

Toll-like receptor 3 (TLR3) activation induces microRNA-dependent reexpression of functional RAR β and tumor regression

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Toll-like receptor 3 (TLR3) is a key effector of the innate immune system against viruses. Activation of TLR3 exerts an antitumoral effect through a mechanism of action still poorly understood. Here we show that TLR3 activation by polyinosinic:polycytidylic acid induces up-regulation of microRNA-29b, -29c, -148b, and -152 in tumor-derived cell lines and primary tumors. In turn, these microRNAs induce reexpression of epigenetically silenced genes by targeting DNA methyltransferases. In DU145 and TRAMP-C1 prostate and MDA-MB-231 breast cancer cells, we demonstrated that polyinosinic:polycytidylic acid-mediated activation of TLR3 induces microRNAs targeting DNA methyltransferases, leading to demethylation and reexpression of the oncosuppressor retinoic acid receptor beta (RAR β). As a result, cancer cells become sensitive to retinoic acid and undergo apoptosis both in vitro and in vivo. This study provides evidence of an antitumoral mechanism of action upon TLR3 activation and the biological rationale for a combined TLR3 agonist/retinoic acid treatment of prostate and breast cancer.

inflammation | combined chemotherapy

Toll-like receptors (TLRs) are a family of transmembrane receptors that recognize conserved ligands of microbial origin called pathogen-associated molecular patterns and provide the first line of defense against pathogen-induced inflammatory responses (1, 2). In addition, TLRs play an important role in tissue repair and tissue injury-induced inflammation (1, 2). The role of TLRs in cancer is still being debated; their pro- or anticancer effect depends on the TLR stimulated and the tumor type (3). We and others previously demonstrated that activation of Toll-like receptor 3 (TLR3) by the specific agonist polyinosinic:polycytidylic acid [Poly(I:C)], exerts an antitumoral effect by inducing apoptosis (4, 5) and recruiting an antitumoral immune response (6–8). In Western countries, prostate and breast cancers are the most common malignant tumors in men and women, respectively (9, 10). MicroRNAs (miRNAs) are small noncoding RNAs with gene regulatory function, and their dysregulation has been demonstrated in all human malignancies (11). Recently, several studies have shown that miRNAs have a role in regulating immune response and inflammation (12). In particular, TLR signaling can modulate miRNA expression (13). Here we demonstrate that TLR3 stimulation induces up-regulation of four miRNAs (has-miR-29b, -29c, -148b, and -152) on DU145 and TRAMP-C1 (TRAMP), human and mouse prostate cancer cell lines, respectively, and MDA-MB-231, a human breast cancer cell line. Interestingly, all these miRNAs directly target effectors of the epigenetic machinery: miR-148b and -152 directly target the maintenance DNA methyltransferase 1 (DNMT1) (14), whereas miR-29b and -29c directly regulate the expression of the de

novo DNMTs 3A and 3B (15) and indirectly affect the expression of DNMT1 (16).

As a result of this miRNA-mediated DNMT silencing, Poly(I:C) induces partial demethylation and reexpression of retinoic acid receptor beta-2 (RAR β), a tumor suppressor gene often silenced by promoter hypermethylation in prostate and breast cancer cells (17–19). We also demonstrate that RAR β reexpression is functional and responsive to 9-*cis*-retinoic acid (RA) both in vitro and in vivo. Taken together, these data identify an miRNA-mediated antitumoral mechanism of action of TLR3 activation and provide the rationale for a Poly(I:C)-retinoic acid combined treatment of breast and prostate cancer.

Results and Discussion

Poly(I:C) Treatment Affects miRNA Expression in Prostate Cancer Cell Lines.

Previous publications reported that TLR stimulation induces miRNA modulation (20–22). In particular, Liu et al. (23) demonstrated that TLR3 stimulation induces up-regulation of the miR-148b/152 family in dendritic cells, and this affects cytokine production. To determine whether Poly(I:C) treatment can induce changes in the miRNome of prostate cancer cells, we used DU145, which is the only validated human prostate cancer cell line that expresses high levels of TLR3 but does not undergo a strong apoptotic effect upon Poly(I:C) stimulation (4, 6). By performing a NanoString assay on Poly(I:C)-treated cells, we found that miR-29b, -29c, -148b, and -152 were among the most significantly up-regulated miRNAs at different time points (Fig. 1). We validated these results by performing quantitative real-time PCR (qRT-PCR) (Fig. S1A). Consistent with the NanoString data, all four miRNAs were significantly up-regulated by Poly(I:C) treatment. To validate these data on other cellular models, we used TRAMP and MDA-MB-231 cells, two cell lines previously described to express TLR3 and to be resistant to Poly(I:C)-induced apoptosis (8, 24). We treated these cell lines with Poly(I:C) and observed increased expression of all four miRNAs of interest at different times (Fig. S1A). The observed rapid up-regulation of the miRNAs

