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The RhoA nuclear localization changes in replicative senescence: New evidence from *in vitro* human mesenchymal stem cells studies

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Abstract: All non-immortalized mesenchymal stem cells have a limited proliferative potential, that is, replicative senescence (RS) is an integral characteristic of the life of all mesenchymal stem cells (MSCs). It is known that one of the important signs of RS is a decrease of cell motility, and that violations of migration processes contribute to the deterioration of tissue regeneration. Therefore, the characterization of the properties of the cell line associated with RS is a prerequisite for the effective use of MSCs in restorative medicine. One of the key proteins regulating cell motility is the small GTPase RhoA. The main purpose of this work was to study the nuclear-cytoplasmic redistribution of the RhoA protein during RS in MSC lines recently obtained and characterized in our laboratory. The study found that a comparative analysis of the intracellular localization of RhoA in three cell lines (MSCWJ-1, FetMSC, DF2) showed a decrease in the nuclear localization of RhoA during RS.

Introduction

Recently, there has been a significant expansion of biomedical research using mesenchymal stem cells (MSCs) of various origins (Adak *et al.*, 2021; Albu *et al.*, 2021; Merimi *et al.*, 2021; Taei *et al.*, 2021; Wangler *et al.*, 2021; Xiao *et al.*, 2021; Zhang *et al.*, 2022). The status of human MSCs of different origins is determined by a number of characteristics that are postulated in the requirements of the International Society of Cellular Therapy (Dominici *et al.*, 2006; Sensebé *et al.*, 2010). Moreover, all MSCs have a limited proliferative potential, that is, RS is an integral characteristic of the life of all MSCs. RS is a complex dynamic process, induced by genetic and epigenetic alterations, which begins at early passages and gradually intensifies during long-term cultivation, passing into the active stage at the end of which cell proliferation stops (Hayflick, 1965). In addition to decrease in proliferative activity, RS is characterized by other significant changes in cellular properties, including, in particular, morphological changes, an increase in the activity of the SA-β-galactosidase enzyme; lipofuscin accumulation; p16INK4A and p21WAF1/Clip1 -positive staining; negative staining for proliferation markers and lamin B1; decrease in cell motility; an increase in the tumor suppressor genes expression (Gorgoulis *et al.*, 2019; Bobkov and Poljanskaya, 2020).

Thus, the properties of MSCs at different stages of RS, i.e., passed different numbers of passages after isolation from tissue, can differ significantly, which must be taken into account both in the development of biomedical applications and in the interpretation of experimental results. In this regard, it becomes necessary to deepen fundamental research of various characteristics of these cells at different periods of their life, especially the RS associated changes. Along with the listed markers, there is the need to identify other factors that alter in specific senescence contexts. Among these factors, we would like to highlight the activity of intracellular signaling pathways, especially the role of small GTPases in RS of MSCs.

Rho family GTPases and cell motility

It is known that one of the important signs of RS is a decrease of cell motility, and that violations of migration processes contribute to the deterioration of tissue regeneration (Turinetto *et al.*, 2016). Accordingly, assessing the extent to which cell motility can be impaired by prolonged culture is an important step in determining how aging can affect the suitability of cells for biomedical applications. In order to understand changes in cell motility, it is necessary to investigate the intracellular signaling hubs that regulate it.

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Hence, the need to study small GTPases is due to the fact that they are the central regulators of the actin cytoskeleton organization and cell motility (Spiering and Hodgson, 2011), that is in its turn, underlies the ability of MSCs to migrate to the damaged zones to carry out reparative functions. Cell migration occurs through close contact with the extracellular matrix and depends on the organization of the actin cytoskeleton. It is a complex dynamic process that involves permanent remodeling of the cellular architecture, which is necessary for the cell to move and adapt to changes in the environment. This requires rapidly activated and spatio-temporal regulated signaling networks that allow cells to respond to external signals. The Rho family small GTPases are key components of such signaling networks (Sadok and Marshall, 2014; Hervé and Bourmeyster, 2015).

