Effect of miR-195 inhibition on human skeletal muscle-derived stem/progenitor cells

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ABSTRACT

Background: The application of a circulating miR-195 inhibitor could be a helping factor in the *in vitro* model of human skeletal muscle-derived stem/progenitor cells (SkMDS/PCs). Previously, microRNA-195 (miR-195) expression has been reported to be a negative factor for myogenesis.

Aims: The study aimed to obtain anti-apoptotic and anti-aging effects in *in vitro* cultured myoblasts and to improve their ability to form myotubes by suppressing miR-195 expression.

Methods: Human wild-type (WT) SkMDS/PC cells incubated with control (nonspecific) miRNA inhibitor and miR-195-inhibited SkMDS/PCs were studied. Functional assays (myotube formation and cell aging), antioxidant, and myogenic gene expression analyses were performed at two time points, at the seventh and eleventh cell passages.

Results: Myotube formation was found to be almost 2-fold higher in the miR-195-inhibited SkMDS/PCs population (P <0.05) compared to WT cells. miR-195 inhibition did not appear to affect cell aging or rejuvenate human SkMDS/PCs. Antioxidant (*SOD3* and *FOXO*) gene expression was augmented in the miR-195-inhibited SkMDS/PCs population, but no positive effect on the remaining antioxidant genes (*SOD1*, *SOD2*, and catalase) was observed. A significant increase in *MyoD* gene expression with a concomitant decrease in *MyoG* (P <0.05) was further documented in miR-195--inhibited SkMDS/PCs compared to WT cells (the eleventh cell passage).

Conclusions: The performed studies may lead to the preconditioning of myogenic stem cells to extend their potential for pro-regenerative activity. The miR-195 inhibitor may serve as a conditioning factor augmenting selective antioxidant gene expression and proliferative potential of SkMDS/PCs, but it does not have an impact on cell aging and/ or apoptosis.

Key words: miR-195 inhibitor, human skeletal muscle-derived stem/progenitor cells (SkMDS/PCs), apoptosis, oxidative stress, regenerative medicine

INTRODUCTION

Over the past 15 years, stem cell therapy has attracted considerable interest. There is a consensus that injection or tissue-based implantation of various stem/progenitor cell types (such as myoblasts, mesenchymal cells, and induced pluripotent stem cells) may exert some beneficial therapeutic effects, and there is a general agreement that additional fundamental, translational, and clinical studies are required to define the optimal cell source and optimal method of cell delivery, for cell pro-regenerative activity [1]. There are many ways to modify cells for application in heart regeneration. Cells intended to be transplanted should first demonstrate cell vitality, proliferative potential, resistance to hypoxia [2], and pro-regenerative ability.

Significant evidence has been accumulated in the last few years showing the fundamental role of microRNAs (miRNAs) in the onset of many diseases [3, 4]. MiRNAs can also be considered a key mediator in cell adaptation to hypoxia by taking part in the hypoxic response pathway.

Particularly noteworthy is microRNA-195 (miR-195), which is a member of the micro15/107 family and is activated in multiple diseases, such as heart failure, cancer, and schizophrenia. The miR-195 regulates a pleth-

WHAT'S NEW?

SkMDS/PCs (skeletal muscle-derived stem/progenitor cells) appear to be regulated by microRNA-195 (miR-195). The miR--195 inhibitor increases functional capacity of human myogenic cells. miR-195 inhibition does not appear to affect SkMDS/PCs aging. *SOD3* and *FOXO* gene expression have been augmented by miR-195 inhibition.

ora of target proteins involved in the cell cycle, proliferation, and apoptosis [5]. The genes encoding a specific kinase (*WEE1*), cyclin-dependent kinase 1 (*CDK6*), and *Bcl-2* (B-cell lymphoma 2) are confirmed targets of miR-195 that mediate cell cycle and apoptotic pathways [6, 7]. However, the mechanism of miR-195 action has not been completely understood yet.

Some reports have shown that overexpression of miR-195 might be a cause of insufficient myocardial regeneration [8] while miRNA studies in heart attacks showed its biological role in the functioning of the heart. For example, overexpression of miR-195 in the developing heart caused a cardiomyocyte cell cycle arrest at the G2 phase and led to hypoplasia and/or congenital heart abnormalities, such as ventricular septal defects; it was also shown to prevent spontaneous heart regeneration from myocardial infarction [9]. Conversely, inhibition of miR-15 family members using locked nucleic acid (LNA)-modified anti-miRNAs led to an increase in cardiomyocyte mitosis in neonatal mice, promoted adult cardiomyocyte proliferation, and improved cardiac function and contractile capacity after ischemia-reperfusion injury [10]. *In vitro* studies have been conducted to determine the potential of miR-195 inhibitors for future benefit in post-infarction regeneration. Okada et al. (2016) [11] were the first to demonstrate the potential of a miR-195 inhibitor in the development of young mesenchymal stem cells (YMSCs) from old bone marrow, which demonstrated high anti-aging properties, as well as excellent cardiac repair efficacy. They indicated that miR-195 overexpression in MSCs induced stem cell senescence, further deteriorated their regenerative ability and deactivated telomerase reverse transcriptase (TERT) [11]. Our research approach was based on these studies. We aimed to replicate the effect reported in human skeletal muscle-derived stem/progenitor cells (SkMDS/PCs) by *in vitro* culture with an appropriate miR- inhibitor. This phenomenon was first observed in a model of C2C12 cells (an immortalized mouse myoblast cell line) [12]. Therefore, we decided to check whether a similar effect is present in a primary cell suspension of human skeletal muscle stem/progenitor cells.

In another analysis based on mesenchymal stem cells (MSCs), miR-195 inhibition significantly induced telomere re-lengthening in old MSCs along with reduced expression of senescence-associated β-galactosidase [13]. Moreover, silencing miR-195 in old MSCs by transfecting cells with a miR-195 inhibitor significantly restored the expression of anti-aging factors, including *TERT* and sirtuin 1 (*SIRT1*), and the phosphorylation of *AKT* and *FOXO1* genes [11]. Thus, the growing interest in miR-195 inhibition is well-founded because it could be a novel factor in improving a protocol of preparation of cells for regeneration of the post-infarction heart.

This study aimed to assess anti-apoptotic, anti-aging, and pro-regenerative abilities of human SkMDS/PCs cultured *in vitro* by suppressing miR-195 expression. We examined the potential application of a miR-195 inhibitor as a possible factor to be used in future myocardial regeneration attempts with human skeletal myoblasts by studying basic SkMDS/PCs biological features while subjecting them to miR-195 inhibition.

