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Antitumor activity induced by regulatory RNA: possible role of RNA-dependent protein kinase and nuclear factor-κB

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Abstract

Regulatory RNAs are noncoding RNAs that can regulate gene expression. Our previous results showed that regulatory RNAs can induce the production of interleukin-1, interleukin-2, interleukin-8, tumor necrosis factor-α (TNF-α), interferon-γ, and Fas ligand (FasL). These cytokines and FasL are involved in host defense mechanisms against tumors. B16-F10 melanoma cells are highly metastatic to the lungs and we showed that lymphocytes treated with the regulatory B16-RNA reduce significantly the number of metastatic nodules. We also found that B16-RNA activates RNA-dependent protein kinase (PKR) and the active B16-RNA fraction is polyadenylated with a sedimentation coefficient of 18*S*. Our findings suggest that the antitumor activity of B16-RNA is mediated by PKR through activation of the transcription factor NF-κB. Thus, B16-RNA may act as a regulatory RNA and may regulate gene expression at transcriptional level. This study provides the rationale for the use of B16-RNA as an immunomodulator in melanoma.

Keywords: RNA, regulatory; RNA, noncoding; PKR (RNA-dependent protein kinase); NF-κB (nuclear factor-κB); B16-F10 melanoma; TNF-α (tumor necrosis factor-α)

1. Introduction

Recent studies have shown that noncoding RNAs, named regulatory RNAs, are remarkably versatile molecules, play important roles in eukaryotic cells, and exert their actions at the RNA level (Erdmann et al., 2001; Eddy, 2001; Szymanski and Barciszewski, 2002). One striking observation was that regulatory RNAs are involved in gene regulation and it is now an area of intensive research (Lau et al., 2001; Storz, 2002; Szymanski and Barciszewski, 2002).

We have demonstrated that regulatory RNAs induce the expression of cytokine genes whose promoters contain binding sites for the transcription regulator, nuclear factor-κB (NF-κB). Thus, we observed the production of interleukin-1, interleukin-2, interleukin-8, tumor necrosis factor-α (TNF-α), and interferon-γ by lymphoid cells treated with regulatory RNAs (Ribeiro et al., 1993, 1995; De Lucca et al., 2002a,b). We have also obtained evidence for the expression of Fas ligand (FasL) in cytotoxic T lymphocytes against a peptide of HIV-1 generated by the regulatory p9-RNA (De Lucca et al., 2002c). These findings suggest that the transcription factor NF-κB and RNA-dependent protein kinase (PKR) are involved in the induction of these genes (Karin and Ben-Neriah, 2000).

The most classical form of NF-κB is a heterodimer of p50 and p65 that is sequestered in the cytosol by the I-κB proteins, which inhibit NF-κB nuclear translocation and DNA binding activity. In response to a variety of stimuli, the prototypic member of this family I-κBα is phosphorylated and degraded via the ubiquitin–proteasome pathway (Karin and Ben-Neriah, 2000).

PKR is a serine–threonine protein kinase, functionally divided in a C-terminal kinase domain and an N-terminal regulatory region containing two RNA binding motifs. The main mechanism of PKR activation is mediated by its interaction with RNA. After RNA binding, PKR dimerizes and is activated through autophosphorylation (Clemens and Elia, 1997). It was found that PKR activates NF-κB either by directly phosphorylating its inhibitor I-κBα (Kumar et al., 1994) or indirectly by activating the I-κB kinase (IKK) complex (Gil et al., 2000).

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Accumulating evidence reveals that the cytokines interleukin-1, interleukin-2, TNF-α, and interferon-γ are involved in host defense mechanisms against tumors (Raitano and Korc, 1993; Eton et al., 2002; Ikeda et al., 2002; Lejeune, 2002; Street et al., 2002). Over the past few years, much has been learned about the way in which cytotoxic T lymphocytes destroy their targets cells. One major mechanism is the expression of the membrane-bound Fas ligand that binds its receptor Fas, thus inducing apoptosis of Fas-bearing cells (Nagata and Golstein, 1995). The fact that our previous results showed that regulatory RNA is active in inducing the production of interleukin-1, interleukin-2, TNF-α, interferon-γ, and FasL prompts us to examine its ability to induce antitumor activity. This study was performed with the regulatory B16-RNA, obtained from lymphocytes of animals immunized with B16-F10 cells, a variant cell line of B16 melanoma, which are highly metastatic to the lungs when injected intravenously into C57BL/6 mice. This experimental metastasis model was used to test the antitumor activity of lymphocytes treated with the regulatory B16-RNA. We also investigated whether this effect could be mediated by PKR through NF-κB activation.

