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The alterations in SATB1 and nuclear F-actin expression affect apoptotic response of the MCF-7 cells to geldanamycin

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Abstract

Introduction. The function and localization of actin in the nucleus have not yet been fully described. However, actin seems to be a key protein in nuclear processes interacting with chromatin and matrix proteins. The aim of the study was to evaluate the effect of controlled expression of nuclear pool of F-actin and special AT-rich sequence-binding protein 1 (SATB1) on the *in vitro* induction of active cell death by geldanamycin (GA).

Material and methods. The expression of SATB1 was regulated by the transfection of non-aggressive breast cancer MCF-7 cells with siRNA against SATB1 or expression plasmid with cloned cDNA of SATB1. The altered expression of cofilin-1 in these cells was used to regulate the nuclear expression and localization of F-actin. The effect of GA was analyzed in the context of cell death induction and cell cycle alterations.

Results. Our studies revealed that the targeted regulation of SATB1 and cofilin-1 expression changed the apoptotic response of the MCF-7 cells to GA. The overexpression of these proteins potentiated GA-induced arrest of the cells in the G1 phase of cell cycle and increased the population of the hypodiploid cells.

Conclusion. The alterations in the nuclear expression of SATB1 and F-actin in MCF-7 cells may affect their active cell death in response to GA. *(Folia Histochemica et Cytobiologica 2015, Vol. 53, No. 1, 79–87)*

Key words: SATB1; F-actin; cofilin-1; geldanamycin; apoptosis; MCF-7 cells; siRNA; transfection

Introduction

It has been suggested for decades that nuclear actin exists only in a globular form (G-actin). The early studies that reported the presence of polymerized form of nuclear actin were treated with skepticism and this issue has been questioned [1]. However, increasing body of evidence showing the nucleoplasmic localization of F-actin suggested that it may interact with other nuclear matrix proteins [1–6]. Moreover, there are a number of reports linking nuclear actin with chromatin remodeling during several nuclear processes [5, 7–13]. For instance, the association of nuclear actin, chromatin remodeling and RNA polymerase II transcription has been demonstrated [9]. It has also been speculated that under stress conditions, cytoplasmic actin may translocate into the cell nucleus to function as a modulator of gene transcription [14–16]. Furthermore, numerous proteins which interact with G- and F-actin have been detected in the nucleus [17, 18]. Nevertheless, the direct involvement of actin filaments in transcription and chromatin remodeling has been questioned [19]. Some authors have shown that nuclear actin filaments are too short to serve as tracks for chromatin dynamics

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in the nucleus [6, 20]. They have also referred to the lack of directed movement of nuclear actin filaments and the lack of colocalization between nuclear actin filaments and nuclear myosin [6]. Results of other studies suggested that the conformational alterations and some post-translational changes of nuclear actin may be responsible for differences in its polymerization propensities [21–23].

SATB1 (Special AT-rich Sequence-Binding Protein 1) is a nuclear matrix protein that binds specifically to the AT-rich sequences of DNA by recognizing the base unpairing regions (BURs) [24]. This protein coordinates the control of the expression of various genes even in distant parts of the genome through the regulation of chromatin-loop architecture and transcription. SATB1 forms three-dimensional functional cage-like structures inside the cell nucleus which anchor chromosomes by tethering the BUR regions of the selected genes [25]. Some authors implicated SATB1 also in the regulation of apoptotic cell death [26–29], since degradation of SATB1 was found in cells undergoing apoptosis [27, 28, 30]. We have previously shown that SATB1/nuclear F-actin complex is localized at the border of condensed and decondensed chromatin, which suggests its involvement in the process of transcription [31]. We have also demonstrated F-actin presence in the cell nucleus of different cell lines treated with anti-cancer drugs and speculated that nuclear F-actin may be involved in chromatin remodeling processes during apoptosis and mitotic catastrophe [32–36]. To date, no studies have described the possible association between the apoptotic process and the alterations in SATB1 expression. Moreover, effects of the altered SATB1 expression on the cell cycle have not yet been studied.