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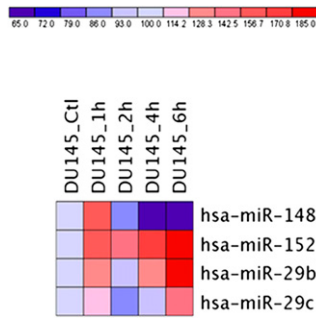


Fig. 1. miRNA up-regulation after Poly(I:C) treatment in DU145 cells. DU145 prostate cancer cells were treated with 25 $\mu\text{g}/\text{mL}$ Poly(I:C) from 1 to 6 h, the RNA was extracted, and NanoString assay was performed. Untreated cells were used as a control. The heat map shows the results of NanoString for miRNA-29b, -29c, -148b, and -152 at the indicated time points.

of interest suggests a direct mechanism of signal transduction. More complex mechanisms involving the secretion of IFN- β , known to often be part of the response to TLR3 stimulation, may be excluded

because, for example in breast cancer, this molecule is secreted 18 h after the stimulation (5).

To confirm the direct involvement of TLR3 in the Poly(I:C)-induced miRNA up-regulation, we transfected DU145, TRAMP, and MDA-MB-231 cells with an empty vector, used as a control, or with a vector encoding a dominant-negative (DN) form of TLR3. In the presence of Poly(I:C), TLR3-DN treatment abolished the up-regulation of miR-29b, -29c, -148b, and -152, suggesting that miRNA up-regulation induced by Poly(I:C) is strictly TLR3 dependent (Fig. S1B).

Effect of Poly(I:C)-Induced miRNAs on DNMTs. Because miR-148b and -152 are known to target DNMT1 (14), whereas the miR-29 family targets all DNMTs (15, 16), we hypothesized that Poly(I:C) treatment would inhibit DNMT activity by inducing these miRNAs. Therefore, we treated DU145, TRAMP, and MDA-MB-231 cells with Poly(I:C) for 24 or 48 h and assayed DNMT activity. Poly(I:C) treatment caused a strong reduction of DNMT activity (49%, 31%, and 37%, respectively, for DU145, TRAMP, and MDA-MB-231 cells), albeit with different timing (Fig. S2). In fact, the effect on DNMT activity is faster in DU145 and MDA-MB-231 (after 24 h of treatment) than in TRAMP cells (after 48 h of treatment). Then, we transfected DU145, TRAMP, and MDA-