2. Materials and methods

2.1. Animals

C57BL/6 mice, weighing 20–25 g, and adult Hartley guinea pigs were used. The animals were raised at the Central Animal Laboratory of the School of Medicine of Ribeirão Preto, SP, Brazil. All experiments with animals were approved by our institutional animal care committee.

2.2. Culture of tumor cells

B16-F10 melanoma cells were adapted to growth in vitro using RPMI medium supplemented with 10% fetal calf serum and antibiotics. Cultures were maintained at 37 °C in an atmosphere of 5% CO_2 and the RPMI medium was replaced at intervals of 3 days and the cells were detached by treatment with EDTA.

2.3. Immunization of RNA donors

B16-F10 cells were prepared by suspension in phosphate-buffered saline (PBS) at a concentration of 40% (vol/vol). This tumor cell suspension was emulsified in an equal volume of complete Freund's adjuvant. Hartley guinea pigs were immunized by injecting 0.25 ml of this mixture into each foot pad and 1 ml of tumor cell suspension without complete Freund's adjuvant intraperitoneally. Two weeks after immunization, the animals were killed and the lymph nodes were removed and used for RNA extraction.

2.4. Preparation of RNA

RNA was extracted from the lymph nodes of immunized and nonimmunized guinea pigs as described by De Lucca et al. (2002b). The RNA preparations extracted from the guinea pigs immunized with B16 melanoma cells and nonimmunized animals are referred to B16-RNA and N-RNA, respectively. The integrity of RNA preparations was routinely evaluated by electrophoresis on 1% agarose gel.

2.5. Fractionation of B16-RNA by oligo(dT) cellulose chromatography

The poly A(−) B16-RNA and the poly A(+) B16-RNA fractions were separated from the bulk of the total B16-RNA by affinity chromatography on a cellulose column as described earlier (De Lucca et al., 2002a).

2.6. Fractionation of B16-RNA by centrifugation on 5–20% sucrose gradient

The B16-RNA was collected by centrifugation (10,000×*g* at 4 °C for 25 min) and resuspended in 0.02 M Tris–HCl (pH 7.6), 0.1 M NaCl, and 0.001 M EDTA. Samples (100 μl) of B16-RNA (1 mg/ml) were layered on a linear 5–20% sucrose gradient prepared in 0.02 M Tris–HCl (pH 7.6), 0.1 M NaCl, and 0.001 M EDTA. The gradients were centrifuged at 53,000 × *g* for 5 h at 4 °C and B16-RNA fractions were obtained using a density gradient fractionater. The gradients were divided into six regions and the fractions corresponding to each region were pooled. The RNA fractions were precipitated and collected by centrifugation.

2.7. Treatment of B16-RNA with ribonuclease

Pancreatic ribonuclease (type I-A; Sigma) was added to a solution of B16-RNA in 0.85% NaCl in the ratio of 1 μg ribonuclease/10 μg B16-RNA, and the mixture was incubated at 37 °C for 30 min.

2.8. Treatment of lymphocytes with B16-RNA

The lymph nodes of normal C57BL/6 mice were teased apart in RPMI-1640 medium and rinsed through mesh stainless steel screens. Erythrocytes were lysed by exposure to Tris–NH_4Cl solution for 3 min. Lymphocytes were washed three times and suspended in RPMI at a concentration of 10^6 cells/ml. The cells were incubated with medium, N-RNA, or B16-RNA (150 μg RNA/10^6 cells) at 37 °C for 30 min. RNA-treated lymphocytes were used for adoptive cellular immunotherapy.