The Rho GTPase subfamily is a member of the Ras-related small GTP-binding protein family that includes RhoA, Cdc42, and Rac1/2 (Wennerberg and Der, 2004). They perform several important cellular functions, including cytoskeleton rearrangement, regulation of cell morphology and motility, and regulation of transcription (Hill *et al.*, 1995; Moorman *et al.*, 1999; Marinissen *et al.*, 2001; Tkach *et al.*, 2005). Most members of this family act as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) forms. The activity of Rho family GTPases is tightly regulated by the guanine nucleotide exchange factors (GEFs), which promote the release of GDP, which makes it possible to bind GTP. GTPase activating proteins (GAPs) inactivate Rho family GTPases by stimulating GTP hydrolysis. Inactive Rho GTPases are isolated in the cytosol by Rho-specific guanine nucleotide dissociation inhibitors (GDIs), which prevent their membrane association. The molecular switching characteristic of Rho GTPases allows them to perform spatio-temporal regulation of signals critical for efficient cell migration (Sadok and Marshall, 2014; Mosaddeghzadeh and Ahmadian, 2021).

RhoA localization changes in MSCs during RS

In this work, we focus on the impact of RS on RhoA distribution between nuclear and membrane associated cytoplasmic fractions. Using the methods of immunofluorescence and confocal microscopy, followed by analysis of colocalization in the obtained images, we carried out a comparative study of the intracellular localization of the RhoA protein in four different lines of human MSCs during long-term cultivation. The following cell lines were used in the work: the DF2 cell line is a non-immortalized human cell line with the status of mesenchymal stem cells and was obtained from the eyelid skin of an adult donor (Krylova *et al.*, 2016); the FetMSCs are mesenchymal stem cells derived from bone marrow of

TABLE 1

The proportion of cells with pronounced β-galactosidase (β-gal) activity during cultivation

Cell line	Passage	Cells count	β-gal, %
DF2	8	1080	4.00 ± 0.60
	13	1045	21.60 ±1.27
	26	856	49.20 ± 1.71
FetMSC	8	1069	3.70 ± 0.60
	16	1606	16.80 ± 0.90
	24	1051	36.50 ± 1.50
MSCWJ-1	8	1025	5.30 ± 0.72
	15	1149	18.98 ± 1.57
	38	1312	57.40 ± 3.06
ADH-MSC	8	2362	10.2 ± 0.6
	12	1718	35.0 ± 1.2
	16	1405	66.6 ± 1.3

Note: β-gal, Beta-galactosidase enzyme activity measured in cells grown and fixed in culture dishes as described in Bobkov *et al.* (2020). Binomial proportion ± 95% CI were computed following the Wilson method (Wilson, 1927).