Therefore, the aim of this study was to evaluate the effect of controlled expression of the nuclear pool of F-actin and SATB1 protein on the *in vitro* induction of active cell death by geldanamycin (GA), a HSP90 blocker.

Material and methods

Cell culture and treatment. Human breast adenocarcinoma MCF-7 cell line was purchased from American Type Culture Collection (ATCC; HTB-22). The cells were grown in cell culture flasks or 6- and 12-well plates (BD Biosciences, Franklin Lakes, NJ, USA) in a monolayer culture in Minimum Essential Medium Eagle (MEM; Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (FBS; Gibco/Life Technologies, Carlsbad, CA, USA) and 50 μg/ml gentamycin (Sigma-Aldrich) at 37°C in a humidified CO_2 incubator (5% CO_2). 72 hours after transfection with siRNA or expression plasmid, the cells were incubated with 2 μM GA (Sigma-Aldrich) for another 24 hours. Stock solutions of GA were prepared in 100% dimethyl sulfoxide (Sigma-Aldrich), stored at –25°C and serially diluted in complete growth medium immediately before use. The control cells were grown under the same conditions without addition of GA.

Transfection by nucleofection. For the nucleofection of MCF-7 cells, the cells were grown in cell culture flasks (BD Biosciences) up to 80–90% confluence in MEM with the addition of FBS (Gibco/Life Technologies) and 50 μg/ml gentamycin (Sigma-Aldrich). After trypsinization, a total of 2×10^6 cells were transfected using SE Cell Line 4D-Nucleofector X Kit according to the manufacturer's instructions. Briefly, the cells were suspended in 100 μl of the mixture containing SE Nucleofector Solution, together with 3 pmol siRNA against human SATB1 (Hs_SATB1_3; Qiagen, Hilden, Germany), 3 pmol siRNA against human cofilin-1 (Hs_CFL1_3), 2 μg human cDNA of SATB1 cloned into pCMV6-XL5 expression plasmid vector (NM_002971; OriGene, Rockville, MD, USA) or 2 μg human cDNA of cofilin-1 cloned into pCMV6-XL5 expression plasmid vector (NM_005507; OriGene, Maryland, USA), respectively. Then, the mixture was transferred into transfection cuvettes. The electroporation was done using 4D-Nucleofector device (Lonza, Verviers, Belgium) under the program EN-130. As a negative control, the commercially designed AllStars negative control siRNA (Qiagen) or pCMV6-XL5 control plasmid vector (OriGene) were used. After transfection, the cells were grown in medium without antibiotics for 72 h and then they were used for further experiments. The downregulation or overexpression of SATB1 and cofilin-1 by $siRNA_{SATB1}$, $siRNA_{CFL1}$, $pCMV6\text{-}XL5_{SATB1}$ expression plasmid or $pCMV6\text{-}XL5_{CFL1}$ expression plasmid were confirmed by Western blot method.

Western blotting. The semi-quantitative analysis of the post-translational expression of SATB1 and cofilin-1 was performed by Western blot analysis. After transfection and treatment of MCF-7 cells, they were lysed with RIPA buffer (Sigma-Aldrich). After normalization of protein concentration by BCA Protein Assay Kit (Thermo Scientific Pierce, Rockford, IL, USA), the 15 μg of total protein per lane was separated by 4–12% NuPage Bis-Tris gel (Novex/ /Life Technologies, Carlsbad, CA, USA) and transferred to the nitrocellulose membrane using iBlot dry Western blotting system (Invitrogen/Life Technologies). Pre-stained molecular weight markers (Novex/Life Technologies) were used to estimate the position of protein bands. After that, the membrane was processed using WesternBreeze Chromogenic Western Blot Immunodetection Kit (Invitrogen/Life Technologies, Carlsbad, CA, USA) by BenchPro 4100 card processing station (Invitrogen/Life Technologies) according to manufacturer's instructions. Briefly, the membranes were blocked with WesternBreeze Blocking Solution for 30 min

