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RADIOSENSITIZING EFFECT OF RHOB PROTEIN IN MELANOMA CELLS

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ABSTRACT

Melanoma cells are highly resistant to chemo or radiotherapy. DNA damage agents such as ionizing radiation induce apoptosis involving RhoB protein. In a great variety of tumors the levels of this protein decrease along tumor progression. RhoB is considered a tumor suppressor gene due to its antiproliferative and proapoptotic effect. Considering the aforementioned, the aim of this study was to characterize the radiobiological response of different human melanoma cell lines, and to evaluate the possible correlation between RhoB expression and radiosensitivity.

The human melanoma cell lines A375, MELJ and SB2 were gamma-irradiated (^{137}Cs). Survival curves were obtained by clonogenic assay and fitted to the Linear-Quadratic (LQ) model. Radiosensitivity was evaluated by surviving fraction at 2 Gy (SF2). Results showed that MELJ was significantly more radioresistant (SF2=0.71) than A375 and SB2 (0.29 and 0.21 respectively). Expression levels of RhoB, evaluated by western blot, increased in all lines vs. non-irradiated control. SB2, the most radiosensitive cells, showed a greater induction ($p<0.05$) of RhoB. Finally, to study whether RhoB has a radiosensitizing effect, these cell lines were stably transfected with a wild type RhoB construction, a constitutively active RhoB mutant V14, or with the empty plasmid as control. For all cell lines higher expression level of this protein was found in RhoB or V14 transfected cells ($p<0.05$). Sensitization was evaluated by SF2. Significant radiosensitization was demonstrated in clones derived from A375 and SB2 ($p<0.05$), while for MELJ cells, radio-sensitization was only found in clones overexpressing V14.

In conclusion, the increase of RhoB in melanoma cell lines, either by radiation or transfection has a radiosensitizing effect. Thus, we propose RhoB modulation as a potential therapeutic tool to improve the radiation response of radioresistant melanoma.

1. INTRODUCTION

Melanoma is a type of skin cancer resulting from the malignant transformation of melanocytes, melanin producing cells. Melanin is the substance that provides skin its color [1]. Advanced melanoma is the most aggressive type of skin cancer, in part due to its lack of successful treatment. While this tumor is less common than other types of skin cancer, is more metastatic being the main responsible of skin cancer death. The current treatments for cancer include radio and chemotherapeutic agents that damage DNA.

RhoB, a protein that belongs to the family of RhoGTPases, can be induced by physical (γ and UV radiation) and chemical (H_2O_2 and cisplatin) agents [2; 3]. Members of this family have been originally associated with a dynamic role in the regulation of actin cytoskeleton and vesicle transport. However, during the last 10 years researchers in cancer have shown interest in Rho proteins. This is partly due to the fact that these proteins can modulate processes such

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as proliferation, survival, apoptosis, etc. [4]. In this regard, RhoB has a different role to other members of this family. While RhoA, Rac1 and Cdc42 promote oncogenesis, invasion and metastasis [5; 6; 7], RhoB has a function as a tumor suppressor gene [8; 9; 10]. RhoB for example, but not RhoA, inhibits proliferation, induces apoptosis, inhibits tumor growth in a nude mice model [8; 11] and could be involved in the apoptotic process induced by stress. Furthermore, consistently with its tumor suppressor activity, a dramatic decrease in RhoB expression has been observed in tumor biopsies from lung, head and neck, and brain as tumors become more aggressive [12; 13; 14]. During the last years progress has been made in understanding the molecular mechanisms responsible for cellular radiosensitivity through the identification of differentially modulated genes in cells resistant to radiation. Most of the identified genes are related to apoptosis, DNA repair, cell cycle and cell adhesion [15].

In order to know whether this differential behavior in gene expression exists or not in melanoma cells, this work aimed to characterize the effect of radiation in several melanoma cell lines. Besides, considering the regulation of RhoB and its role in apoptosis induced by radiation, the relationship between RhoB and radiosensitivity was studied.

2. MATERIALS AND METHODS

2.1 Cell lines and culture

Human melanoma cell lines A375, SB2 and MELJ were used. The cells were cultured in standard conditions. A375 and SB2 were grown in DMEM:Ham-F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS), 50 µg/ml streptomycin, 50 U/ml penicillin, 5 µg/ml insulin, 1.76 µg/ml ascorbic acid, 150 µg/ml pyruvic acid and 300 µg/ml galactose. MELJ cell line was cultured in DMEM:Ham-F12 (1:1) supplemented with 10% FBS, 50 µg/ml streptomycin, 50 U/ml penicillin and 2.5 µg/ml insulin.