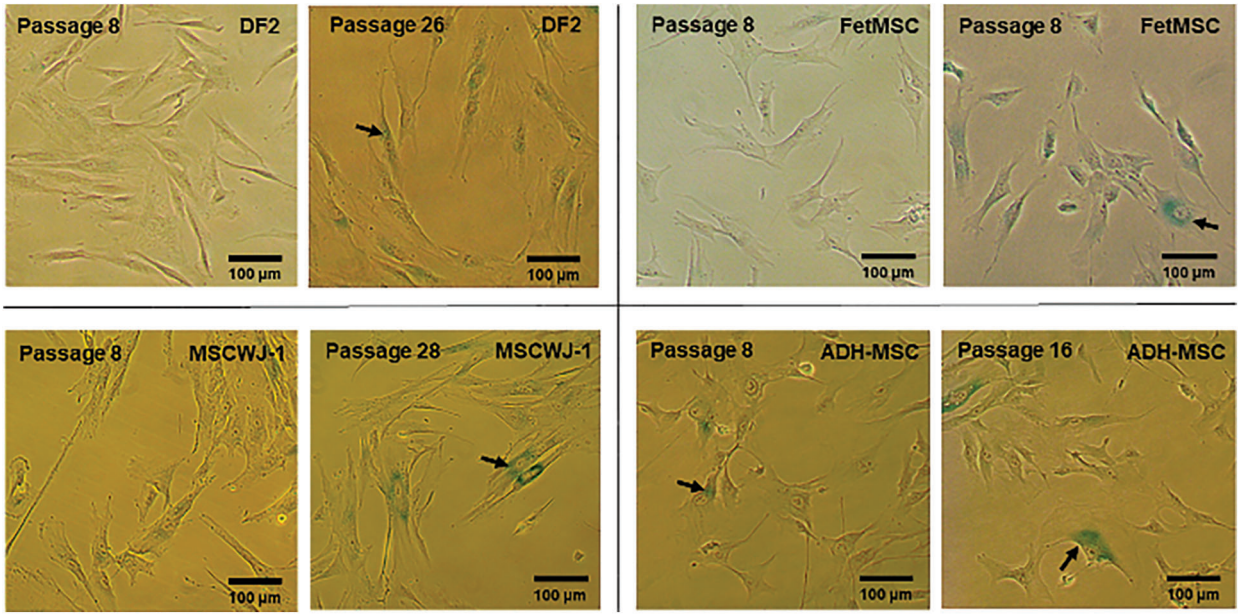

FIGURE 1. Peculiarities of staining for β-galactosidase in human MSCs at different stages of replicative aging. Bright-field images of cells grown in culture flasks and fixed at two passages: the initial (8th passage) and late stages of replicative senescence (the passage number is individual for each line). Images were taken with an inverted light microscope. Cell line names and passage numbers are written on each image. Arrows indicate example cells with pronounced β-galactosidase activity. Scale bar = 100 µm.

5–6 week embryo (Krylova *et al.*, 2012); the MSCWJ-1 is a human MSC line obtained from Wharton's jelly of human umbilical cord (Koltsova *et al.*, 2018); the ADH-MSC line obtained from the heart of an adult donor during coronary artery bypass grafting (Musorina *et al.*, 2019). All cell lines used in this work were obtained from the Center for Shared Use "Collection of Vertebrate Cell Cultures" (Institute of Cytology, Russian Academy of Sciences, St. Petersburg, Russia).

The existence of RS during the cultivation of each cell line was confirmed by assessing the activity of β-galactosidase in cells at different passages (Fig. 1). For convenience, previously unpublished data of this type (DF2 and FetMSC cells) are combined with data that we previously published (ADH-MSC and MSCWJ-1 cells) and shown in Table 1, which demonstrates results indicating a significant increase in β-galactosidase activity in the cells during long-term cultivation (Musorina *et al.*, 2019; Bobkov *et al.*, 2020). Each cell line was cultured for 2 weeks after thawing, and then the experiment was started, during which the cells were in culture for the following number of days: DF2 for 54 days, FetMSC for 21 days, MSCWJ-1 for 84 days, and ADH-MSC for 27 days.

In a series of recent works we performed a comparative analysis of the nuclear-cytoplasmic distribution of RhoA, carried out during long-term cultivation of two different lines of MSCs (MSCWJ-1 and ADH-MSC) obtained from