2.9. Adoptive cellular immunotherapy

C57BL/6 mice received intravenously 10^5 of B16-F10 melanoma cells in 0.1 ml of PBS on day 0. Following

inoculation of tumor cells, each mouse received intravenously and intraperitoneally injections of 10^6 of normal lymphocytes previously incubated with B16-RNA or N-RNA (150 μg RNA/10^6 cells) on days 3, 6, and 9. On day 18, the mice were sacrificed and the pulmonary tumor nodules were counted under a dissecting microscope.

2.10. PKR assay

Lymphocytes from normal C57BL/6 mice were washed with ice-cold PBS and lysed with 3 vol of lysis buffer consisting of 20 mM Tris–HCl (pH 7.6), 50 mM KCl, 400 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, aprotinin (2 μg/ml), leupeptin (2 μg/ml), 1 mM dithiotreitol, 1% Triton X-100, and 20% glycerol. The lysates were centrifuged at 10,000 × *g* for 20 min and the supernatant (S-10) was stored at − 70 °C. The PKR assay was carried out in the conditions described by De Lucca et al. (2002b). Quantification of PKR phosphorylation was determined by using ImageQuant software, version 3.3 (Molecular Dynamics), and the results were expressed in terms of arbitrary units. The same amount of lymphocyte extract (50 μg of protein) used in PKR assay was also subjected to immunoblotting using anti-PKR (sc no. 6282; Santa Cruz) as a loading control.

2.11. Statistical analysis

Differences between values were analyzed using the Mann–Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

Fig. 1 shows that a significant reduction in number of metastatic nodules occurs in C57BL/6 mice that had received lymphocytes incubated with B16-RNA. We also found that the pretreatment of B16-RNA with ribonuclease abolishes its antitumor activity whereas lymphocytes treated with N-RNA had no effect.

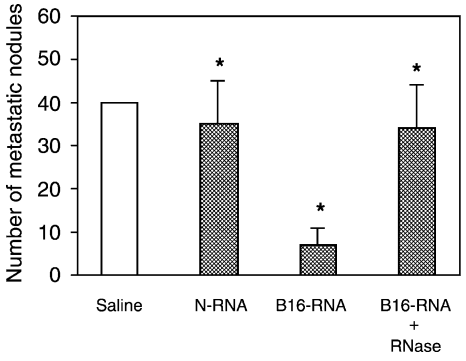

Fig. 1. Effect of normal lymphocytes treated with B16-RNA on the pulmonary colonization of B16-F10 melanoma cells in C57BL/6 mice. The injection of B16-F10 cells into mice and the adoptive cellular immunotherapy were as described in Materials and Methods. B16-RNA was also pretreated with ribonuclease (RNase) and normal lymphocytes incubated with saline or N-RNA were used as controls. Values represent mean ± S.E.M. of three independent experiments. Mice were used in groups of 10. *Not significant as compared to saline-treated mice. **$P<0.01$ as compared to saline-treated mice.

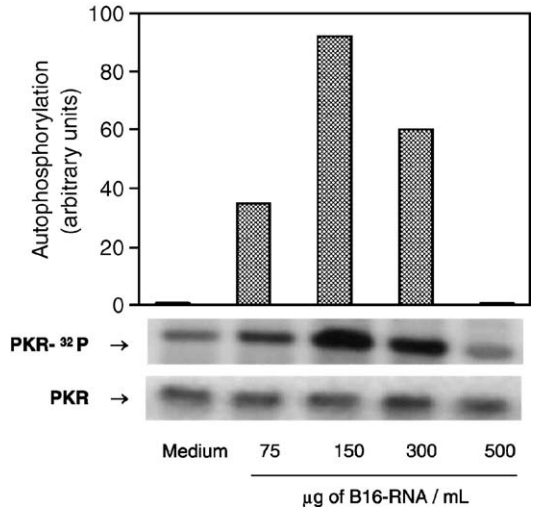

Fig. 2. Effect of B16-RNA on the activity of PKR. Mouse lymphocytes were lysed and PKR assay was carried out at different doses of B16-RNA as described in Materials and Methods. The immunoprecipitate was subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. The same amount of lymphocyte extract (50 μg of protein) used in the PKR assay was also subject to immunoblotting using anti-PKR as a loading control. The results shown in this figure are representative of three independent experiments.