METHODS

Human SkMDS/PCs isolation

Human skeletal muscle-derived stem/progenitor cells (SkMDS/PCs) were isolated from tissue fragments derived from residual muscles after surgical intervention in the abdominal rectus muscle area. For this purpose, approval from the Bioethical Local Committee (Medical University of Poznan, no. 818/13) and written consent from patients was obtained.

Isolation of SkMDS/PCs was conducted according to the technique previously described [14]. The cell isolation method was optimized for its best efficiency [15, 16]. Several steps were diligently tested before further use, including cutting the tissue into small pieces with a scalpel, incubation with the digesting enzyme, filtration of cells from the remaining debris. The culture medium (standard Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and supplemented with 20% fetal bovine serum [Lonza Group, Basel, Switzerland]), 1% antibiotics (Lonza Group, Basel, Switzerland), 1% ultraglutamine (Lonza Group), and basic fibroblast growth factor (bFGF) (Sigma-Aldrich, Saint Louis, MO, US) was changed every other day, and the cells were passaged using 0.25% trypsin in phosphate buffered saline (PBS) (Lonza Group). All performed tests and final analyses were made after the seventh and/or eleventh cell passages when the cell confluence reached approximately 75%–90% when microscopically assessed. Cells used in the current study were derived from a single subject, and experiments were performed in 2 (fusion index, X-gal assay) or 3 (real-time polymerase chain reaction [RT-PCR], annexin test) technical replicates.

The passage number represented a population of young and old cell populations. This selection was based on our previous laboratory experience and observations [15]

indicating the best proliferation potential around passage 7 and the onset of differentiation and changes in cell morphology around passage 11 when the cell divisions began to be exhausted, which was also confirmed by staining for β-galactosidase activity.

Transfection of SkMDS/PCs with the miR-195 inhibitor

The oligonucleotide lipofection was performed using a Viromer® BLUE kit (Lipocalyx, Halle, Germany). Single stranded and chemically modified (2'-O-Methyl-RNA) for mature miRNA knockdown was transferred: HmiR-AN0282-SN-5 (GGAGCAGCACAGCCAAUAUUGG) (miArrest™ miRNA Synthetic inhibitors, GeneCopoeia, Rockville, MD, US). The experimental procedures of transfection (lipofection) were performed when the cells were at 75% confluence in the fourth cell passage (Supplementary material 1).

miRNA expression analysis

The miR-195p expression was assessed using quantitative reverse transcription PCR (qRT-PCR) (Supplementary material 2).

Evaluation of CD56-positive cells

Flow cytometry was used to determine the purity of the human skeletal SkMDS/PC populations under study three days after lipofection. The SkMDS/PC transfections were first evaluated using anti-CD56 PC5 conjugate (Beckman Coulter, Brea, CA, US). Briefly, 2.5×10^5 cells were harvested, centrifuged, and resuspended in 100 µl of PBS with 2% fetal bovine serum (FBS) and 10 µl of antibodies (1:200 dilution). After 20 minutes of incubation, the cells were centrifuged, resuspended in PBS with 2% FBS, and further analyzed (Beckman Coulter, Brea, CA, US).

Differentiation potential for myotube formation

To estimate human SkMDS/PC differentiation, *in vitro* cultures were kept under the regime of the cell differentiation protocol. Cells were cultured in 6-well plates, and 1 ml of the cell sample per studied variant was examined. The fusion index (FI) of all the cell populations under study was determined. The differentiated cell populations were fixed in freezing methanol:acetic acid (3:1) solution and stained using a Giemsa solution (Merck, Darmstadt, Germany). Photographs were taken using a standard light microscope. All the cell nuclei shown in the microscopy images were counted (≥450 nuclei). FI was defined as the ratio of the number of nuclei present in the differentiated myotubes (Nd) vs. the total number of nuclei × 100: FI = Nd/ /(Nd + Nnd) × 100.

Cell senescence assay

Senescence-associated β-galactosidase (SA-β-Gal) activity was evaluated using a cell senescence detection kit (BioVision, Milpitas, CA, US) according to the manufacturer. The kit was designed to chemically detect SA-β-Gal activity in cells cultured *in vitro*. The SA-β-Gal is present only in senescent cells and has not been found in pre-senescent, quiescent, or immortal cells. SkMDS/PCs were observed and counted under a light microscope DMi8 (Leica, Wetzlar, Germany). Advanced senescent cells (dark blue), senescent cells (light blue), and young cells (pink) were counted using the Java-based imageJ processing program and converted to percentages.

Detection of apoptosis

To study the SkMDS/PC populations (wild type [WT], non-specific fluorescent dyes; fluorescent aptasensor for miRNA [FAM], miR-195 inhibitor), we used the Annexin V-FITC Kit (Beckman Coulter, Fullerton, CA, US). The cells were washed twice with cold PBS and then resuspended in a binding buffer at a concentration of 5×10^6 cells/ml. Then, 100 µl of cell sample was incubated with 1 µl of Annexin-V-FITC solution and 5 µl of propidium iodide (PI) for 15 min in the dark and analyzed via flow cytometry (Beckman Coulter, Brea, CA, US). The main assumption of the test was the ability of Annexin V to bind inversely located phosphatidylserine. Annexin V is linked to fluorescein (FITC) and labels apoptotic cells. Apoptotic cells were stained with Annexin V+/ /PI– for early apoptosis or Annexin V+/PI+ for late apoptosis.

Gene expression analysis: Real-time polymerase chain reaction (RT-PCR)

Gene expression analysis was in detail described in Supplementary material 3. The qPCR experiments were performed using the iCycler detecting system (BioRad, Hercules, CA, US), and mean cycle threshold (Ct) values were estimated with BioRad CFX Manager 3.1 software. Relative expression levels were calculated using the 2–DCt formula.

Statistical analysis

Each experiment was performed at least twice, and each experimental variant was reproduced in triplicate. GraphPad Prism7 statistical software was used for statistical analysis. Data were analyzed using one-way ANOVA: Tukey's Multiple Comparisons tests concerning CD56 presence, apoptosis, gene expression, and miR-195 expression while Bonferroni multiple comparison test was used with respect to Fusion Index. Two-way ANOVA with Bonferroni multiple comparisons test was applied for X-gal data. Results were presented as mean and standard deviation (SD).

RESULTS

Characteristics of human muscle-derived stem/ /progenitor cell samples

The percentage of CD56-positive cells was defined in a human skeletal SkMDS/PC population by flow cytometry using an anti-CD56-specific antibody and described in detail in Supplementary material, *Figure S1*.