and incubated with primary rabbit monoclonal anti-SATB1 (1:1,000; Abcam, Cambridge, MA, USA), rabbit anti-cofilin 1 (1:1,000; Sigma-Aldrich) or rabbit polyclonal anti-GADPH (1:2,000; Sigma-Aldrich) antibodies diluted in blocking solution for 2 h in room temperature (RT). After washing, the membranes were incubated for 1 h in RT with ready-to-use solution of alkaline phosphatase-conjugated anti-species IgG (Invitrogen/Life Technologies). The immunoreactive bands were then visualized using ready-to-use solution of BCIP/NBT substrate for alkaline phosphatase (Invitrogen/ /Life Technologies). After scanning, the densitometry of the bands was quantified using the Quantity One Basic software (Ver. 3.6.5; Bio-Rad, Hercules, CA, USA).

Cell death analysis. The analysis of cell death was performed using Tali Image-based cytometer (Invitrogen/Life Technologies) and Tali Apoptosis Kit (Invitrogen/Life Technologies) according to manufacturer's instructions. Briefly, after transfection and treatment, the cells were harvested and resuspended in Annexin Binding Buffer (ABB) at a concentration of ~5×10^5 to 5×10^6. After that, to each of 100 μL sample, 5 μL of Annexin V Alexa Fluor 488 were added and incubated at RT in the dark for 20 min. Then, the cells were centrifuged at 300 *g* for 5 min and resuspended in 100 μL of ABB. After the addition of 1 μL of propidium iodine (PI) to each sample, the cells were incubated at RT in the dark for 3 min. 25 μL of stained cells were then loaded into a Tali Cellular Analysis Slide (Invitrogen/Life Technologies). The data were analyzed using FlowJo software (Ver. 10; Tree Star, Ashland, OR, USA) on assumption that viable cells are both Annexin V Alexa Fluor 488- and PI-negative cells. Cells in various stages of apoptosis were Annexin V Alexa Fluor 488-positive and PI-negative or PI-positive, whereas necrotic cells were Annexin V Alexa Fluor 488-negative and PI-positive.

Cell cycle analysis. The analysis of cell cycle was performed using Tali Image-based cytometer (Invitrogen/Life Technologies) and Tali Cell Cycle Kit (Invitrogen/Life Technologies) according to manufacturer's instructions with some modifications. Briefly, after transfection and treatment, the cells were harvested and resuspended in PBS. After centrifugation (300 *g*, 5 min), the cells were fixed in 70% ice-cold ethanol and incubated overnight. The next day, the cells were washed in cold PBS, resuspended in 200 μL of Tali Cell Cycle Solution and incubated for 30 min at RT in the dark. 25 μL of stained cells were then loaded into a Tali Cellular Analysis Slide (Invitrogen/Life Technologies). The data were analyzed using FlowJo software (Ver. 10; Tree Star).

Statistical analysis. The data were shown as mean ± SEM. Statistical comparisons between two groups of cell death or cell cycle data were performed with Mann-Whitney U test. The differences between the groups were considered significant at $p \leq 0.05$. The GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses.

Results

Analysis of post-translational down-regulation and overexpression of SATB1 and cofilin-1 in the MCF-7 cells

Western blot analysis (Figure 1A, B) confirmed altered expression of both SATB1 and cofilin-1 in comparison with appropriate control after the transfection of MCF-7 cells with $siRNA_{SATB1}$, $siRNA_{CFL1}$, $pCMV6\text{-}XL5_{SATB1}$ expression plasmid or $pCMV6\text{-}XL5_{CFL1}$ expression plasmid.

Densitometric analysis showed a decrease in SATB1 (34.51% of initial expression) and cofilin-1 (26.75% of initial expression) post-translational expression in the cells transfected with $siRNA_{SATB1}$ and $siRNA_{CFL1}$, as compared with the cells transfected with non-targeting siRNA, respectively (Figure 1A, B).