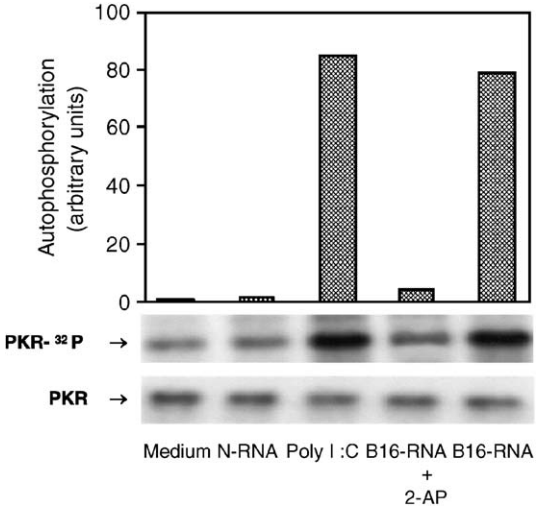

Fig. 3. Effect of the 2-aminopurine on activation of PKR by B16-RNA. Mouse lymphocytes were lysed and PKR assay was performed using B16-RNA alone or in the presence of its inhibitor, 2-aminopurine (2-AP), as described in Materials and Methods. The immunoprecipitate was subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. PKR was identified by Western blot analysis and used as loading control. N-RNA extracted from nonimmunized C57BL/6 mice was used as a negative control and the polyinosinic/polycytidylic acid as a positive control. The results shown in this figure are representative of three independent experiments.

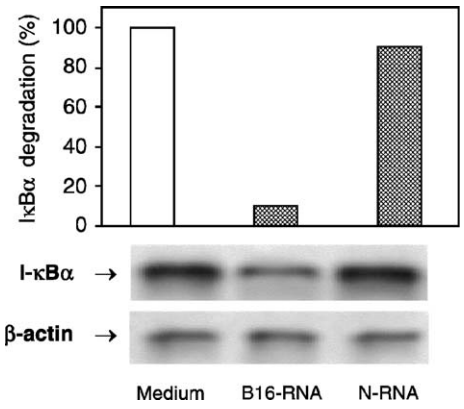

Fig. 4. Degradation of the I-κBα induced by treatment of mouse lymphocytes with B16-RNA. Lymphocytes were treated with B16-RNA, lysed, and the proteins were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and degradation of I-κBα was evaluated by Western blot analysis. N-RNA extracted from nonimmunized C57BL/6 mice was used as control. Western blotting was performed with antibodies directed against I-κBα (upper panel) or β-actin (lower panel). The immunoblot with anti-β-actin was used as a loading control. The percentage of I-κBα degradation was determined using ImageQuant software, version 3.3, and the band corresponding to medium alone was used as control. The results shown in this figure are representative of three independent experiments.

We demonstrated that B16-RNA activates PKR from lymphocytes of C57BL/6 mice in a dose-dependent manner and maximum activation was achieved at 150 μg of B16-RNA/ml (Fig. 2). Our results indicated that this effect was abrogated by the 2-aminopurine and that N-RNA has no effect on PKR activity (Fig. 3).

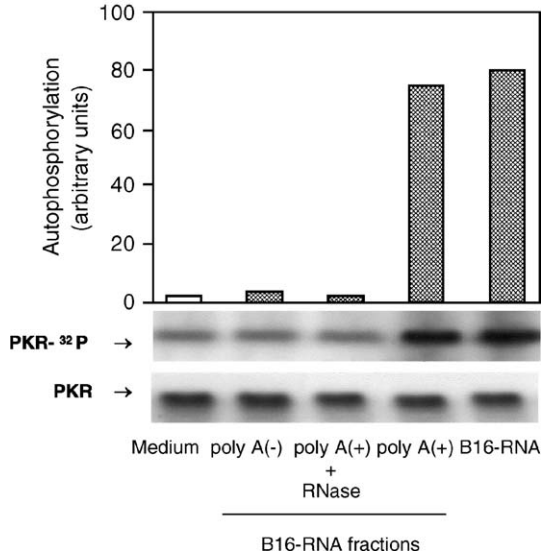

Fig. 5. Effect of poly A(−) and poly A(+) B16-RNA fractions on PKR activity. Mouse lymphocytes were lysed and PKR assay was performed using B16-RNA, poly A(−) B16-RNA, or poly A(+) B16-RNA as described in Materials and Methods. The immunoprecipitate was subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography. PKR was also identified by Western blot analysis and used as loading control. The results shown in this figure are representative of three independent experiments.