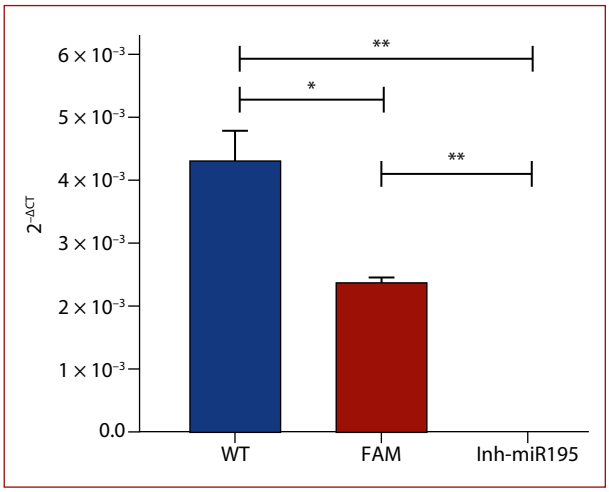

Figure 1. The expression level of miR-195 in SkMDS/PC populations *in vitro* at 72 hours after lipofection (transfection) — from the fourth cell passage. WT-wild type SkMDS/PC population, FAM-myoblasts lipofected wich control FAM molecule (FAM™ dye-labeled synthetic nonspecific miRNA inhibitor), Inh.miR195 — SkMDS/PCs lipofected with miR-195 inhibitor. Values are given as mean and standard deviation (SD)

*P <0.05; **P <0.01
Abbreviations: FAM, non-specific fluorescent dyes; fluorescent aptasensor for miRNA; WT, wild type

The miRNA expression analysis

Based on the real-time PCR analysis, we have demonstrated that the SkMDS/PCs with the inhibitor showed lower, virtually undetectable, expression of miR-195 when compared to the control population with non-specific inhibitor (FAM) or the wild-type cells population (P <0.01) (Figure 1). The efficiency of miR inhibitor transfection has been presented in Supplementary material, *Figure S1*.

Myotube formation

The population of miR-195-inhibited SkMDS/PCs exhibited a superior ability for myotube formation, as shown in Figure 2. The fusion index is a ratio of the nuclei-fused cells to the number of non-fused SkMDS/PCs cells (× 100). Lipofection of human SkMDS/PCs with a miR-195 inhibitor generated an almost 2-fold higher percentage of fused nuclei (P <0.001) at the seventh *in vitro* cell culture passage (Figure 2A). After the eleventh passage, the cells exhibited a 2-fold reduced fusion ability in general; however, in the miR-195-inhibited SkMDS/PCs population, a 2-fold higher level of fused nuclei, compared to WT cells, was observed (P <0.05) (Figure 2B). We observed a ratio of 11% fused nuclei in the WT population, 17% in the FAM transfected population, and 22% in the SkMDS/PCs transfected with miR-195 inhibitor.

Senescence analysis based on SA-β-gal activity

Higher percentages of the senescent and advanced senescent SkMDS/PCs (SA-β-gal-positive cells) were observed at the eleventh passage vs. the seventh passage, as expected (Figure 3). There was no evidence that the miR-195 inhibitor reversed cell senescence. Except for a slightly higher (approx. 7%) level of senescent miR-inhibited cells at the 11th culture passage than in the WT SkMDS/PCs population (P <0.01), the miR-195 inhibitor did not affect cell aging in human SkMDS/PCs cultured *in vitro*.

Apoptosis

We detected slightly higher levels of cell mortality in the SkMDS/PC population lipofected with miR-195 inhibitor than in the WT cells, as shown in Figure 4.

The percentage of apoptotic SkMDS/PCs in the miR-195-inhibited population was almost 4% higher at the seventh cell passage (P <0.001) (Figure 4A) and 2% higher at the eleventh cell passage (P <0.01) (Figure 4B) compared with the one observed in the nontreated SkMDS/PC population (WT). However, this cell population presented a lower level of apoptosis at the seventh cell passage (P <0.001) and at the eleventh cell passage (P <0.001) when compared to FAM-control lipofected cells.

The gene expression analysis

The expression of antioxidative (*SOD1, SOD2, SOD3, CAT*) and anti-aging (*FOXO, SIRT1*) genes, but also myogenic (*MyoD, MyoG*) genes, was examined in all the cell populations under study. We did not observe a *TERT* gene expression in the SkMDS/PC population (as opposed to the iPS population to which we have access) (Supplementary material, *Figure S3*). Therefore, we used indirect targets (*SIRT, FOXO* gene) as anti-aging markers for the miR195 inhibitory effect on the SkMDS/PCs population. The expression levels of extracellular dismutases, catalase, sirtuin, and *FOXO* genes are shown in Figure 5. We observed no significant differences in the expression level of superoxide dismutase 1 [Cu-Zn] (*SOD1*) between the WT and miR-195 inhibitor-transfected SkMDS/PC populations (Figure 5A). The miR-195 inhibition caused a more than 2-fold decrease in the manganese-dependent superoxide dismutase 2 (*SOD2)* expression level at the seventh passage (2.7-fold; P <0.05) compared to FAM treated cells, and WT SkMDS/PC population and at the eleventh passage (2.3-fold decrease at P <0.01) compared with the one obtained for the WT SkMDS/PC population (Figure 5B). However, extracellular superoxide dismutase 3 (*SOD3*) expression increased by 1.9-fold (P <0.05) in miR-195-inhibited SkMDS/PCs at the 11th cell culture passage (Figure 5B) compared with the one observed for the WT population. The expression levels of the genes encoding catalase (*CAT*) and *SIRT1* remained at similar levels in all the studied SkMDS/PC populations at both cell passages (Figure 5D, E). The expression level of the forkhead box O3 (*FOXO*) gene increased by 1.6-fold in treated with miR-195 inhibitor at the seventh *in vitro* cell passage compared to WT cells (P <0.05) (Figure 5F).