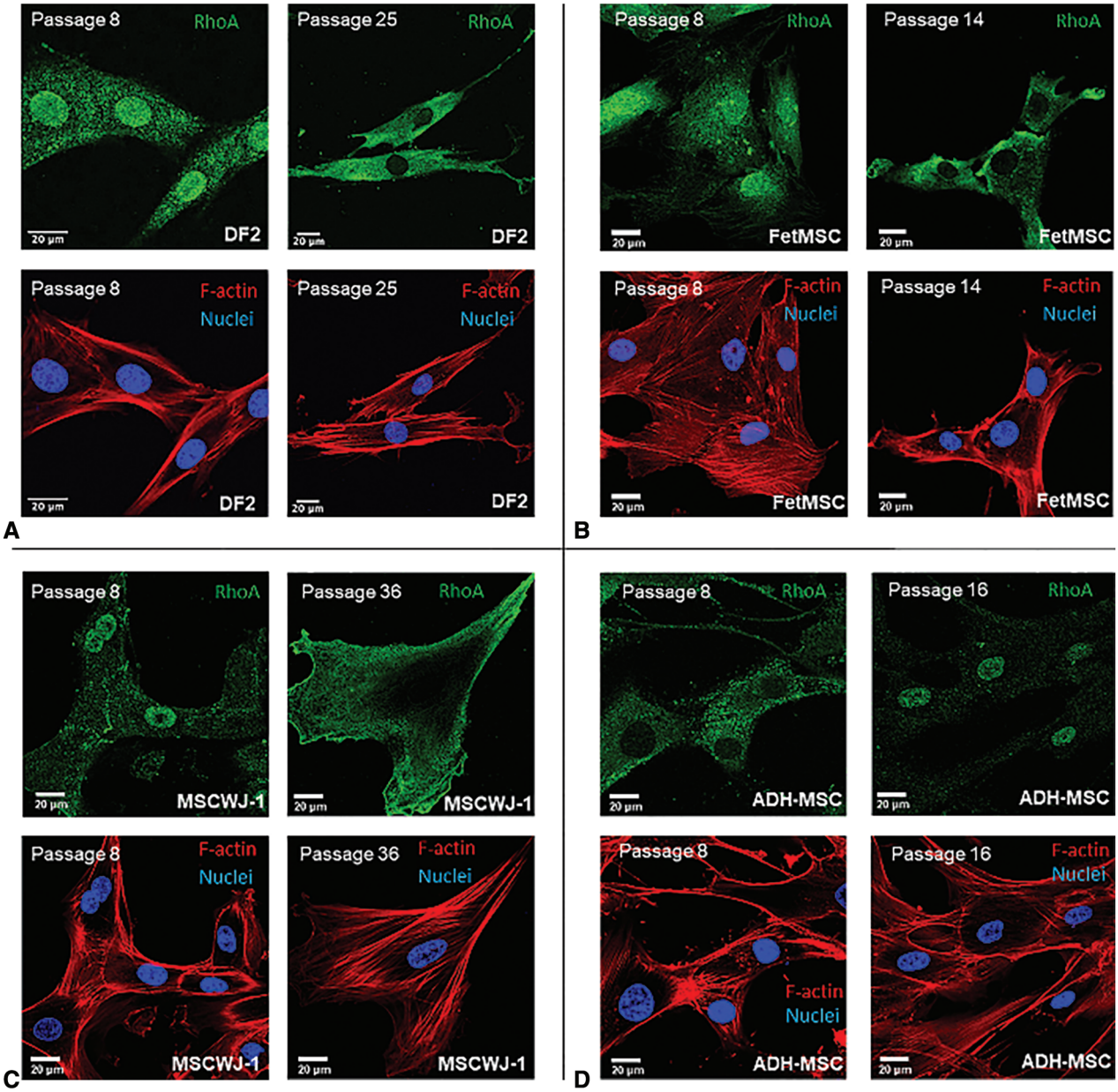

FIGURE 2. Features of the nuclear-cytoplasmic distribution of RhoA in human MSCs at different stages of replicative senescence. The immunofluorescent images of four cell lines (A, DF2; B, FetMSC; C, MSCWJ-1; D, ADH-MSC) grown on glass coverslips and fixed at two passages: the initial (passage 8) and late stages of replicative senescence (the passage number is specific for each line). Cells were stained with anti-RhoA polyclonal antibodies and Alexa 488 second antibodies (green), Rhodamin phalloidin (red), and Hoechst 33342 (blue). The images are optical sections made at the mid-nucleus level using confocal laser scanning microscopy. Cell line names and passage numbers are written on each image. Scale bar = 20 μm.

various sources (Bobkov *et al.*, 2020; Goncharova *et al.*, 2021). The main results, together with the latest data obtained on the FetMSC and DF2 lines are summarized in Figs. 2 and 3.

Using anti-RhoA polyclonal antibodies, we performed an initial screening to identify features that may be associated with RS and to identify key time points that may be useful for further analysis. RhoA/nuclei colocalization analysis was carried out during long-term cultivation, including the stage of active RS in 3 lines: DF-2, FetMSC, and MSCWJ-1, which were obtained from healthy donors, but isolated from different organs (Krylova *et al.*, 2012, 2016; Koltsova *et al.*, 2018). Another cell line, ADH-MSC, was obtained from an unhealthy donor and isolated from an unhealthy organ (heart) during coronary artery bypass grafting (Musorina *et al.*, 2019).