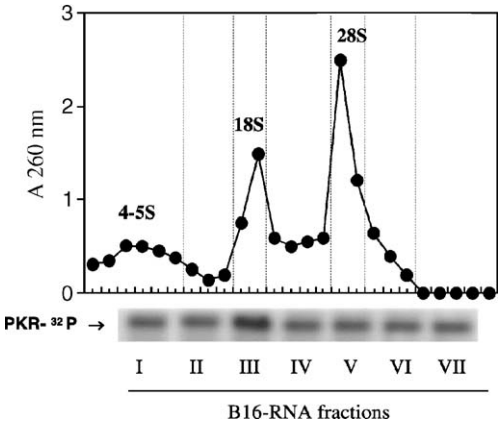

Fig. 6. Effect of B16-RNA fractions obtained by sucrose gradient centrifugation on PKR activity. Total B16-RNA was separated by centrifugation on a 5–20% sucrose gradient and PKR assay with B16-RNA fractions was performed as described in Materials and Methods. The region of the gradient (group VII) containing only sucrose was used as a control in the PKR assay. The results shown in this figure are representative of three independent experiments.

Fig. 4 depicts the results of a typical experiment in which I-κBα is degraded after incubation of mouse lymphocytes with B16-RNA but not with N-RNA.

The fractionation of B16-RNA by affinity chromatography on an oligo(dT) cellulose column indicated that the poly A(+) B16-RNA is the fraction responsible for PKR activation (Fig. 5). Moreover, the fractionation of B16-RNA by centrifugation on a linear 5–20% sucrose gradient showed that B16-RNA fraction was responsible for PKR activation sediments in the 18*S* region of the gradient (Fig. 6).

4. Discussion

The first step was to examine the ability of B16-RNA to induce antitumor activity in lymphocytes from normal C57BL/6 mice. We have demonstrated that a significant reduction in number of metastatic nodules only occurs in the lungs of C57BL/6 mice that had received normal lymphocytes incubated with B16-RNA. The pretreatment of B16-RNA with ribonuclease abolishes this effect, indicating that the integrity of the polynucleotide chain is critical for the antitumor activity of B16-RNA.

The next step was to investigate the molecular events involved in the induction of antitumor activity in lymphocytes incubated with B16-RNA. We hypothesized that B16-RNA preparations contain noncoding RNA molecules that are able to activate PKR of lymphocytes from C57BL/6 mice and, therefore, act as regulatory RNAs. It is known that PKR is activated by low concentrations of double-stranded RNA but inhibited by higher concentrations, giving a characteristic bell-shaped curve (Clemens and Elia, 1997). Our results indicated that the activation of mouse PKR with different doses of B16-RNA also gave a bell-shaped curve.

We demonstrated that this effect of B16-RNA was significantly reduced by 2-aminopurine, an inhibitor of PKR (Hu and Conway, 1993).

It was found that PKR activates NF-κB through the phosphorylation of its inhibitor I-κBα (Kumar et al., 1994) and by activating the I-κB kinase (IKK) complex (Gil et al., 2000). It should be emphasized that phosphorylation of I-κBα serves a molecular tag, leading to its ubiquitination and degradation by components of the ubiquitin–proteasome system (Karin and Ben-Neriah, 2000). It is well established that the nuclear translocation of NF-κB is preceded by a decrease in the level of cytoplasmic I-κBα, indicating that its degradation is a key step in NF-κB activation (Karin and Ben-Neriah, 2000). An important observation was that I-κBα is degraded in lymphocytes treated with B16-RNA, suggesting that this regulatory RNA is able to activate NF-κB. It is reasonable to assume that NF-κB activation results in production of interleukin-1, interleukin-2, TNF-α, interferon-γ, and FasL since the promoters of these cytokine genes and FasL gene have binding sites for NF-κB (De Lucca et al., 2002a,b,c) and these molecules could mediate the antitumor activity of normal lymphocytes treated with B16-RNA.