Transfection with the miR-195 inhibitor did not increase the expression of the *MyoD* gene in early *in vitro* culture (seventh passage) compared to WT cells. Even though lipofectamine itself affected *MyoD* expression (Figure 6A), such

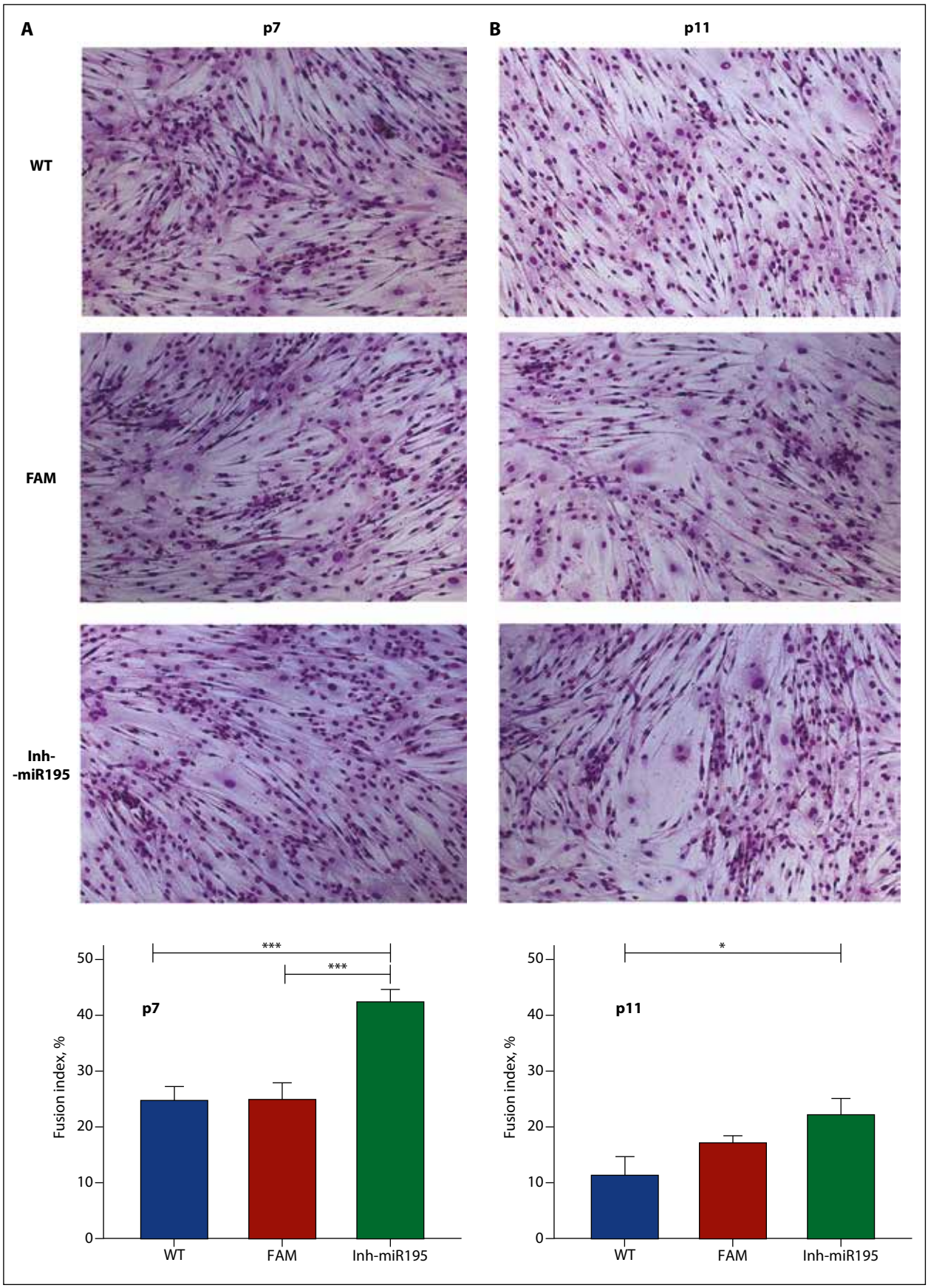

Figure 2. Percentages of fused nuclei in myoblast-originated myotubes at (**A**) the seventh cell culture passage and (**B**) the eleventh cell culture passage. Values are given as mean and standard deviation (SD)