To assess the nuclear localization of RhoA, we used the Kendall's tau correlation coefficient (bTau) calculated between image channels corresponding to RhoA and nuclei staining, this method was described by us in detail earlier in Bobkov *et al.* (2020). The original data of bTau and bGal, together with the scripts necessary for calculations and drawing of graphs, are available online under the MIT license at https://github.com/dan609/biocell.

A decrease in the level of RhoA in the nucleus and its accumulation in the cytoplasm during RS were shown in DF2 and MSCWJ-1 lines. The lowest level in the nucleus is observed at the stage of active RS. A similar picture of a decrease in the RhoA level in the nucleus also takes place in the FetMSC line during cultivation up to passage 14 (Fig. 3); in the future, the study of this parameter will be continued in FetMSC line, including active stage of RS.

A completely different pattern of RhoA redistribution between nuclei and cytoplasm is observed in the ADH-MSC line. With prolonged cultivation, including the stage of active RS, an increase in the RhoA level in the nucleus and a decrease in the cytoplasm are observed (Figs. 2 and 3). In ADH-MSC line, other cellular parameters are also changed, in particular, early RS occurs, during which karyotypic heterogeneity increases, including clonal chromosomal rearrangements, compared to MSCs from healthy sources (Musorina *et al.*, 2019). Previous studies indicate the presence of dysfunctions in different MSCs obtained from patients with chronic diseases (Costa *et al.*, 2021). In addition, it was shown that cultures obtained from donors with cardiovascular diseases show a lower replicative potential than healthy controls of the same age (Karavassilis and Faragher, 2013).

We have done some additional analysis of the correlation between the levels of nuclear localization of RhoA (bTau) and β-galactosidase. The results presented in Table 2 show that there is a statistically significant negative correlation between the above parameters in cell lines DF2, FetMSC, and MSCWJ-1. The statistically significant positive correlation was found in the ADH-MSC cells.