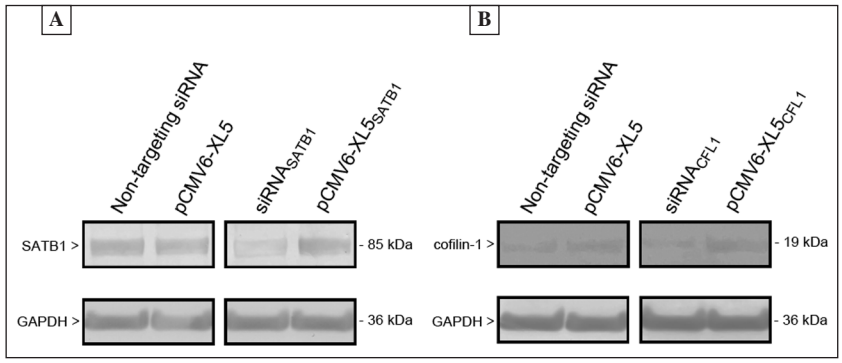

Figure 1. Post-transcriptional expression of SATB1 and cofilin-1 in the MCF-7 cells. **A.** Post-transcriptional expression of SATB1 in the cells transfected with siRNA against SATB1 or pCMV6-XL5 expression plasmid with cloned cDNA of SATB1; **B.** Post-transcriptional expression of cofilin-1 in the cells transfected with siRNA against cofilin-1 or pCMV6-XL5 expression plasmid with cloned cDNA of cofilin-1