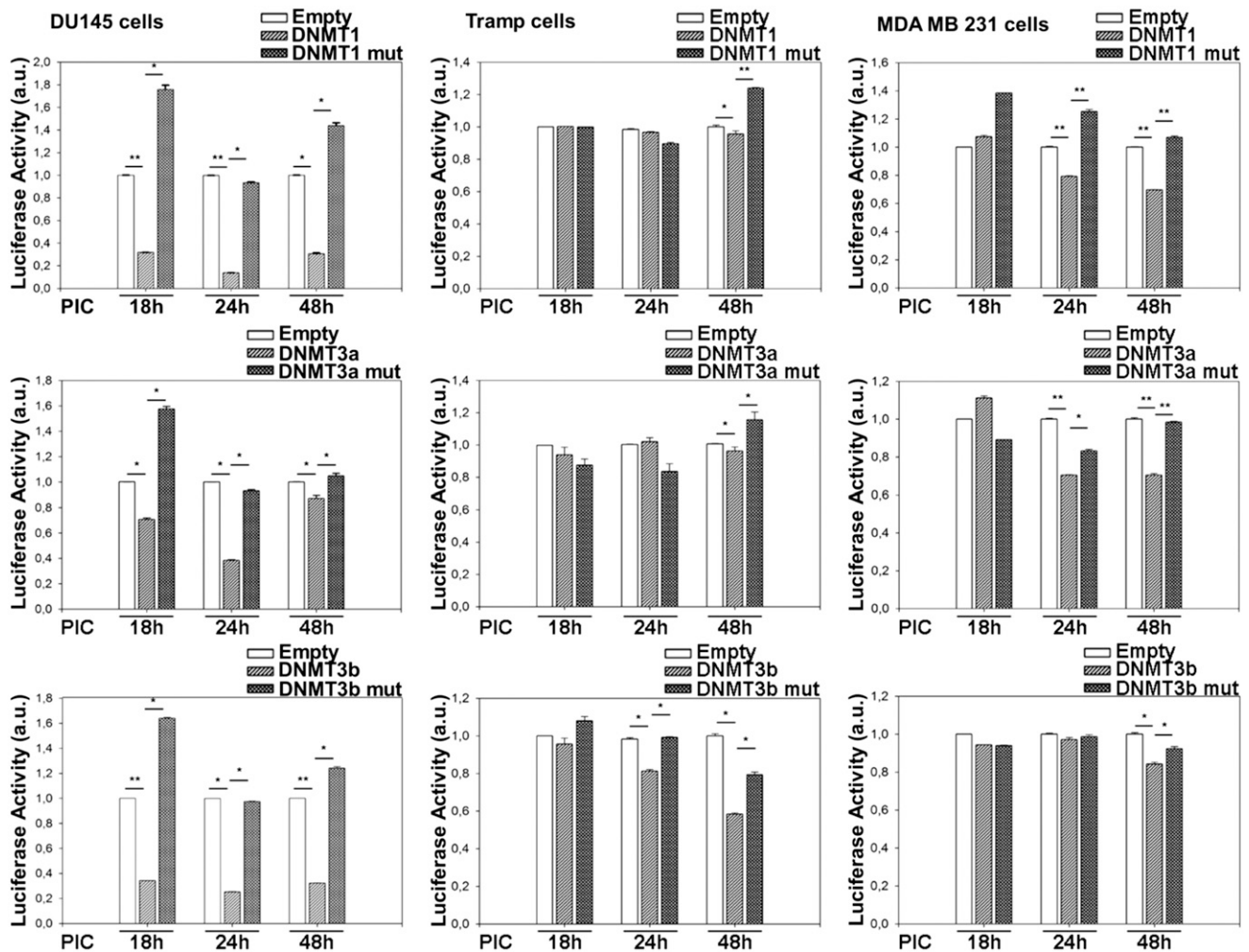


Fig. 2. Effect of Poly(I:C)-induced miRNAs on DNMT expression in prostate and breast cancer cells. DU145, TRAMP, or MDA-MB-231 cells were transfected with the Renilla luciferase expression construct pRL-TK and one of the following constructs: the luciferase construct PGL3-DNMT1WT-3'-UTR-luc (DNMT1) or PGL3-DNMT1MUT-3'-UTR-luc (DNMT1 mut), PGL3-DNMT3AWT-3'-UTR-luc (DNMT3A) or PGL3-DNMT3 AMUT-3'-UTR-luc (DNMT3A mut), PGL3-DNMT3BWT-3'-UTR-luc (DNMT3B) or PGL3-DNMT3BMUT-3'-UTR-luc (DNMT3B mut). In the "mut" constructs, the mutations were introduced in DNMT target sites for a specific miRNA. Twenty-four hours after transfection, the cells were treated with 25 $\mu\text{g}/\text{mL}$ Poly(I:C) for 18, 24, or 48 h before the luciferase assay was performed. All data represent mean and SD from four determinations from three independent experiments. * $P < 0.05$; ** $P < 0.01$. a.u., arbitrary units.

MB-231 cells with plasmids containing the 3'-UTR of DNMT1, -3A, or -3B genes cloned downstream of the firefly luciferase gene (14, 15). Cells were treated with Poly(I:C), and a luciferase reporter assay was performed. Poly(I:C) treatment significantly reduced the luciferase reporter activity in the cells transfected with each plasmid, and this effect was reversed by site-specific mutagenesis of the miRNA binding sites on DNMTs (Fig. 2 and Fig. S3).

RAR β Demethylation After Poly(I:C) Treatment. To investigate the functional implications of Poly(I:C) treatment on our model, we treated DU145 cells with Poly(I:C) for 48 h and performed a Methyl-Profiler analysis designed specifically for prostate cancer. Among the 24 genes analyzed, RAR β was the most significantly demethylated (Fig. 3A). Interestingly, the promoter of this gene is one of the most commonly hypermethylated in prostate and breast cancer patients and cell lines (17, 19, 25). The results were validated by methylation-specific PCR (MSP) and Pyrosequencing assay. Using 5-azacytidine (5-AZA) as a positive control, we observed that Poly(I:C) induced a partial demethylation of RAR β promoter after 48 h of treatment (Fig. 3B and C). Poly(I:C) treatment of DU145 cells also resulted in increased levels of RAR β protein, with an expression peak detected after 48 h (Fig. 3D). We also performed MSP assay on TRAMP and MDA-MB-231 cells and showed that Poly(I:C) could induce a partial demethylation of RAR β promoter on both cell lines (Fig. 3E and Fig. S4). Moreover, we demonstrated that RAR β protein was reexpressed in both cell lines after 48 h of Poly(I:C) treatment (Fig. 3F).