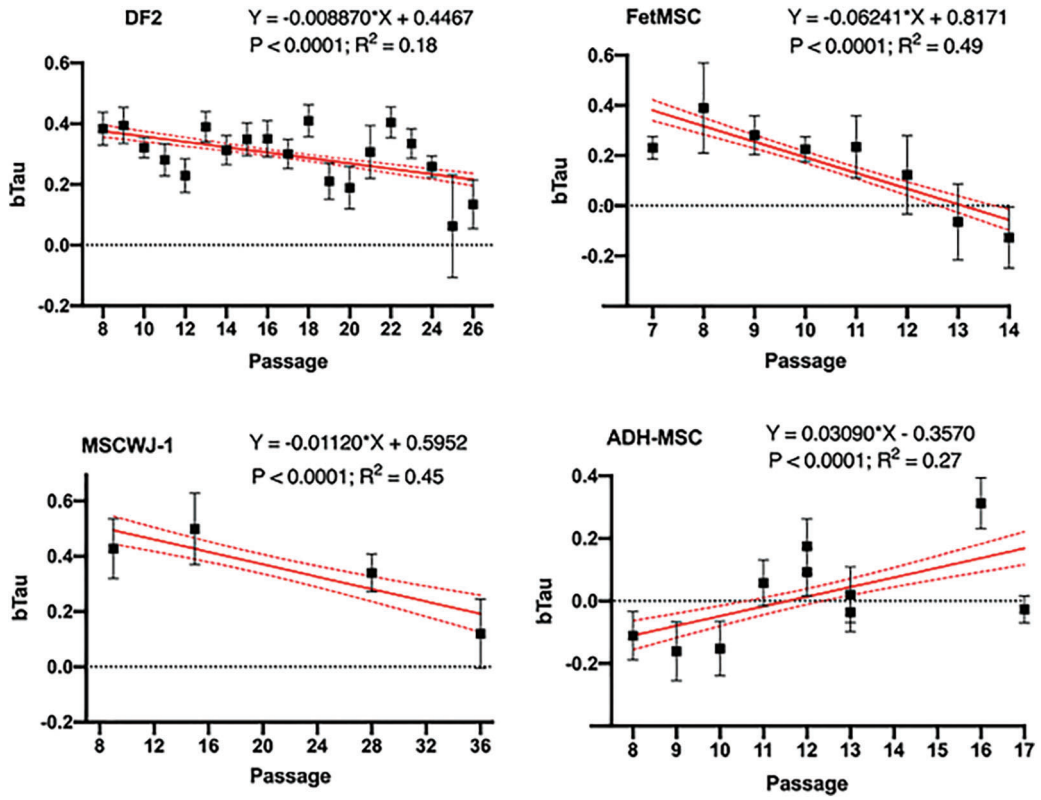

FIGURE 3. Nuclear RhoA localization is passage-dependent in long-term cultivation. Quantification of RhoA/nuclei colocalization performed in four cell lines: DF2, FetMSC, MSCWJ-1, ADH-MSC. bTau, Y–The Kendall's tau correlation coefficient calculated between green (RhoA) and blue (nuclei) channels as described in Bobkov *et al.* (2020). A cell was manually marked as ROI in ImageJ software and used for colocalization analysis, which was one observation. Grouped observations are shown as black squares with whiskers (n = 20, Median ± SD). Linear regression lines (red) with 95% CI (dashed red) were calculated for passage number as predictor and bTau as estimator. Passage, X-number of passages from cell culture isolation to measurement. The linear regression equations, along with the P and R squared values are shown above each plot.

TABLE 2

The results of calculating the correlation between bTau and β-galactosidase levels for four studied cell lines: DF2, FetMSC, MSCWJ-1, and ADH-MSC

bTau/β-gal Correlation Cells	Pearson		Spearman		Kendall	
	Coef.	*P*	Coef.	*P*	Coef.	*P*
DF2	–0.41	<2.2e-16*	–0.38	6.7e-15*	–0.27	1.2e-14*
FetMSC	–0.66	<2.2e-16*	–0.65	<2.2e-16*	–0.49	<2.2e-16*
MSCWJ-1	–0.52	8.2e-08*	–0.48	7.4e-07*	–0.35	4.8e-06*
ADH-MSC	0.43	2.9e-10*	0.50	3.6e-14*	0.34	7.8e-12*

Note: bTau, the Kendall's tau correlation coefficient calculated between green (RhoA) and blue (nuclei) channels in confocal microscopy immunofluorescent images; β-gal, Beta-galactosidase enzyme activity measured in cells grown and fixed in culture dishes as described in Bobkov *et al.* (2020). The values of the Pearson's, Spearman's and Kendall's correlation coefficients (Coef.) as well as their *P*-values are shown. When calculating the correlations, the methods of linear extrapolation and interpolation for missing values were used. Asterisks indicate the following level of statistical significance: *, $P < 0.00001$. Extreme low values are shown in exponential notation.

Conclusion

Thus, based on the results obtained, a preliminary conclusion can be drawn about the presence of a general mechanism of RhoA redistribution during long-term cultivation, including the stage of active RS, in human MSCs isolated from different but healthy sources. There is required further expansion and deepening of research to confirm this assumption. The role of the RhoA protein in cellular processes is multifaceted-on the one hand, it is one of the main regulators of the actin cytoskeleton (Ridley, 2006), on the other hand, its participation in gene expression and DNA damage response is found (Rajakylä and Vartiainen, 2014; Magalhaes *et al.*, 2021, Cheng *et al.*, 2021). It can be assumed that the nuclear or cytoplasmic level of the RhoA protein may change in response to the stress that the cell is experiencing at the moment, depending on external conditions or DNA damage. The question also remains open whether the change in the RhoA concentration in the nucleus/cytoplasm during RS can be programmed by genetic or epigenetic factors. This work shows for the first time the results of a comparative study of changes in the intracellular localization of RhoA occurring in various MSC lines during RS. This opens up perspectives for future research in RS field, aimed at studying the role of signaling pathways associated with RhoA and other small GTPases, in particular, Cdc42 and Rac1, in different cell lines.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: D. Bobkov, G. Poljanskaya; data collection: D. Bobkov, A. Polyanskaya, A. Musorina; analysis and interpretation of results: D. Bobkov, A. Polyanskaya, A. Musorina; draft manuscript preparation: D. Bobkov, G. Poljanskaya. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: Animals and humans did not participate in the experiments.

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