2.2 Clonogenic assay

To evaluate the intrinsic radiosensitivity of each cell line, clonogenic assay was performed after irradiating cells with a gamma ^{137}Cs source (CERBISA, Buenos Aires, Argentina). Briefly, cells were plated on 25 cm^2 culture flask (1000 cells/flask), incubated for 18 h and irradiated with doses of 0.5, 1, 2 and 4 Gy. Control cells were sham irradiated. Cells were incubated for 10-15 days post- irradiation at 37ºC and 5% CO_2. Colonies were fixed with methanol-acetic acid (3:1) and stained with 0.5% crystal violet in 25% methanol. The fraction of clonogenic cells was determined by scoring colonies containing at least 50 cells. Two independent experiments including all the cell lines were performed in triplicates for each condition. Survival curves were fitted to the linear quadratic model using GraphPad Prism 5 program for processing the data (Eq. 1):

$$S = \exp-(\alpha D+\beta D^2) \quad (1)$$

The parameter α and the survival fraction at 2Gy (SF2) were analyzed.

2.3 Western blot

For immunoblot analysis, cells were lysed (lysis buffer: 20 mM Tris-HCL, pH.7.5; 150 mM NaCl; 10% glycerol; and 1% Triton X-100), and cell extracts were analyzed by SDS-PAGE followed by nitrocellulose membrane blotting with specific rabbit polyclonal antibodies for detecting RhoB and actin proteins.

Finally, bands density was quantified using Image J. The density values obtained from the RhoB band were relativized to its actin density band, and the results were expressed as the ratio RhoB/actin.

2.4 Phalloidin staining

It has been described that RhoB overexpression inhibits cellular migration, invasion and metastasis [16], all mechanisms mediated by actin cytoskeleton dynamics. In order to study the cellular actin organization in control and irradiated cells, the presence of actin fibers was evaluated and qualitatively compared in all the cell lines under the different treatments. Cells were plated on 60 mm diameter cell culture dishes and allowed to grow to 70 % confluence. They were washed with PBS, fixed with 4% paraformaldehyde in PBS and washed again with PBS. Cells were permeabilized with triton X-100 0.5% in PBS for 15 minutes on ice, and nonspecific binding sites were blocked with FBS 5% in PBS for 30 min at room temperature. Then, cells were incubated with conjugated phalloidin isothiocyanate tetramethylrhodamine (Sigma) 1:100 in PBS for 1 h in a humid chamber and washed with PBS. Samples were observed with an Olympus BX51 fluorescence microscope using a 100X objective (UPlan Fl 40 X / 0.75).

2.5 RhoB stable overexpression

Cell lines A375, MELJ and SB2 were stably transfected with a plasmid pcDNA3 containing the coding sequence of human RhoB. Also, the different cell lines were transfected with a plasmid containing a constitutively active mutant version of RhoB. As a negative control, each cell line was transfected with empty plasmid. All the transfections were performed using cationic lipids (Lipofectamine 2000 ®, Invitrogen) according to manufacturer's instructions. Geneticin resistant clones were selected by clonal dilution and subsequently analyzed for their expression of RhoB by western blot.

3. RESULTS

3.1. Characterization of the intrinsic radiosensitivity of human melanoma cell lines.

Figure 1 shows the survival curves obtained after clonogenic assay of the three cell lines studied. It can be observed that the curves fall from 1 Gy dose for A375 and SB2 cell lines, while for MELJ the curve presents a smaller slope, indicating its higher radioresistance. This behavior is also shown by the surviving factor at 2 Gy (SF2) as a sensitivity parameter (Table 1).

Table 1. SF2 parameter obtained from the fitted survival curves of the cell lines MELJ, A375 and SB2 gamma irradiated.