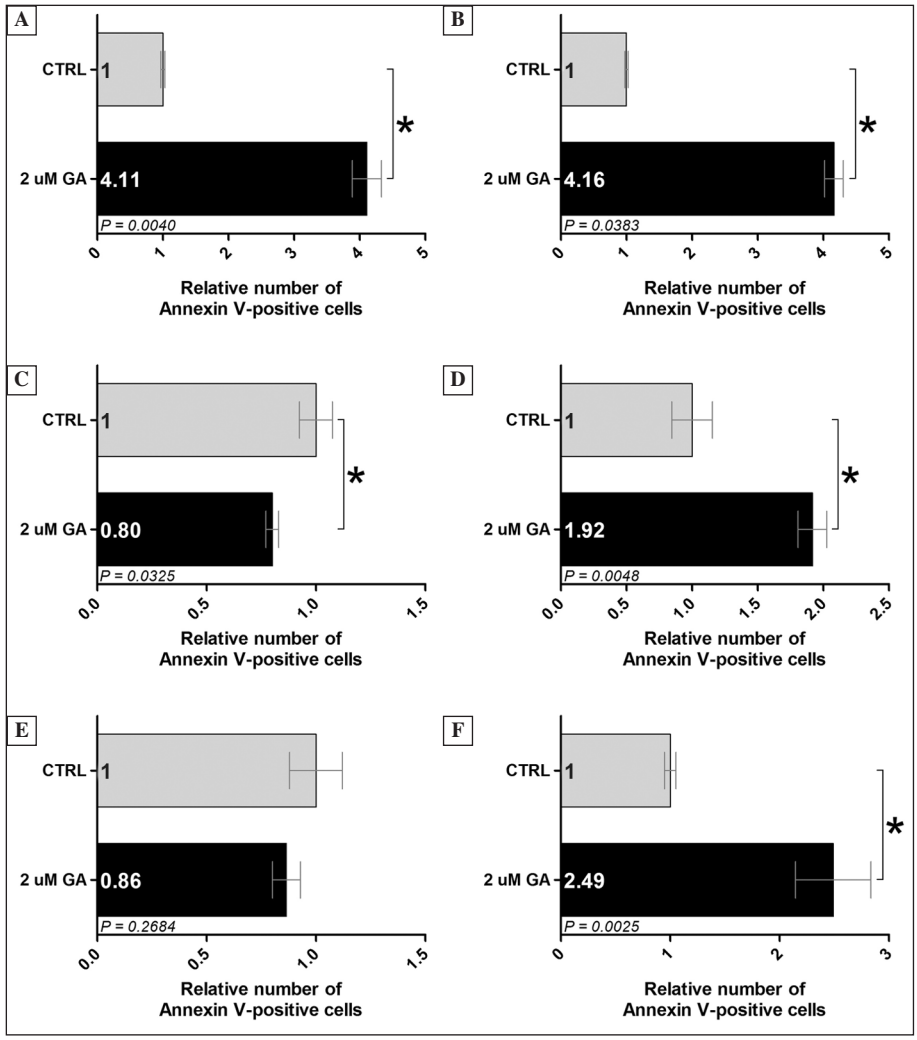

Figure 2. Relative number of the Annexin V-positive MCF-7 cells after the 24 h treatment with 2 μM geldanamycin. **A.** Cells transfected with non-targeting siRNA; **B.** Cells transfected with pCMV6-XL5 empty plasmid; **C.** Cells transfected with siRNA against SATB1; **D.** Cells transfected with pCMV6-XL5 expression plasmid with cloned cDNA of SATB1; **E.** Cells transfected with siRNA against cofilin-1; **F.** Cells transfected with pCMV6-XL5 expression plasmid with cloned cDNA of cofilin-1. Data are expressed as the ratio of the percentage of Annexin V-positive cells after exposition to 2 μM GA to the percentage of Annexin V-positive control cells

Transfection of the cells with pCMV6-XL5$_{SATB1}$ and pCMV6-XL5$_{CFL1}$ increased post-translational expression of SATB1 (134.3% of initial expression) and cofilin-1 (127.8% of initial expression) in comparison with the cells transfected with pCMV6-XL5 plasmids without cloned cDNA of SATB1 and CFL1 (Figure 1A, B).

Alterations in SATB1 and cofilin-1 expression change apoptotic response of MCF-7 cells to geldanamycin

The quantitative analysis of cell death was performed using Tali Image-based cytometer after Annexin V Alexa Fluor 488 and propidium iodide double staining. To avoid the miscount of spontaneous apoptosis induced by the regulation of SATB1 and cofilin-1 expression, the data were presented as relative Annexin V-positive cell counts (ratio of the percentage of Annexin V-positive cells after exposition to 2 μM GA to the percentage of Annexin V-positive control cells).

As shown in Figure 2, treatment of the MCF-7 cells transfected with non-targeting siRNA (Figure 2A) or empty plasmid (Figure 2B) with 2 μM GA increased the relative number of Annexin V-positive cells.

After down-regulation of SATB1 expression by siRNA$_{SATB1}$, the relative population of apoptotic cells following exposition to 2 μM GA significantly decreased (Figure 2C). In turn, overexpression of SATB1 by pCMV6-XL5$_{SATB1}$ in MCF-7 cells treated with