Effects of miRNA and of DNMT Modulation of RAR β Expression. To demonstrate that the expression of RAR β was correlated with the overexpression of Poly(I:C)-induced miRNAs, we transfected

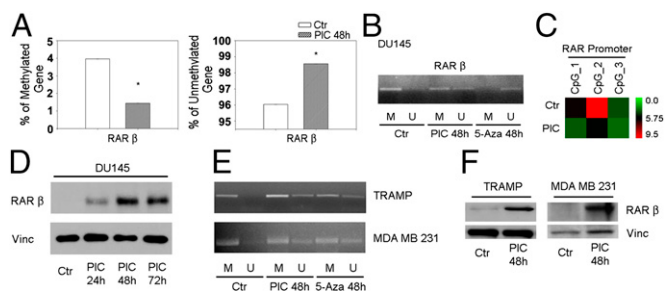


Fig. 3. Poly(I:C) induces RAR β promoter demethylation and protein reexpression. (A) DU145 cells were treated for 48 h with 25 μ g/mL Poly(I:C), then DNA was extracted and a Methyl-Profiler PCR assay was performed. The histograms show the levels of methylated (Left) and unmethylated (Right) RAR β promoter. $P = 0.04393$. (B) DU145 cells were treated with 25 μ g/mL Poly(I:C) for 48 h, then genomic DNA was extracted and used to perform MSP analysis for RAR β using primers specific for unmethylated (U) or methylated (M) gene after sodium bisulfite treatment. Cells treated with 10 μ M 5-Aza-cytidine were used as a positive control. (C) Graphical representation of the quantitative DNA methylation data for the RAR β promoter region in DU145 cells, obtained through the Pyrosequencing system. Each square represents a single cytosine-phosphated-guanin sequence (CpG) or a group of CpGs analyzed. The data presented are from five independent replicates for the sample. $P = 0.009$ for CpG₁ and $P = 0.05$ for CpG₂ and CpG₃. (D) Whole-cell lysates obtained from DU145 after Poly(I:C) treatment (25 μ g/mL) from 24 to 72 h were subjected to Western blot analysis using polyclonal Ab against RAR β . The same filter was reincubated with an antivinculin Ab as a control for an equal amount of protein loaded. (E) TRAMP and MDA-MB-231 cells were treated with 25 μ g/mL Poly(I:C), and the genomic DNA extracted was used to perform MSP analysis for RAR β , as for DU145 cells in B. (F) TRAMP and MDA-MB-231 whole-cell lysates obtained after 25 μ g/mL Poly(I:C) treatment for 48 h (the maximum time point of RAR β expression observed in DU145 cells) were subjected to Western blot analysis by using polyclonal Ab against RAR β . The same membranes were reincubated with an antivinculin Ab to verify the equal amount of protein loaded. All data are representative of three independent experiments. * $P < 0.05$.

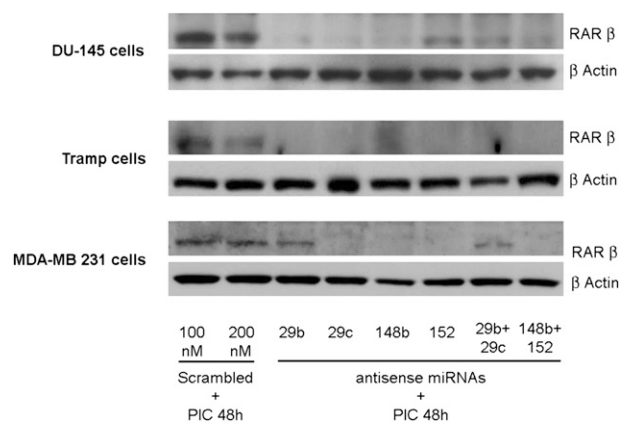


Fig. 4. RAR β protein expression is miRNA dependent. DU145, TRAMP, or MDA-MB-231 cells were transfected with antisense molecules of miR-29b, -29c, -148b, or -152 individually (100 nM) or as a family (miR-29b + miR29c and miR-148b + miR-152, 100 nM each), or with a scrambled molecule (100 nM or 200 nM). Twenty-four hours after transfection, the cells were treated with 25 μ g/mL Poly(I:C) for 48 h. Whole-cell lysates from transfected cells were used to perform Western blot analysis using a polyclonal Ab against RAR β . Data were normalized using β -actin density values. All data are representative of three independent experiments.