Línea celular	SF2
MELJ	0,71
A375	0,29
SB2	0,21

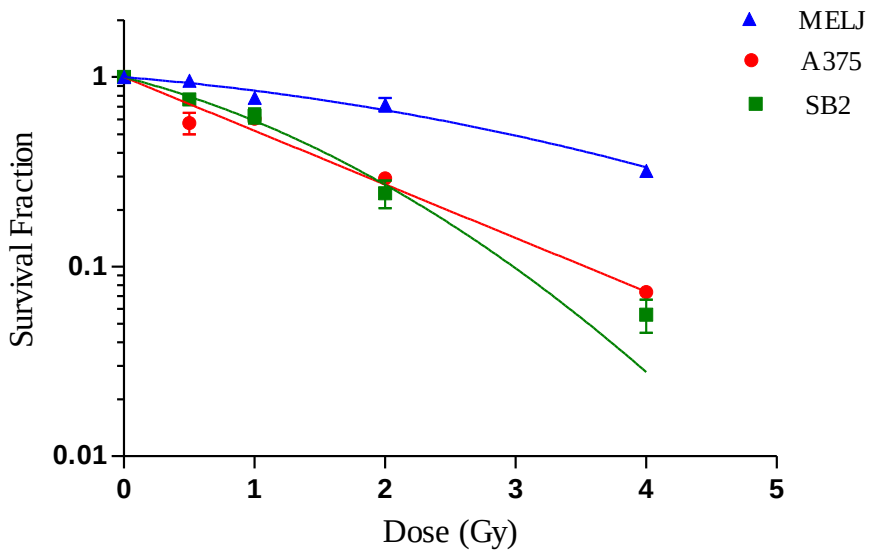

Figure 1. Survival curve of human melanoma cell lines MELJ, A375 y SB2 gamma irradiated (^{137}Cs) and adjusted to the linear quadratic model.

3.2 The radioresistance of the different cell lines and RhoB level expression are not correlated.

It has been described that RhoB protein has tumor suppressor activity in lung and head and neck cancer [8; 10; 11], and that its levels decrease dramatically with tumor aggressiveness [17]. Considering this, the basal expression levels of RhoB were analyzed in A375, SB2 and MELJ human melanoma cell lines (Figure 2).

RhoB expression level for MELJ, the most radioresistant cell line, far exceeds the one observed for SB2, while RhoB level for A375 is much closer to MELJ than SB2. This result indicates that the radioresistance of the cells is not related to RhoB protein levels.

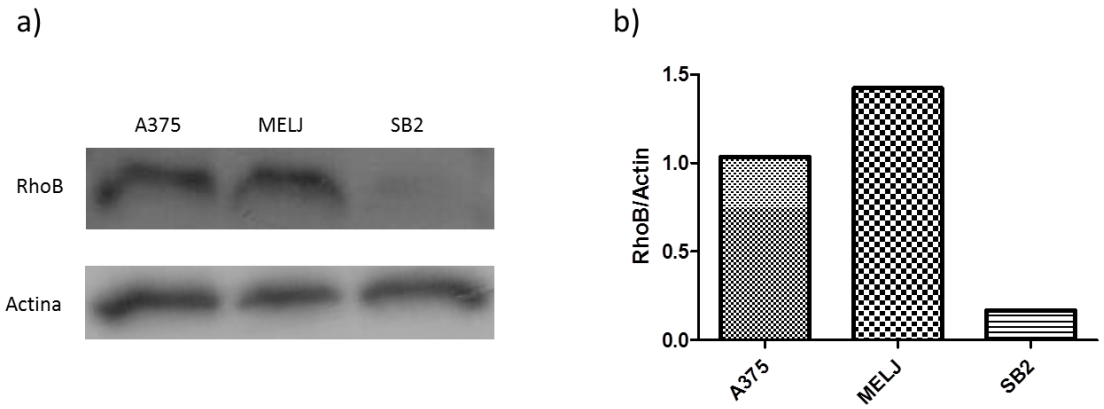

Figure 2. Expression level of RhoB protein. Western blot analysis in A375, MELJ and SB2 cell lines. a) Representative western blot image. b) Densitometric analysis of RhoB / Actin.

3.3 Effect of radiation on RhoB expression

It is known that certain DNA damaging agents, such as radiation, lead to an increase in RhoB levels [18]. The possible induction of RhoB expression by radiation was tested by western blot in protein extracts obtained from irradiated cells.

