT,FGA,FGF,FGG
Embryonic cell migration	0.00214	Activation	4	FGA,IGF2,NR4A1,VTN
Exocytosis	0.00306	Activation	4	FGA,FGF,FGG,SCGN
Apoptosis	0.00110	Inhibition	29	AFP,AGT,ALB,ALDH1A1,ATP2B2,CDH1, CRYAA/CRYAA2,DUSP1,EGR3,ERBB3,EREG,F7,FGFR3,FGFR4, FOSB,HES1,HPN,ID1, IGF2,MT1F,NR1H4,NR4A1,PRAME,PROC,RBP4,ST6GAL1,TF, VSNL1,VTN

strong paracrine effect and can change the composition of the released cytokines during the differentiation process [87–90]. The increase in the lipid droplet content in AD-MSCs induced by AGF9 corresponds to an increase in leptin secretion under the influence of AGF in three out of the five donors, which corresponds to others' observation about AGF and leptin relationships [85,91]. In addition to leptin, an increase in the concentration of angiogenesis stimulating factors (NGF, HGF) and pro-inflammatory factors (IL-6, IL-8, MCP-1) has been demonstrated. Other authors also pointed out the high secretory activity of AD-MSCs [26–28,61,92]. A few studies have shown the effect of AGF on other differentiation-related processes (osteogenesis, chondrogenesis) [47,48,93]. Oike et al., reported skin wound healing and blood vessel development, in an ear pinna punch wound model in a transgenic mouse with enhanced AGF expression [47,55]. In connection with this observation, in this study, the effect of the AGF9 peptide on the AD-MSCs cultured for 6 weeks in a chondrogenic medium and under conditions of appropriate cell density (3D culture, a large number of cells lying close to each other), which ensures adequate production of extracellular matrix, was assessed. An extensive extracellular matrix was observed in Alcian blue-stained histological slides, and the analysis of AGF9-stimulated cultures showed an increase in glycosaminoglycan deposition compared to controls. Thus, AGF9 appears to influence chondrogenesis in addition to its effect on adipocyte differentiation. Moreover, AGF9 decreased the secretion of alkaline phosphatase of AD-MSCs in the process of osteogenesis ($p < 0.05$). It could indicate an inhibition of the process of AD-MSCs differentiation into osteocytes. Interestingly, AGF9 in the non-differentiation medium did not stimulate AD-MSCs to secrete the enzyme related to the mineralization process. Transcriptomic studies enable the assessment of dynamic phenomena occurring in the cell under the influence of chemical substances [94]. The AGF9 peptide influenced transcriptome changes related to biological processes, including the activation of migration, fatty acid metabolism, protein secretion or inhibition of apoptotic pathways. Among activated genes were genes for fibrinogens (FGA, FGB, FGG), angiotensinogen (AGT), and sialyltransferase (ST6GAL1). Fibrinogens control cell proliferation, angiogenesis, thereby promoting revascularization and wound healing [95]. The renin-angiotensin system regulates stem cells, thus involving haematopoiesis and tissue regeneration by progenitor cells and MSC differentiation to adipocytes [96,97]. ST6GAL1 catalyses the addition of α 2,6-linked sialic acids to terminal N-glycans and it can modify glycoproteins and/or glycolipids and plays an important role in the regulation of pluripotency and differentiation of cells [98,99]. Thus, it seemed that AGF9 during two days of culture influenced general cell processes. Longer incubation is needed to notice the increase in specific proteins related to chondrogenesis and osteogenesis, which is consistent with the observations of other authors [100–102]. The enhanced AD-MSCs migratory response to AGF9 could be mediated by the RGD motif within this peptide. AGF9 and 27 contain the RGD motif, thus it seems that, as for AGF [50] the integrin receptor signalling pathway could be the target for these derivatives. These receptors are widely present in a variety of cells, including AD-MSCs, and activate many pathways leading to migration, proliferation, metabolic activity, and differentiation [103–106]. Flow cytometry analysis of our AD-MSCs pointed out that over 95 % of them have integrin beta 1 (CD29) and about 90 % integrin alpha 4 (CD49d) (data not shown). Thus, we conjecture that the integrin receptor signalling pathway could be the target for AGF9 and 27 derivatives but it needs to be examined in future.

Thus, to sum up, based on the observation of *in vitro* culture and the results of the conducted analyses of viability, migration, chemotaxis, differentiation, cytokine secretion, and transcriptomic responses to AGF, it is concluded that the AGF9 peptide has the potential to modulate the biological properties of AD-MSCs. Further studies are warranted to elucidate the underlying molecular mechanisms and to explore the full extent of AGF9's impact on AD-MSCs. AGF9 could work as a factor stimulating chondrogenesis, thus, it is predicted for further examination for applications in regenerative medicine.

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CRediT authorship contribution statement

Paweł Sachadyn: Writing – review & editing, Conceptualization. **Anna Wardowska:** Resources, Investigation. **Agata Tymińska:** Investigation. **Natalia Filipowicz:** Investigation, Formal analysis. **Alina Mieczkowska:** Investigation, Formal analysis. **Mirosława Cichorek:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Adriana Schumacher:** Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Piotr Mucha:** Writing – original draft, Investigation, Funding acquisition, Conceptualization. **Izabela Puchalska:** Investigation. **Milena Deptuła:** Visualization, Methodology, Investigation. **Arkadiusz Piotrowski:** Conceptualization. **Michał Pikula:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: M. Pikula, P. Sachadyn, A. Piotrowski reports financial support was provided by National Centre for Research and Development. P. Mucha, I. Puchalska, M. Pikula, A. Schumacher, M. Cichorek, A. Wardowska, M. Deptuła, A. Piotrowski, N. Filipowicz, P. Sachadyn has patent #Patent number: PL236332 B1, 28 Dec 2020 issued to assignee. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2024.117052](https://doi.org/10.1016/j.biopha.2024.117052).

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