DU145, TRAMP, and MDA-MB-231 cells with antisense molecules for miR-29b, -29c, -148b, and -152 individually or in combination (miR-29b/29c, miR-148b/152), and we treated cells with Poly(I:C) for 48 h. Poly(I:C)-induced RAR β expression was blocked in the presence of all miRNA antisense molecules, except miR-29b in MDA-MB-231 cells (Fig. 4). These data suggest that all four miRNAs are directly involved in RAR β reexpression modulation, although with different efficiency. Moreover, DU145, TRAMP, and MDA-MB-231 cells were transfected with antisense molecules for miR-147 and -574-5p (miRNAs not involved in DNMT targeting) as negative controls and for miR-29c as a positive control, then the cells were treated with Poly(I:C) (or its solvent as a control). No induction of RAR β expression occurred in any of the analyzed conditions without Poly(I:C) treatment (Fig. S5A). Conversely, after Poly(I:C) treatment, we observed RAR β expression in cells transfected with scrambled and antisense molecules for miR-147 and -574-5p, but not in the cells transfected with the antisense molecule for miR-29c, excluding off-target effects of the involved miRNAs. We also transfected DU145, TRAMP, and MDA-MB-231 cells with each of the specific miRNA mimics alone or in the previously described combination, although with different efficiency, the transfection of each of the four miRNAs induces partial reexpression of RAR β in all three cell lines, except miR-29b in MDA-MB-231 cells (Fig. S5B). It may be postulated that Poly(I:C)-induced up-regulation of miR-29b might not be necessary for DNMT targeting in MDA-MB-231 cells, because miR-29b previously was reported to be up-regulated in this cell line (26). The combination of miR-29b/29c is the most efficient to induce RAR β reexpression in DU145 and TRAMP cells, whereas for MDA-MB-231 cells, the best result is obtained using the miR-148b/152 combination.

To investigate the robustness of the epigenetic control of RAR β in prostate and breast cancer, we transfected DU145, TRAMP, and MDA-MB-231 cells with the specific siRNA for DNMT1, -3A, or -3B alone; with a combination of these; or with an siRNA for the methylation-unrelated proteins Rab27-a and Rab27-b as negative controls. After checking for the efficiency and specificity of the DNMT silencing by using qRT-PCR (Fig. 5A), we observed reexpression of RAR β 48 h after treatment with all three DNMT siRNAs (Fig. 5B) but not with the Rab27-a, -27-b, or control siRNA. To confirm these data, we performed a rescue experiment (Fig. S5C). We transfected DU145, TRAMP, and MDA-MB-231 cells with a plasmid encoding for each specific DNMT, with an

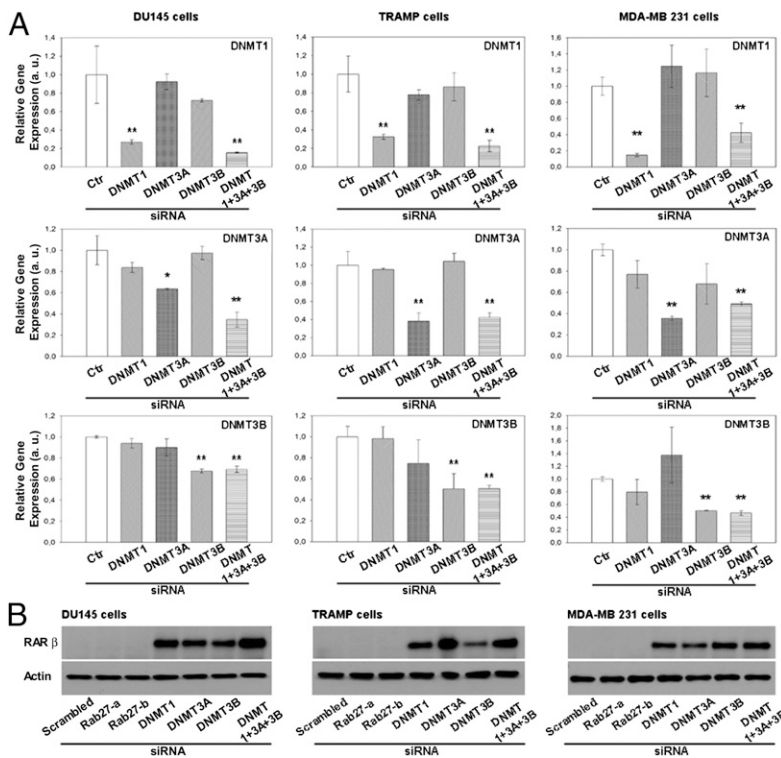


Fig. 5. RAR β protein expression is DNMT dependent. (A) DU145, TRAMP, or MDA-MB-231 cells were transfected with antisense molecules for DNMT1, -3A, or -3B (200 nM each); a combination of the three siRNAs (200 nM each); or a scrambled molecule (600 nM). Forty-eight hours after transfection, RNA was extracted and qRT-PCR was performed to verify the efficiency of siRNA transfection. Data represent the mean of triplicate samples from three independent experiments and are expressed as mean \pm SD. * P < 0.05; ** P < 0.01. a.u., arbitrary units. (B) Whole-cell lysates from cells transfected as in A plus cells transfected with Rab27-a and Rab27-b siRNA used as negative controls were used to perform a Western blot analysis using a polyclonal Ab against RAR β . * P < 0.05. All data represent typical experiments that were repeated three times with similar results.

siRNA against the DNMT, or with a combination of the plasmid and the siRNA for the same DNMT. Our data show that DNMT down-regulation induces RAR β expression. In the cells transfected with the combination of each DNMT plus the relative siRNA, we observed a reduced expression level of RAR β with respect to the cells transfected with the siRNA alone. Overall, these data indicate that all three main DNMTs are involved in the epigenetic control of RAR β expression.