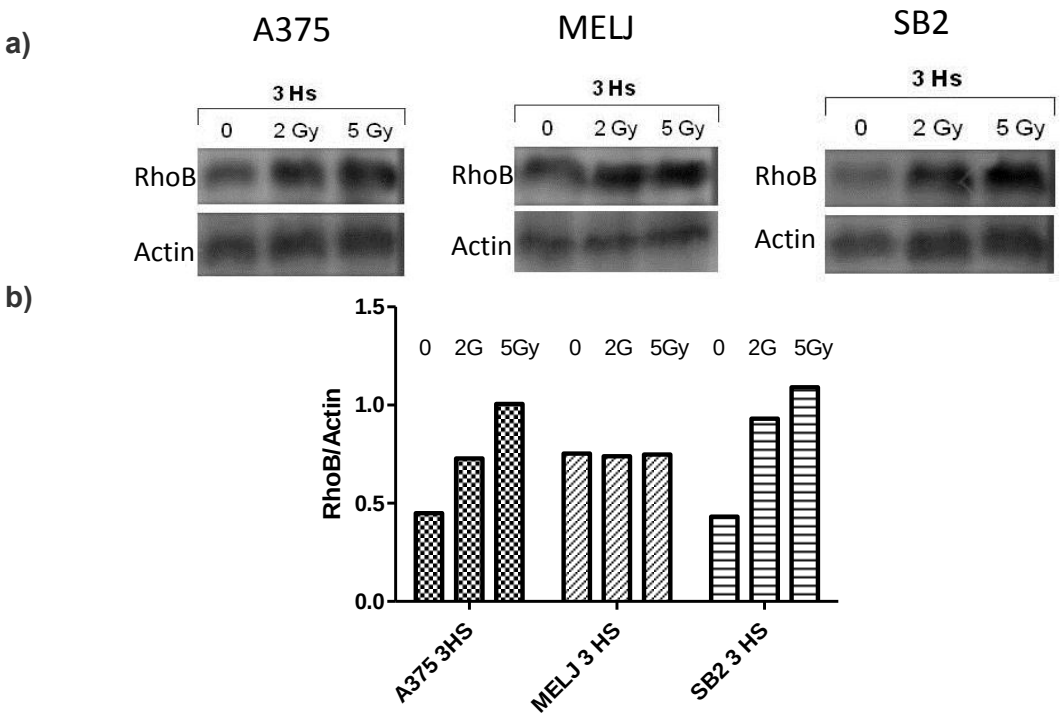

Figure 3. Profile expression of RhoB. a) Western Blot analysis on cell lines A375, MELJ and SB2 irradiated at 2 and 5 Gy, 3 hs post irradiation. b) Densitometric analysis of the RhoB western blot bands normalized respect to each corresponding actin bands.

Both cell lines A375 and SB2 showed a similar expression profile, where RhoB levels increased with radiation dose at 3 hours post irradiation (Figure 3). On the other hand, for MELJ cell line no differences in RhoB expression were detected for the different doses and compared to unirradiated control (Figure 3).

3.4 Radiation effect on actin cytoskeleton

RhoB has a specialized role in intracellular receptor trafficking [18], and activation of Rho proteins induces the appearance of large actin filaments known as stress fibers [19]. Considering this, it might be speculated that alterations in the actin cytoskeleton may play an active role in the sensitization of neoplastic transformed cells to DNA damage [18]. In order to evaluate whether radiation leads to a reorganization of actin fibers, the three cell lines in study were stained with phalloidin (an organic actin-binding molecule) and DAPI to identify cell nuclei.

Figure 4 shows stress fibers in each cell line. Regarding the basal levels of RhoB and stress fibers in the different cell lines, the higher the expression level of RhoB the higherthe stress fibers content. In this sense, MELJ is the cell line that exhibits more stress fibers, and their

presence in SB2 is insignificant. In turn, it can be qualitatively seen that irradiation induces these fibers formation on A375 and SB2 lines, while in MELJ this induction is not observed.