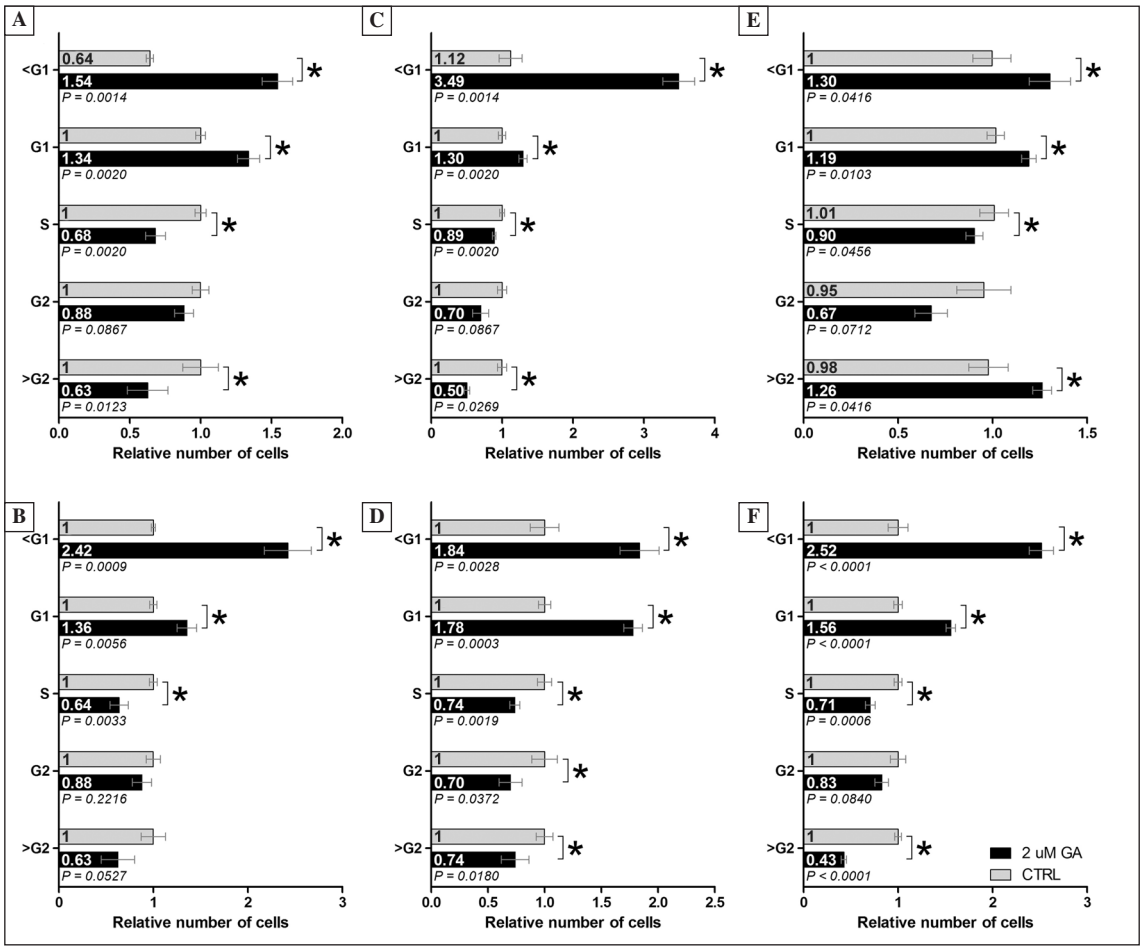

Figure 3. Relative number of the MCF-7 cells in particular phases of cell cycle after the 24 h treatment with 2 μM geldanamycin. **A.** Cells transfected with non-targeting siRNA; **B.** Cells transfected with pCMV6-XL5 empty plasmid; **C.** Cells transfected with siRNA against SATB1; **D.** Cells transfected with pCMV6-XL5 expression plasmid with cloned cDNA of SATB1; **E.** Cells transfected with siRNA against cofilin-1; **F.** Cells transfected with pCMV6-XL5 expression plasmid with cloned cDNA of cofilin-1. Data are expressed as the ratio of the percentage of cells in appropriate phase of the cell cycle after exposition to 2 μM GA to the percentage of control cells in the same phase of the cell cycle

GA almost doubled the relative number of Annexin V-positive cells (Figure 2D).

The down-regulation of cofilin-1 expression by $siRNA_{CFL1}$ in the cells incubated with GA, did not change the relative number of apoptotic cells (Figure 2E). However, the overexpression of cofilin-1 by expression plasmid $pCMV6\text{-}XL5_{CFL1}$ increased by a factor of 2.5 the relative number of apoptotic cells after their treatment with 2 μM GA (Figure 2F).

Overexpression of SATB1 and cofilin-1 in MCF-7 cells augments geldanamycin-induced arrest in the G1 phase of the cell cycle and increases population of hypodiploid cells

The image-based cytometry analysis using the DNA intercalator dye, propidium iodide, was applied to determine the percentage of cells in each phase of the cell cycle. To avoid the miscount of spontaneous changes in the cell cycle distribution induced by the alterations in SATB1 and cofilin-1 expression, the data were presented as relative numbers (ratio of the percentage of cells in appropriate phase of the cell cycle after exposition to 2 μM GA to the percentage of control cells in the same phase of the cell cycle).