*P <0.05; ***P <0.001
Abbreviations: see Figure 1

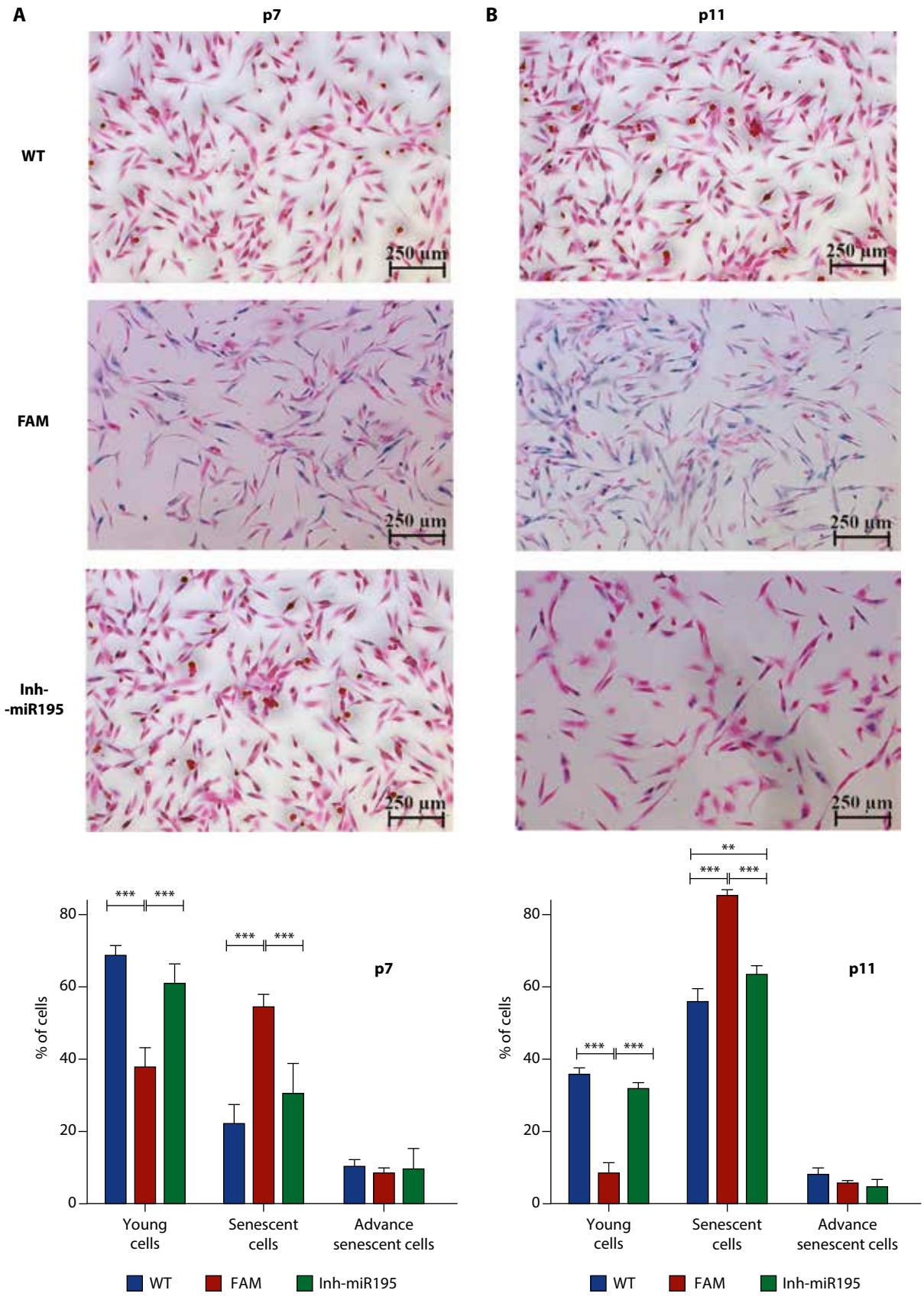

Figure 3. X-gal staining of SkMDS/PCs at the seventh (**A**) and eleventh (**B**) *in vitro* cell culture passages. Percentages of young, senescent, and advanced senescent cells in the WT, FAM, and inh-miR-195 SkMDS/PC populations at the seventh (p7) and eleventh (p11) *in vitro* cell culture passages. Values are given as mean and standard deviation (SD)

***P* <0.01; ****P* <0.001

Abbreviations: see Figure 1

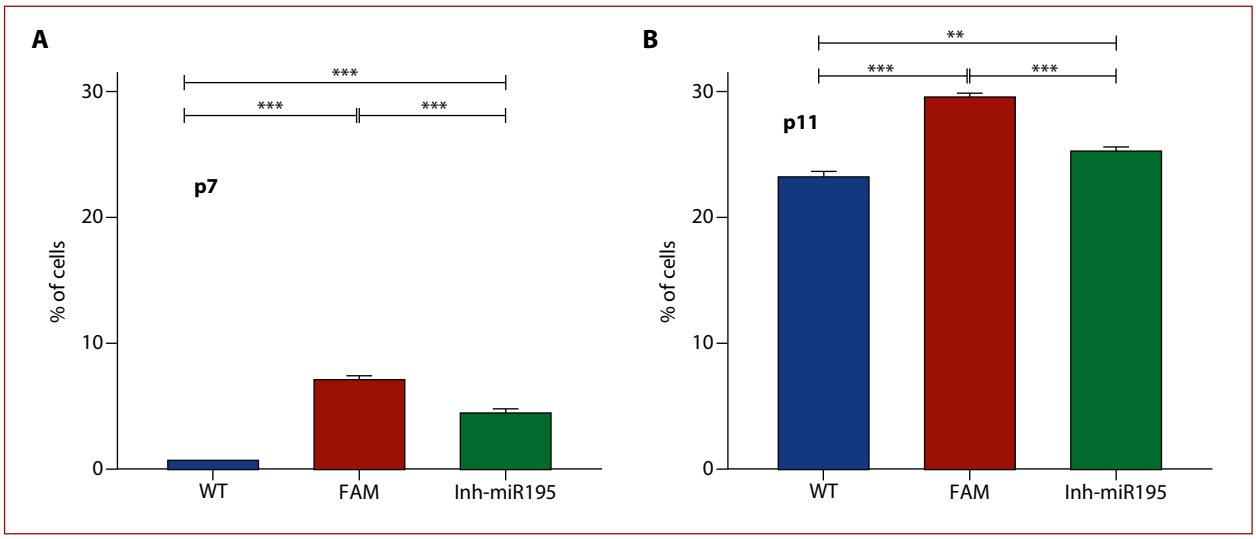

Figure 4. Cell apoptotic rate in the SkMDS/PC populations under study: WT, FAM, and miR-195 inhibitor-treated cells at the seventh (**A**) and eleventh (**B**) *in vitro* culture passages. Values are given as mean and standard deviation (SD)

***P* <0.01; ****P* <0.001
Abbreviations: see Figure 1

effect was eliminated in the miR-195-inhibited cell population, and the level was similar to that one observed in WT SkMDS/PCs. However, the expression of *MyoD* was significantly higher in miR-195 inhibitor-transfected myoblasts at the eleventh *in vitro* cell culture passage (more than 2-fold) (P <0.05) (Figure 6B). In late SkMDS/PC cultures (eleventh passage), *MyoG* expression decreased (almost 4-fold) in the miR-195 inhibitor-transfected population compared with that one observed in the WT culture (P <0.01) (Figure 6D), in contrast to that seen at the seventh cell passage (not statistically significant).

DISCUSSION

The present study aimed to assess the biological effect of miR-195 inhibition on *in vitro* cultured human SkMDS/PC properties with a prospective application for post-infarction heart regeneration.

From our previous experience, we knew that SkMDS/PCs at the seventh passage have been most frequently used for cell therapy, as they divided intensively and presented a myoblast-representative morphology, with low levels of myogenin gene expression indicating a non-initiated differentiation process, marked at the seventh passage (as "young"). Subsequent passages demonstrated a deteriorating quality of cells due to hyperoxic stress of standard *in vitro* culture, and at the eleventh passage, the cells were elongated while differentiating and dividing very slowly; they were marked as "old" in our study.

The cell modifications were required to adapt the cells to cooperate with recipient cardiomyocytes. Without such modifications, native myoblasts may become senescent, cause arrhythmia, and migrate out from the delivery site, thereby not fulfilling their function [17] Modifications have been also required to extend their "parental potency". Lipofection is a common method of transferring a genetic construct into the cell genome [18], with DNA inclusion in lipids or polymer nanoparticles [19].

There is some information regarding the influence of miR-195 inhibition on human myogenicity. To the best of our knowledge, we are the second group to evaluate such a scenario. It was important that miR-195 inhibition had no negative impact on the purity of the SkMDS/PC populations under study and did not affect the SkMDS/PC differentiation process into myotubes. The presence of the CD56 marker typical of SkMDS/PCs remained similar, with approximately 90% of CD56-positive cells being maintained in the treated cell populations (Figure 1).