Apoptotic Effect of Poly(I:C)/Retinoid Combined Treatment. Because retinoids are natural ligands of RAR β , we further investigated the effects of retinoids in DU145, TRAMP, and MDA-MB-231 cells by reexpressing RAR β after Poly(I:C) treatment. 9-*cis*-RA is an activator of both RAR and retinoic x receptor (RXR), but the affinity for the first group is at least 20 times higher than for the second (27). Although not as specific for RAR as 13-*cis*-RA or all trans retinoic acid (ATRA), 9-*cis*-RA has been demonstrated to have higher affinity for RAR β (27, 28). All cell lines were treated with Poly(I:C) for 48 h, then a time course of 9-*cis*-RA was performed (Fig. S6A). Cell cycle analysis showed that after 48 h of Poly(I:C) stimulation, there was a significant increase in the sub-G1 apoptotic population only after subsequent stimulation for 72 h with 9-*cis*-RA. A strong apoptotic peak was induced only in the cells treated with the combination of Poly(I:C) and 9-*cis*-RA and not in the cells treated with Poly(I:C) or 9-*cis*-RA alone (Fig. 6A–C). To confirm that the observed apoptotic induction was the result of RAR β activation, an RNA interference against RAR β was used to transfect DU145, TRAMP, and MDA-MB-231 (whereas a scrambled sequence and an RNA interference against Rab27-a and another one against Rab27-b were used as negative controls); then after 24 h, all the cells were treated as previously described. Cell cycle analysis (Fig. S6B) showed that the apoptotic induction was lower in the cells transfected with RNA interference against RAR β than in those transfected with the negative controls, confirming that the apoptotic signal was induced through this receptor. Moreover, to validate the data obtained with cell cycle analysis, we performed a colony assay treating DU145, TRAMP, and MDA-MB-231 cells with Poly(I:C) and 9-*cis*-RA singularly or in combination. The combination of the two drugs induced a strong reduction in colony

number (Fig. S6C). We also found that the Poly(I:C)/9-*cis*-RA combination induced poly ADP ribose polymerase (PARP) precursor cleavage, mediated by caspase cascade activation. Interestingly, only the combined Poly(I:C)/9-*cis*-RA treatment, but not the single agents of this combination, could induce PARP

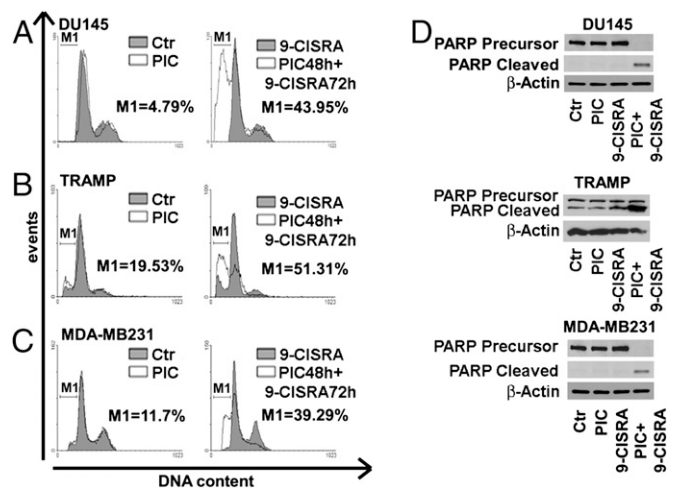


Fig. 6. Apoptotic effect on DU145, TRAMP, and MDA-MB-231 cells after stimulation of RAR β induced by Poly(I:C). DU145, TRAMP, or MDA-MB-231 cells were pretreated with 25 μ g/mL Poly(I:C) for 48 h, then with 10 μ M 9-*cis*-RA for 72 h. Cells untreated or stimulated with 9-*cis*-RA alone were used as a control. The cells then were detached and subjected to propidium iodide PI staining. M1 is the percentage of apoptotic cells (A–C). (D) Western blotting of PARP precursor and PARP cleaved forms of DU145, TRAMP, and MDA-MB-231 cells treated with 25 μ g/mL Poly(I:C) for 48 h or with 10 μ M 9-*cis*-RA for 72 h alone, or pretreated with 25 μ g/mL Poly(I:C) for 48 h and subsequently with 10 μ M 9-*cis*-RA for 72 h. Untreated cells were used as a control. β -Actin was used as loading control. Data represent typical experiments that were repeated three times with similar results. * P < 0.05; ** P < 0.01. a.u., arbitrary units.

precursor cleavage and cleaved PARP appearance (Fig. 6D). Overall, these data indicate that pretreatment with Poly(I:C) induces reexpression of a functionally active RAR β ; however, this is not sufficient per se to induce cell cycle arrest or apoptosis, as reported for different cell types, although it restores DU145, TRAMP, and MDA-MB-231 cell sensitivity to treatment with RAR β agonist 9-*cis*-RA.