Treatment of the MCF-7 cells with 2 μM GA increased the relative number of cells with hypodiploid DNA content (subG1 phase) in the cells transfected with either non-targeting siRNA (Figure 3A) or empty plasmid (Figure 3B). Concomitantly, the population of cells in the G1 phase significantly increased, whereas the proportion of cells in the S phase significantly decreased (Figures 3A and 3B, respectively). Moreover, the exposure of the MCF-7

cells transfected with non-targeting siRNA to 2 μM GA resulted in the reduction of the number of polyploid cells *(*Figure 3A).

Interestingly, similar results were observed in the cells with down-regulated SATB1 expression which were transfected with $siRNA_{SATB1}$. In these cells, after exposure to 2 μM GA, increases in subG1 and G1 population, and decreases in S phase and polyploid populations were observed (Figure 3C). The down -regulation of cofilin-1 did not change the number of cells in G2 phase after GA treatment (Figure 3C).

GA treatment of MCF-7 cells with SATB1 overexpression increased the number of hypodiploid cells that was accompanied by the decreases in the population of polyploid cells and cells in S and G2 phases (Figure 3D).

The down-regulation of cofilin-1 expression in the MCF-7 cells exposed to 2 μM GA increased the proportion of subG1 and polyploid cells, decreased number of cells in the S phase, and did not change the proportion of cells in the G2 phase of the cell cycle (Figure 3E).

Cofilin-1 overexpression in the MCF-7 cells treated with 2 μM GA resulted in the increased population of cells in subG1 and G1 phases, and decreased population of the S phase cells and polyploid cells (Figure 3F).

Discussion

The aim of the study was to evaluate the effect of controlled expression of SATB1 and F-actin in the nucleus of MCF-7 cells on GA-induced cell death and changes in cell cycle. Human breast cancer line used, regarded as a non-aggressive one, has been widely used as a model in the studies of the SATB1 protein due to its easy-to-control expression level [37–40]. There are studies showing that SATB1 reprograms chromatin organization and transcription profiles of breast tumors to promote growth and metastasis [39]. Cancer growth represents an imbalance between cell proliferation and cell death and there is a great need for improving the systemic therapy for breast cancer which is still most frequently diagnosed type of cancer and a leading cause of cancer death among women [41].

Geldanamycin has been often used as a model agent which inhibits Hsp 90 activity. However, the effects of the Hsp90 inhibition are very complex and depend on the biochemical features and molecular background of treated cells [42]. The reported cell cycle effects are mostly G1 and G2/M arrest [43–46]. Here, after GA treatment of MCF-7 cells, we observed the increased incidence of apoptosis and the decreased frequency of polyploid cells with the features of mitotic catastrophe. Moreover, we found that overexpression of SATB1 in these cells promoted the increase in the percentage of subG1 cells and Annexin V-positive cells. A similar effect was observed here after increasing the nuclear transport of actin by cofilin-1 overexpression. These results suggest that in the MCF-7 cells, the SATB1 protein may be directly involved in the apoptotic process through its possible cooperation with nuclear F-actin.

It can be also expected that SATB1 and nuclear F-actin may jointly act to support alterations in chromatin architecture during an active process of cell death. Sun et al. showed that three-dimensional network of SATB1 during early apoptosis was associated with the cleavage of this protein and was accompanied by the nuclear architecture collapse which was mediated by caspase-3 [47]. They also suggested that the architectural changes of chromatin during early apoptosis supported by SATB1 network reorganization could be a mechanism that renders matrix-associated regions of DNA exposed to endonucleolysis [47]. Galande et al., using Jurkat cells as an experimental model, demonstrated that during apoptosis SATB1 is also cleaved by a caspase 6-like protease resulting in the loss of its BUR-binding activity and dissociation from chromatin [27].