To determine which RNA fraction is responsible for PKR activation, B16-RNA was fractionated on an oligo(dT) cellulose column and our results indicate that the poly A(+) B16-RNA is the active fraction. This finding is in accordance with several reports that have described messenger RNA-like transcripts, which are polyadenylated but have no defined open reading frames, indicating that they lack protein coding capacity and, therefore, regulatory RNAs exert their action at the RNA level (Erdmann et al., 2001; Szymanski and Barciszewski, 2002). Moreover, the fractionation of B16-RNA by centrifugation on sucrose gradient reveals that B16-RNA fraction is responsible for PKR activation sediments in the 18*S* region of the gradient. The fact that the 18*S* B16-RNA fraction is polyadenylated ruled out the possibility that this fraction is the 18*S* ribosomal RNA. Further experimental work is necessary to characterize the 18*S* B16-RNA fraction and to test its ability to induce antitumor activity.

Based on our findings, we suggest a model for the mechanism of action of B16-RNA (Fig. 7). According to our model, the incubation of lymphocytes from C57BL/6 mice with B16-RNA results in its uptake by these cells and thus poly A(+) B16-RNA fraction induces PKR activation in the cytoplasm of lymphocytes. Thereafter, PKR can activate NF-κB either by directly phosphorylating its inhibitor I-κBα (Kumar et al., 1994) or indirectly by activating the I-κB kinase (IKK) complex (Gil et al., 2000). Thus, PKR activation leads to the phosphorylation of I-κBα and its subsequent degradation, thereby freeing the NF-κB to translocate to the nucleus of lymphocytes. The NF-κB binds to the promoter of TNF-α, interleukin-2, interferon-γ, and FasL genes, inducing their expression. The newly synthesized cytokines are secreted and the

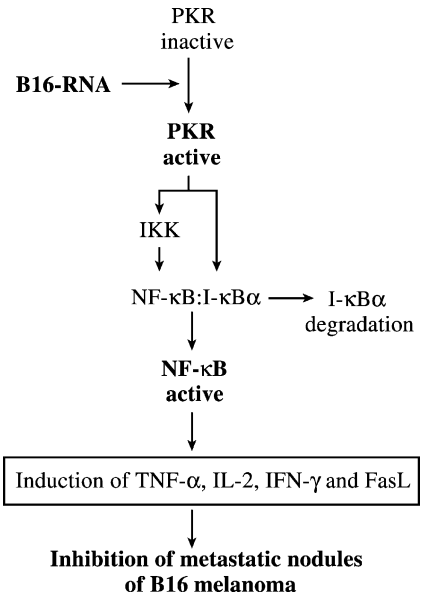

Fig. 7. Diagram of proposed molecular events involved in the induction of antitumor activity by regulatory B16-RNA. The following abbreviations were use: PKR = RNA-dependent protein kinase; NF-κB = nuclear factor-κB; I-κB = inhibitor of NF-κB; IKK = I-κB kinase; TNF-α = tumor necrosis factor-α; IL-2 = interleukin-2; IFN-α = interferon-γ; FasL = Fas ligand.

FasL molecules migrate to membrane of lymphocytes. These molecular events could be involved in the inhibition of tumor nodules in the lungs of mice that had received lymphocytes treated with B16-RNA. Thus, the poly A(+) B16-RNA fraction may act as a regulatory RNA and it regulates the expression of cytokine and FasL genes at transcriptional level, acting as a modulator of protein function via RNA–protein interactions according to the functional classification of noncoding RNAs (Szymanski and Barciszewski, 2002). Since the poly A(+) B16-RNA was obtained from lymphocytes of immunized animals, it is tempting to speculate that immunization with B16 melanoma cells induces the synthesis of regulatory RNAs. In this context, it is noteworthy that expression of the NTT (noncoding transcript in T cells) gene was detected only in activated human lymphocytes and this transcript is polyadenylated and does not encode protein (Liu et al., 1997).

The B16 murine melanoma is a rapidly growing metastatic tumor of spontaneous origin as are human malignant melanomas. Although there are still many unanswered questions, this study provides the rationale for the use of regulatory B16-RNA as an immunomodulator in human melanoma.

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