Poly(I:C)/9-*cis*-RA Effects in Vivo. To investigate the in vivo relevance of our in vitro experiments, we injected DU145 cells s.c. in the backs of 20 nude mice. Two weeks after the injection, we randomly divided the mice into four groups ($n = 5$ per group). All groups were treated as shown in Table S1.

In a first experiment, mice were treated for a total two cycles, with a week of rest between treatments. In the second experiment, we eliminated the week of rest between treatment cycles. In both cases, starting from the second week of treatment, we observed a significant reduction in tumor growth only in group 4 [mice treated with the Poly(I:C)/9-*cis*-RA combination] (Fig. 7A–C). We and others previously reported that Poly(I:C) stimulation may induce an indirect antitumor effect, activating a strong inflammatory response with the recruitment of immune cells acting against the tumor (6, 29). To test the involvement of the immune system in the mechanism described, we injected TRAMP cells in syngeneic C57 black 6 immunocompetent mice. Two weeks after the injection, the mice were divided randomly and treated as described for the experiment in Fig. 7B. After 2 wk of treatment, we observed

a reduction in tumor growth in Poly(I:C)-treated mice compared with PBS-treated mice; however, these data were not statistically significant. We also observed a significant reduction in tumoral mass in mice treated with the Poly(I:C)/9-*cis*-RA combination (Fig. 7D and E). We also analyzed *ex vivo* the xenograft and syngeneic tumors (Fig. 7F, Left and Right, respectively) and observed that Poly(I:C) treatment induced up-regulation of miR-29b, -29c, -148b, and -152, and RAR β up-regulation (Fig. 7G, Upper and Lower, respectively), confirming that TLR3 agonist Poly(I:C) induces reexpression of the epigenetically silenced RAR β through an miRNA-mediated mechanism and restores sensitivity of cancer cells to retinoids.

Quantification of miR-29b, -29c, -148b, and -152 in Prostate and Breast Human Cancer Samples.

To quantify the basal expression level of miR-29b, -29c, -148b, and -152 in human primary prostate and breast cancer patients, we used qRT-PCR on RNA extracted from both normal and tumoral samples, whose clinical characteristics are listed in Fig. S7A and B. We observed strong down-regulation of all four miRNAs of interest in the prostate tumor vs. the normal counterpart (Fig. S7C; $P < 0.01$, Wilcoxon test). No correlation was observed with the Gleason score. Although not statistically significant using the Wilcoxon test, in breast cancer patients we observed down-regulation for all four miRNAs of interest, with median values of 0.79, 0.50, 0.83, and 0.62 for miR-29b, -29c, -148b, and -152, respectively (Fig. S7D). Also, in this case, no correlation