It has been shown that actin may translocate into the cell nucleus to function as a transcriptional modulator of gene expression [5, 7–16]. Our earlier studies have demonstrated not only the translocation of actin into the nucleus but also the involvement of nuclear F-actin in chromatin remodeling during cell death processes [32–36]. The results presented in this paper suggest that SATB1 may be involved together with F-actin in chromatin reorganization during cell death. Indeed, the association between nuclear F-actin and SATB1 and their involvement in chromatin remodeling during cell death was observed by us in CHO AA8 cell line [31] and in Jurkat cells [48]. Our previous observations suggest also that the expression of cofilin-1 is essential for the activation of cell death [35]. Here, after exposure of MCF-7 cell with overexpression of cofilin-1 to 2 μM GA, we observed a significant increase of apoptotic cell population and decrease in the number of polyploid cells. Furthermore, Chua et al. have revealed that cofilin-1 plays a key role during the initiation phase of apoptosis. This type of cell death is mediated by the translocation of cofilin-1 to mitochondria, causing the release of cytochrome c, the activation of caspase-3 and caspase-9 as well as the condensation and fragmentation of chromatin [49, 50]. It has also been suggested that oxidant-induced apoptosis and mitochondrial integrity could be regulated through the

oxidation of cofilin-1 [51]. Other studies have shown the reduced release of cytochrome c and the block of apoptosis upon siRNA-induced cofilin-1 knockdown.

Rehklau et al., studying embryonic fibroblasts deficient for cofilin-1 and actin depolymerizing factor (ADF), showed that ADF/cofilin activity is not essential for the initiation or progress of apoptosis in mammalian cells [52]. In the MCF-7 cells with the decreased expression of cofilin-1, no statistically significant differences were found in the relative value of Annexin-positive cells. The data of this report are also in agreement with our earlier results and indicate that the presence of F-actin in the nucleus is a key event during GA-induced apoptosis in the MCF-7 cells [31]. The direct role of SATB1 in the induction of this process was also confirmed. In the present study, we observed the decrease in Annexin V-positive cells in the response to $siRNA_{SATB1}$ treatment. These observations have also been confirmed by our studies on the model of Jurkat cells [48].

Other reports also link SATB1 protein with early events in apoptosis. Gong et al. suggested that SATB1 cleavage at a very early stage of apoptosis results in down-regulation of Bcl-2 expression and modulates the association of SATB1 with the mbr and the promoter. They also showed that the inhibition of SATB1 cleavage by the treatment of cells with caspase-6 inhibitor repressed the disassembly of the SATB1-mediated chromatin loops and restored Bcl-2 expression level [53]. The correlation between SATB1 and Bcl-2 expression was found in glioblastoma and embryonal carcinoma [54, 55]. Some other reports have presented additional data showing the association between SATB1 and HIF-1a expression together with the co-expression of androgen receptor (AR), estrogen receptor (ER), and Bcl-2 in breast cancer [56]. However, any direct relationship between SATB1 and Bcl-2 immunohistochemical expression was found in a tissue microarray study of a breast ductal carcinoma [56]. Of note, in one study a moderate correlation between the expression of SATB1 and proliferation marker Ki-67 in ER-negative breast tumors was found [57]. Moreover, Kowalczyk et al. demonstrated the lower *SATB1* mRNA content in both tumor tissue and unchanged mucosa of colorectal carcinoma (CRC) patients than in the normal colon mucosa, while SATB1 protein levels in colorectal tissues of CRC patients were significantly higher than those in the colon mucosa of healthy controls. It suggest that post-transcriptional events limit the SATB1 expression in CRC [58].

In summary, we showed here that the alterations in SATB1 and cofilin-1 expression affected apoptotic response of the MCF-7 cells to GA. Moreover, cell cycle studies confirmed that the overexpression of these proteins amplify the response of geldanamycin-induced arrest of cells in G1 phase of cell cycle and increased population of hypodiploid cells. Together with our previous results, we conclude here that the nuclear expression and localization of both SATB1 and F-actin is required for the induction of active cell death.

Acknowledgments

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