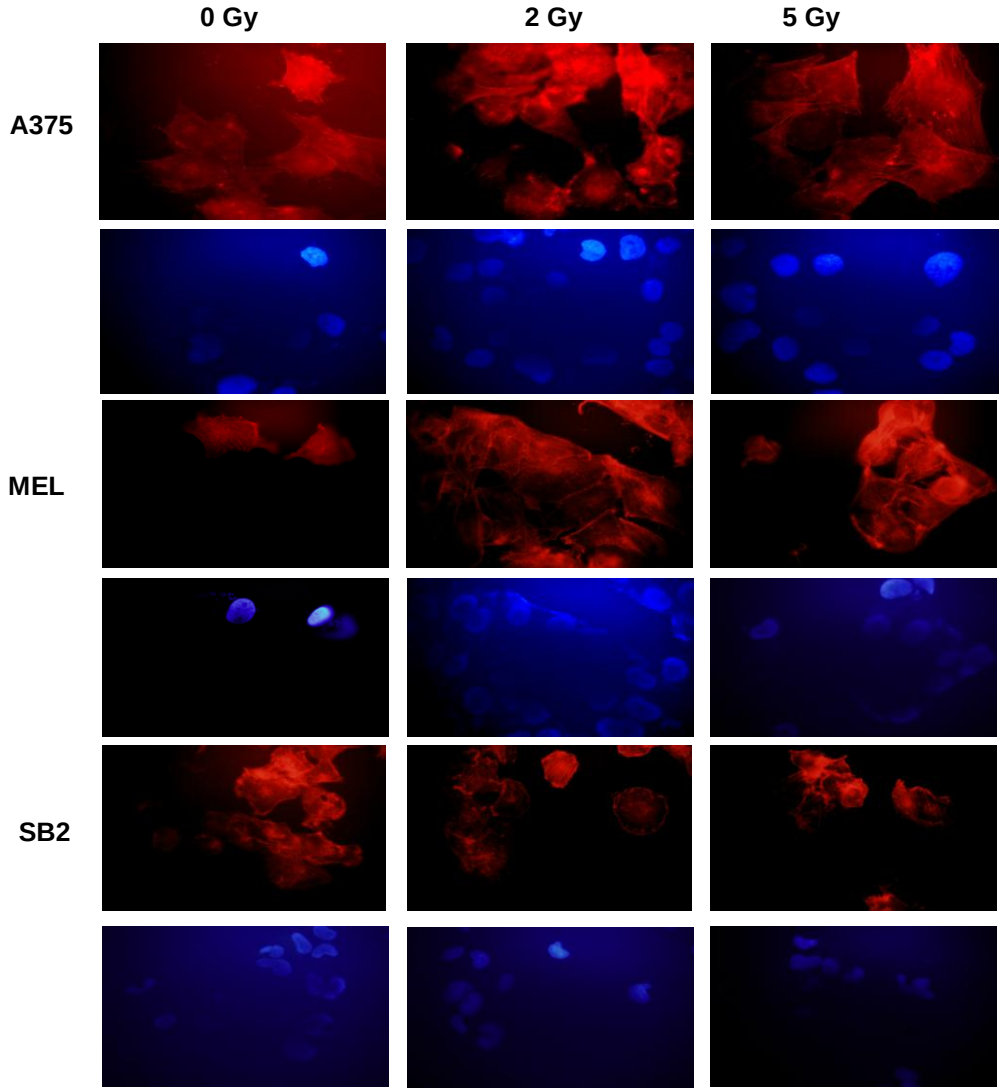

Figure 4. Detection of actin stress fibers. A375, SB2 and MELJ cells control and irradiated with 2 and 5 Gy, 3 hs post-irradiation. Cells were stained with phalloidin (red) and DAPI (blue).

3.5 RhoB overexpression effect on melanoma cells radiosensitivity

In view that SB2 raised the highest levels of RhoB after irradiation, and it turned to be the most sensitive cell line in this study, we wondered what would be the effect on radiosensitivity of overexpressing RhoB in the different melanoma cell lines.
Cell lines A375, SB2 and MELJ were stably transfected with either a RhoB construction, a constitutively active mutant of RhoB (V14) or the empty plasmid as a control (Table 2). As shown in Table 2, for the cells transfected with RhoB, clones were obtained, while for the

negative and positive controls (empty plasmid or V14) we decided to use cell pools. RhoB overexpression in transfected cells was assessed by western blot (data not shown).

Table 2. Clones and pools obtained by stable transfection

	A375	**MELJ**	**SB2**
RhoB Clones	C3, D4	B7, C8	C12, D2, F3
V14	Pool	Pool	Pool
PCDNA3	Pool	Pool	Pool

In order to evaluate RhoB overexpression effect on cellular radiosensitivity, survival curves were done. Each cell line was irradiated with doses of 0, 0.5, 1, 2, 3 and 5 Gy, and 10-15 days later the colonies were stained and counted, fitting the curve to the linear-quadratic model (Figure 5). SF2 was calculated for each condition (Table 3). It can be observed that A375 and SB2 overexpressing RhoB are more radiosensitive than their counterpart transfected with empty plasmid (Figure 5).