The first approach showed significantly higher levels of miR-195 in old SkMs compared with young ones in the Kondo et al.'s study [20] . The study investigated the effect of miR-195 inhibition on cell reprogramming and observed that the inhibition of miR-195 significantly improved genetic reprogramming efficiency in old SkMs. By inducing inhibition of miR-195, we studied this effect on the purity of the SkMDS/PC population and the SkMDS/PC differentiation into myotubes, apoptosis, and possible reversal of cellular aging. We observed the presence of the CD56 marker typical of SkMDS/PCs up to 90%, indicating the high homogeneity of the population of myogenic origin (Supplementary material, *Figure S1*).

Cell fusion is critical for the normal development of certain tissues, yet the nature and degree of conservation of the underlying molecular components remain largely unknown [21]. The potential for cell differentiation in myotubes was assessed using a fusion index (Figure 3). It was shown that the inhibition of miR-195 led to a statistically significant increase in the cell fusion level (Figure 3), and thus, to the ability to form myotubes. Such a feature could be useful for regeneration of the post-infarction myocardium in general and could help treat chronic in-

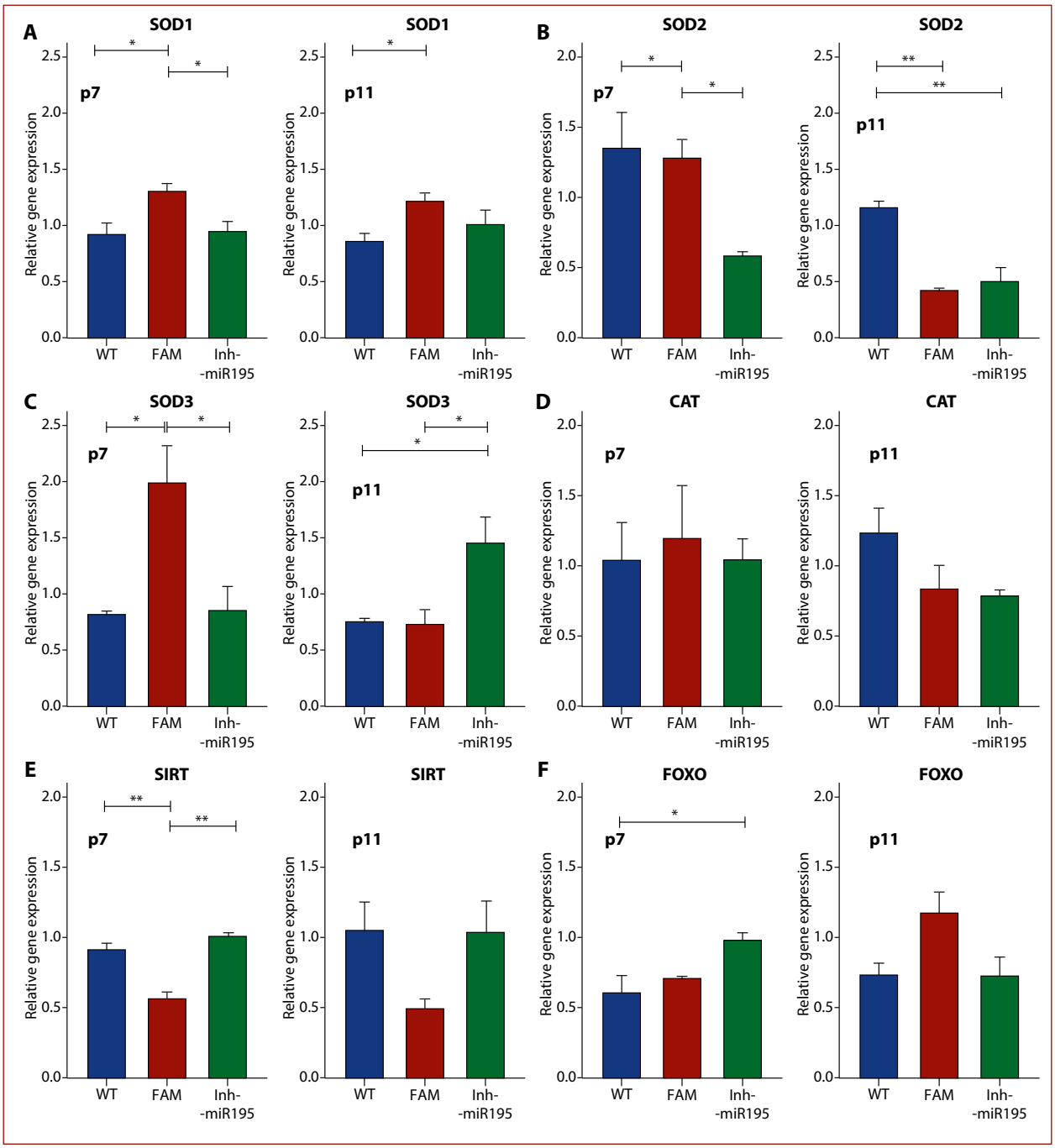

Figure 5. Expression levels of anti-oxidative genes: superoxide dismutase 1 (*SOD1*) (**A**), manganese-dependent superoxide dismutase (*SOD2*) (**B**), extracellular superoxide dismutase 3 (*SOD3*) (**C**), catalase (*CAT*) (**D**); anti-aging genes: sirtuin 1 (*SIRT1*) (**E**); forkhead box O3 (*FOXO*); (**F**) in human SkMDS/PCs at the seventh and eleventh cell culture passages. WT, wild type population; FAM, population treated with miRNAs labeled non-specific inhibitor (carboxyl fluorescein); inh-miR195, cell population treated with miR-195 inhibitor. Values are given as mean and standard deviation (SD)

*P <0.05; **P <0.01

Abbreviations: CAT, catalase; SOD, superoxide dismutase; SIRT, sirtuin; FOXO, forkhead box O3; other — see Figure 1

flammation of the skeletal muscles. However, this effect on SkMS/PCells appears to be different from the observations on cardiac pericardial cells alone. In the study by Dueñas et al. [22], they showed that among micro-RNAs displaying differential expression in the developing epicardium, miR--195 showed a tendency to increase over time. However, we did not evaluate the kinetics of miR195 expression over time but demonstrated the effect of miRNA-195 inhibition on the potential of skeletal muscle-derived cells to fuse. Therefore, the effect of miR-195 inhibition on specific cell line types requires additional studies.