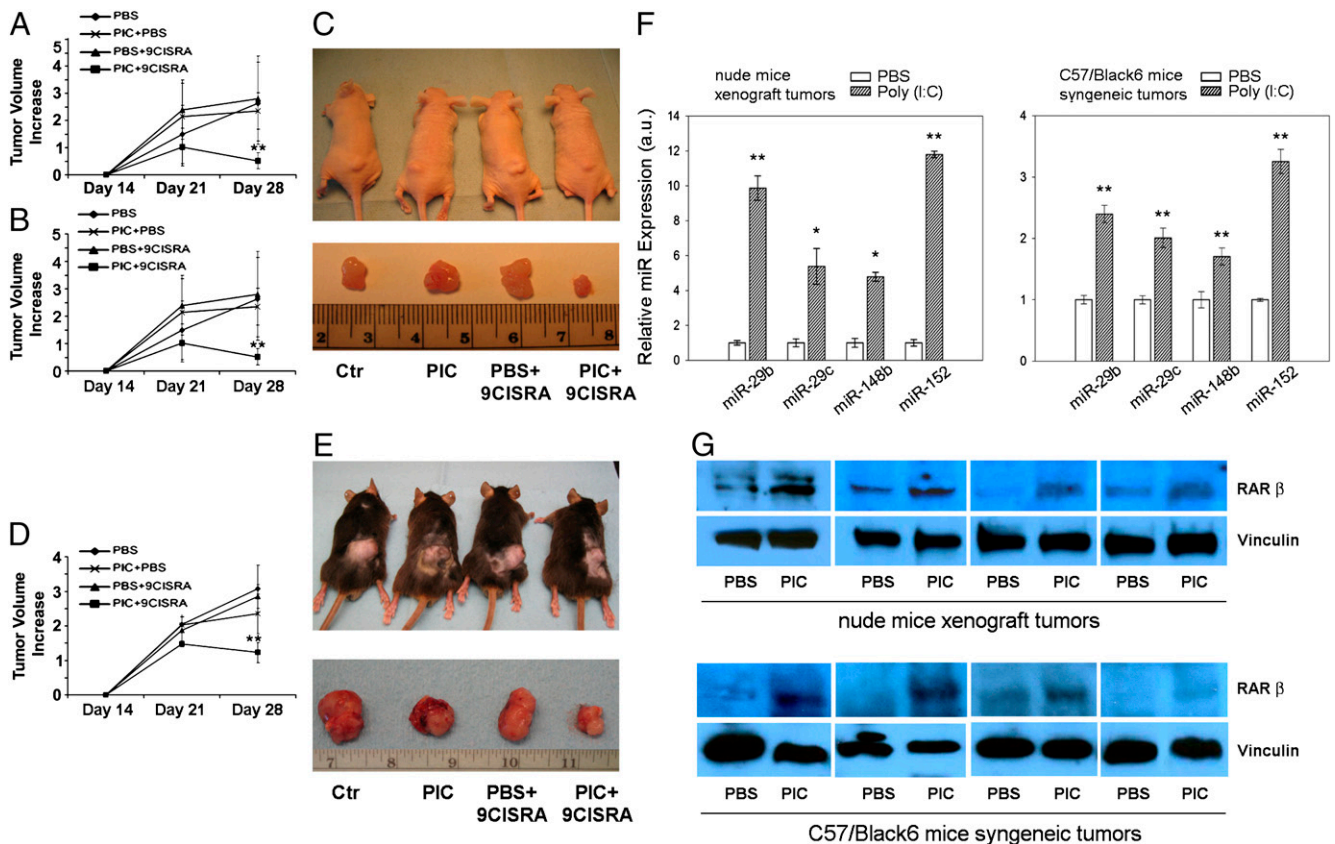


Fig. 7. Poly(I:C) plus 9-*cis*-RA treatment effect on tumoral growth in vivo. Nude mice injected with DU145 cells or C57 black 6 mice injected with TRAMP cells were divided into four groups, then treated only with PBS (i.p., then intratumorally); with Poly(I:C) (250 μ g) i.p., then PBS intratumorally; with PBS i.p., then 9-*cis*-RA (50 μ L of 500- μ M solution) intratumorally; or with Poly(I:C) i.p., then 9-*cis*-RA intratumorally (as described in Table S2). The data in A and B are from two different experiments conducted on nude mice (for details, see Results and Discussion). The data in D are from an experiment conducted on C57 black 6 mice. The graphs represent the averages with SD of size tumors from five mice of each group. $**P < 0.01$. (C and E) Representative images of mice and tumors collected at the end of the experiments presented in A and D. (F) Real-time PCR for miR-29b, -29c, -148b, and -152 performed on RNA extracted from xenograft tumors of nude mice (Left) or C57 black 6 mice (Right) treated only with PBS (i.p., then intratumorally) or with Poly(I:C) i.p., then with PBS intratumorally. $*P < 0.05$; $**P < 0.01$. (G) Western blot for RAR β performed on lysates from eight xenograft tumors of four nude mice (Upper) or C57 black 6 mice (Lower) treated only with PBS or four mice treated with Poly(I:C). Vinculin was used as a control for an equal amount of protein loaded.

was observed with the tumor grade. A recent paper demonstrated that in breast cancer, down-regulation of the miR-29 family induces dedifferentiation of the cells and increases cancer stem cell population, which favors tumor progression (30). Moreover, previously published data from our laboratory demonstrates that the miR-29 family is down-regulated in invasive breast cancer (30, 31). Overall, these data strongly suggest that in prostate and breast cancer, down-regulation of these miRNA levels might be an important condition for the onset of the pathology, and treatment with Poly(I:C) might be beneficial in reversing this condition. In summary, here we describe a mechanism of action through which Poly(I:C) exerts an antitumoral effect on prostate and breast cancer cell lines by modulating the expression of miRNAs, thereby restoring retinoid sensitivity by reexpressing functionally active. The evidence provided by

this study suggests that Poly(I:C) might be used effectively in combination with retinoids, opening possible therapeutic avenues for treating prostate and breast cancer. In addition, the demethylating response to Poly(I:C) might allow stratification of prostate and breast cancer patients based on their responsiveness to retinoid therapy.

Methods

A detailed description of the materials and methods used in this study may be found in *SI Methods*.

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