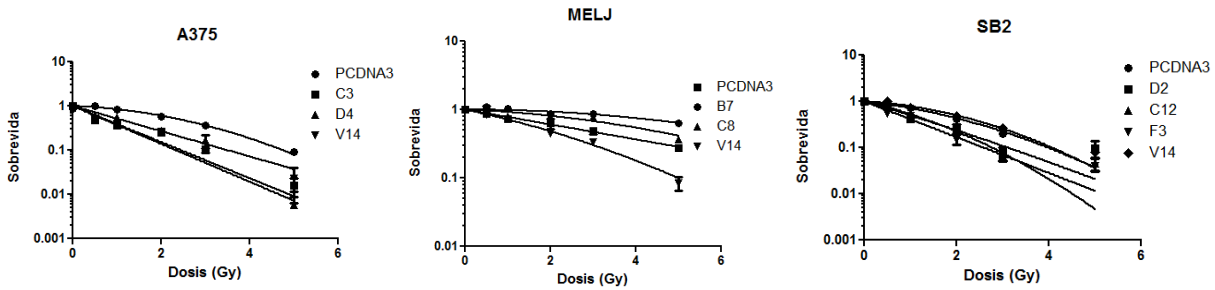

Figure 5. Survival curves of A375, MELJ and SB2 derived cell lines overexpressing RhoB and controls

Table 3. SF2 parameter obtained from the fitted survival curves of the cell lines MELJ, A375 and SB2 overexpressing RhoB or controls and gamma irradiated.

A375		**MELJ**		**SB2**	
PCDNA3	0.58	PCDNA3	0.62	PCDNA3	0.41
C3	0.256	B7	0.87	D2	0.25
D4	0.273	C8	0.79	C12	0.26
V14	0.261	V14	0.44	F3	0.15
				V14	0.47

4. DISCUSSION

Radiotherapy, after surgery, is one of the most effective treatments for cancer. Local treatments, including surgery and / or radiotherapy are successful in 40% of cases while

chemotherapy round 2%. However, only 15% of all cancers can be treated with radiotherapy. For example, melanomas tend to be insensitive to radiation [20], but in turn different types of melanoma differ in the resistance to radiation therapy, as can be observed in the cell lines used in this work, A375, SB2 and MELJ. This study aimed to understand potential mechanisms involved in the modulation of the radiosensitivity of human melanoma cells.

The ability of cells to become oncogenic depends not only on the activation of proliferative pathways but also on the inhibition of tumor suppressor ones. It is known that inhibiting the expression of RhoB is a step for oncogenic cell transformation, and its ectopic expression blocks transformation, resistance to apoptosis induced by anticancer drugs, cell migration, and invasion [17]. In an animal model, ectopic expression of RhoB in the highly metastatic melanoma cell line B16-F10, dramatically inhibited lung metastases [17] suggesting that RhoB functions as a tumor suppressor.

According to the characteristics of the three cell lines in study, and the above background, the highest expression level of RhoB could have been expected for SB2 and the lowest for MELJ. However, the more aggressive melanoma cell line, MELJ, presented a basal expression of RhoB much higher than the detected for the cell line of least malignancy, SB2. Future studies will elucidate the biological significance of this different basal expression.

However, when irradiating the three cell lines studied with a 2 Gy dose, A375 and SB2, the most radiosensitive cell lines, induced RhoB expression, whereas in MELJ this induction was not observed. Taking these results, we can infer that the radiosensitivity in melanoma cells is unrelated to the baseline concentration of RhoB, but rather would be associated with the cells ability to regulate the expression of this protein towards a stress stimulus as gamma radiation.

All Rho family proteins regulate stress fibers to some degree, but how this property works with the differential biological function of each Rho protein is not totally understood. It has been found that one of the consequences of RhoB gain of function is the reorganization of actin cytoskeleton and apoptosis [4]. When the effect of radiation on the induction of actin stress fibers is analyzed in the studied cell lines, a clear correlation is seen between RhoB levels or induction and stress fibers. It was noted that MELJ had the highest baseline levels of stress fibers in non-irradiated cells and presented a lower induction when subjected to radiation while SB2 showed a significant induction of stress fibers formation by radiation.

Finally, RhoB increased levels in melanoma cell lines led to radiosensitization. Thus, modulation of RhoB may constitute a therapeutic tool for radioresistant melanoma treatment.

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