Another identified critical factor in cell therapy is cell aging. Primary cell suspensions cultured *in vitro* normally proliferate for a limited number of divisions before terminal growth arrest and acquisition of a senescent phenotype [23]. The cell population ultimately used for cardiac

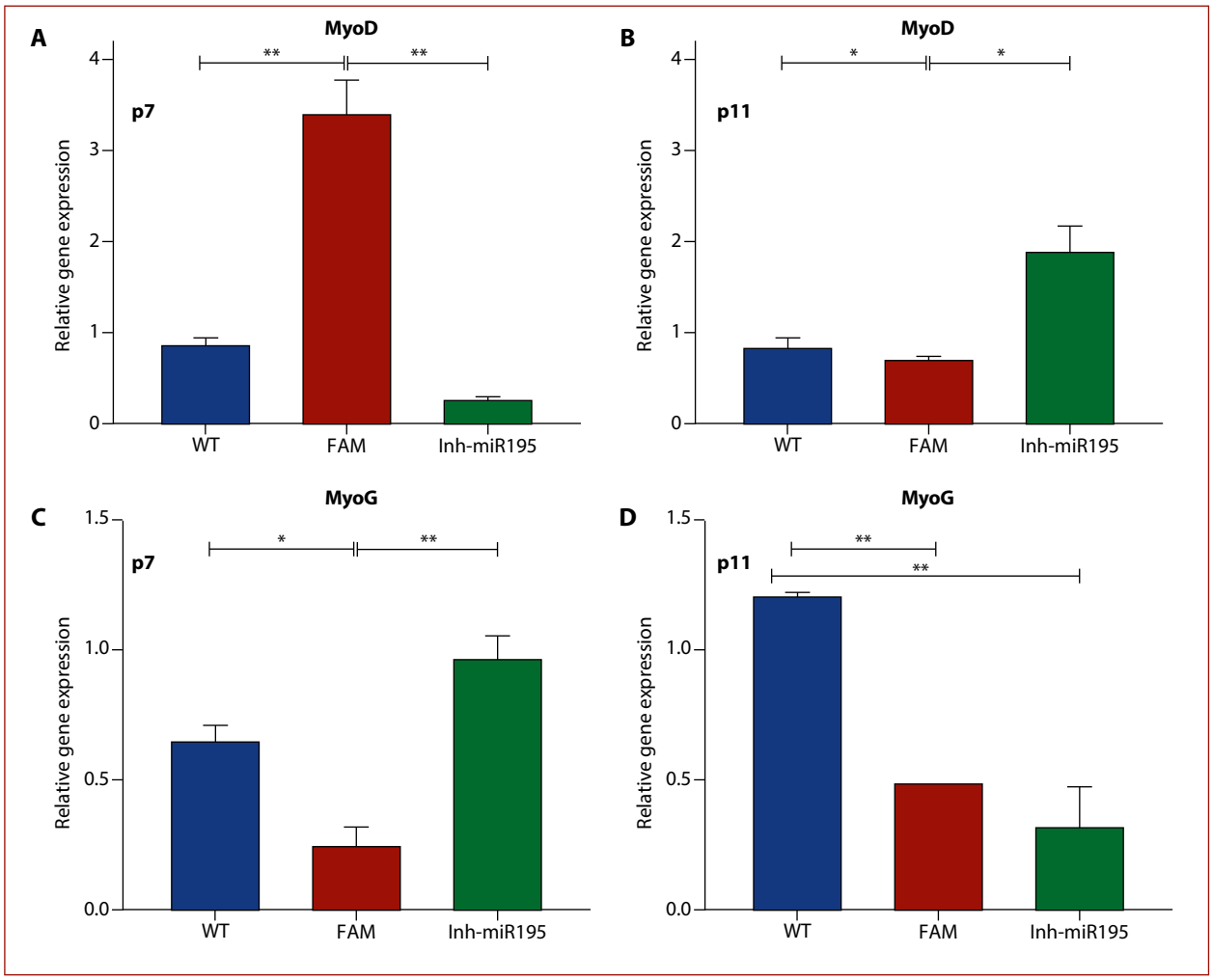

Figure 6. Levels of *MyoD* expression at the seventh (**A**) and eleventh (**B**) *in vitro* culture passages of SkMDS/PCs and the levels of *MyoG* expression at the seventh (**A, C**) and eleventh (**B, D**) *in vitro* culture passages. Values are given as mean and standard deviation (SD)

*P <0.05; **P <0.01
Abbreviations: see Figures 1 and 5

regeneration must be relatively young and capable of division. Therefore, we decided to investigate the effect of miR-195 inhibition on the aging of SkMDS/PC populations *in vitro* using common markers, such as the expression of senescence associated with β-galactosidase (SA-β-Gal) activity. However, we did not observe any significant differences in the percentages of young, senescent, and advanced senescent cells, in the miR-195 inhibited cell population vs the WT population. A higher percentage of senescent and advanced senescent cells were observed in the control-FAM group under study.

Previously, similar studies have been performed on cell populations from young and aged mice. There, the hypothesis of high -galactosidase expression in the aged cell population and significant reduction when miR-195 was inhibited were confirmed [20]. The origin of the cells from donors of different ages appears to be of importance, and the determination of the moment that distinguishes between old and young cell populations *in vitro* has been crucial. For such observations, the distinction into early and late cell passages despite the indications of myogenic gene expression, proliferation potential, and characteristics of undifferentiated myoblasts may not be sufficient for the precise measurement of the aspect of β-galactosidase. In the above-mentioned work, differences were visible when analysis was performed at the same passage of *in vitro* culture in each case.

The overexpression of miR-195-5p and the inhibition of YAP (Yes-associated protein) expression can inhibit cell proliferation and invasion and promote apoptosis. The overexpression of miR-195-5p can inhibit the YAP-mediated Wnt/β-catenin signaling pathway and promote cell apoptosis, so it may be a potential therapeutic target for cancer. Zhao et al.'s results [24] revealed that silencing of miR-195 prevented apoptosis and alleviated cell injury in LPS-induced NCM460 cells [25]. In our study, we observed slight (but statistically important) differences between the levels of apoptosis in the WT SkMDS/PCs population vs. SkMDS/PCs treated with miR-195 inhibitor (Figure 4), which were in favor of the "native" wild-type cells. This ob-

servation may not confirm that inhibition of miR-195 causes lower levels of cell apoptosis.

It was expected that the inhibition of miR-195 would lead to changes in the expression background of anti-apoptotic genes. Visualization of miR-195 regulatory role in skeletal muscle-derived stem/progenitor cells differentiation, targets, and pathway interactions was presented in Supplementary material, *Figure S4*. The *FOXO3* gene is a member of the forkhead family of transcription factors and is important for self-renewal of skeletal muscle stem cells [26]. We demonstrated for the first time an increased expression level of *FOXO3* at the eleventh cell passage caused by miR-195 inhibition (Figure 5F). *FOXO3* was also expected to be involved in protection from oxidative stress by up-regulating antioxidant genes, such as catalase and *MnSOD* (*SOD2*). After inhibition of miR-195, the SkMDS/PCs showed a significant increase in *FOXO3* expression, which likely led to cell maintenance in the G0 phase and thus had a positive effect on muscle regeneration [26]. Therefore, theoretically, overexpression of *FOXO3* should lead to oxidative stress resistance by stimulating the expression of catalase and *SOD1*. Western blot analysis revealed diminished protein levels of SIRT1 and TERT, as well as forkhead box transcription factor O1 (FOXO1) that interacts closely with SIRT1 [27]. However, a study revealed a neutral effect on catalase, the *SOD1* expression level, a visible significant decrease in the expression level of *SOD2*, and a significant increase in the expression level of the *SOD3* gene, showing that the inhibition of miR-195 indirectly affected antioxidant gene expression, which could have a positive impact on the cell microenvironment.

Based on the approach of improving the viability of miR195-inhibited cells [11], we have investigated this relationship. Unfortunately, in the case of the SkMDS/PC population, it was not possible to target the *TERT* gene (Telomerase reverse transcriptase) directly, as the expression of this gene has not been observed in SkMDS/PCs cultured *in vitro* (Supplementary material, *Figure S3*). Therefore, we decided to study indirect gene targets, such as *SIRT* gene expression. It was expected that inhibition of miR-195 would lead to increased expression of *SIRT1*, which is known to protect cells against oxidative stress and promote DNA stability [28]. However, we observed similar *SIRT1* expression levels in miR-195-inhibited SkMDS/PCs at the 7th and 11th *in vitro* cell passages compared with those in the control WT SkMDS/PC population. RT-PCR data showed the mRNA levels of *SIRT1* and *TERT* (targets of miR-195 known as senescence-related genes) were downregulated. It was previously documented that silencing miR-195 increased the protein products of Bcl-2, *SIRT1*, and PIM-1, preventing apoptosis, and reducing some organ injury in mice [29]. However, this could not be confirmed in our SkMDS/PC population.

High expression levels of myogenic genes can mark a certain predisposition of the cells to a particular stage of myogenesis. The *Myf5* and *MyoD* are usually expressed at earlier stages than myogenin [30]. Such sequential expression of myogenic factors defines its early (*Myf5*/*MyoD*) and late (myogenin [*MyoG*] / *MRF4*) stages of myogenesis. *MyoD* plays a role in muscle cell specialization, while myogenin controls the differentiation process and is associated with the formation of myotubes [31]. In the WT SkMDS/PC population, we observed similar expression levels of the *MyoD* and *MyoG* genes at the early stage of *in vitro* culture, whereas, as expected, a high level of *MyoG* expression was observed in the advanced *in vitro* cell culture (Figure 7). The inhibition of miR-195 seemed to reverse this tendency. Thus, we may speculate that inhibition of miR195 can maintain the proliferative potential of the SkMDS/PCs under the presence of *MyoD*, which is quite relevant when planning to use these SkMDS/PCs for the post-infarcted heart regeneration. Also in the other reports, reduced *MyoD* expression in juvenile SkMDS/PC, was a result of miR-195/497 overexpression, and it revealed an intimate link between quiescence and suppression of myogenesis in SkMDS/PC. However, transplantation of cultured miR-195/497-treated SkMDS/PC, resulted in more efficient muscle regeneration in dystrophin-deficient mice, indicating the potential utility of miR-195/497 in stem cell therapy [32]. We might be surprised by the improvement of the cell properties despite the low level of *MyoD*, which may suggest that the cells have entered a later level of differentiation and showed a parallel increase in expression of late myogenic factors (including *MyoG*), which was not examined in the mentioned work. Fortunately, the effect could be explainable because, for muscle regeneration, the cells must first enter the myocyte/myotubes stage and show an increase in *MRF4* and *MyoG* factor expression [33]. In contrast, in our case of *in vitro* culture, we showed increased expression of transcription factor of an early stage of myogenesis, *MyoD* at late *in vitro* 11th passage (Figure 6).

In recent years, *MyoG* studies have indicated that *MyoG* expression precedes final muscle cell differentiation in both *in vivo* and *in vitro* systems and regulates the formation of myotubes [34]. Moreover, myogenin has been found to be expressed in undifferentiated cells, mainly detected in the cytoplasm [31]. The association of miR-195 with myogenesis was also confirmed by Qiu et al. [35]. They observed that upregulation of miR-195/497 is associated with postnatal development. They also confirmed anti-proliferative effects of miR-195/497 *in vitro* by transfecting miRNA mimetics and miR-195/497 inhibitors in C2C12 myoblasts [35]. We have observed downregulation of *MyoG* in the population of miR-195-inhibited SkMDS/PCs, in the elderly *in vitro* cells, which is in line with the results of Qiu et al. [35], showing that as the cell population ages, the *MyoG* factor should increase, but inhibition of miR195 reverses this process. In our study, we purposely delayed the stage of complete differentiation while simultaneously maintaining the myoblasts' ability to proliferate by observed low myogenin

expression in the cells of the advanced *in vitro* culture (11th cell passage).

Despite some data indicating the beneficial effect of miR-195 inhibition on functioning of SkMS/PCs, we should point out a non-uniform reaction towards the antioxidant genes, lack of effect on cell aging, as well as no beneficial influence on apoptosis (contrary to Kondo et al.'s study) [20]. We also did not present an evaluation of gene protein products that may correlate in a different way, but this should be implemented in further studies on the effect of miR-195 inhibition in human stem cells of myogenic origin.

Thus, the use of the miR-195 inhibitor maintains SkMDS/PC myogenicity at the early phase transcriptional level. It improves the ability of cells to undergo myotube formation when induced *in vitro*. However, it does not substantially influence the apoptotic rate nor reverse the cell senescence. Interestingly, it augments extracellular antioxidant activity and may act beneficially *via* a *FOXO*-1-dependent pathway. Therefore, these manipulations may create opportunities for further preclinical studies with preconditioned human skeletal SkMDS/PCs.

Supplementary material

Supplementary material is available at https://journals.viamedica.pl/kardiologia_polska.

Article information

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