

Anti-RhoA and Anti-RhoC siRNAs Inhibit the Proliferation and Invasiveness of MDA-MB-231 Breast Cancer Cells *in Vitro* and *in Vivo*

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Overexpression of RhoA or RhoC in breast cancer indicates a poor prognosis, due to increased tumor cell proliferation and invasion and tumor-dependent angiogenesis. Until now, the strategy of blockage of the Rho-signaling pathway has used either GGTI or HMG-CoA reductase inhibitors, but they are not specific to RhoA or RhoC inhibition. In this study, a new approach with anti-RhoA and anti-RhoC siRNAs was used to inhibit specifically RhoA or RhoC synthesis. Two transfections of either RhoA or RhoC siRNA (8.5 nM) into MDA-MB-231 human breast cancer cells or HMEC-1 endothelial cells induced extensive degradation of the target mRNA and led to a dramatic decrease in synthesis of the corresponding protein. *In vitro*, these siRNAs inhibited cell proliferation and invasion more effectively than conventional blockers of Rho cell signaling. Finally, in a nude mouse model, intratumoral injections of anti-RhoA siRNA (100 μ l at 85 nM) every 3 days for 20 days almost totally inhibited the growth and angiogenesis of xenografted MDA-MB-231 tumors. One may infer from these observations that specific inhibition of the Rho-signaling pathway with siRNAs represents a promising approach for the treatment of aggressive breast cancers.

Key Words: RhoA, RhoC, anti-cancer therapy, siRNA, GGTI, angiogenesis, breast cancer, cancer cell signaling

INTRODUCTION

Cancer aggressivity may be defined as the association of increased cellular proliferation with increased invasiveness, and low-molecular-weight GTP/GDP-binding GTPases of the Ras superfamily, Ras homologous A (RhoA) and Ras homologous C (RhoC), have been

shown to promote both cell proliferation and cell invasion [1,2]. Indeed, accumulating data indicate that Rho-protein-dependent cell signaling is important for malignant transformation [3–5]. Once activated, RhoA triggers a complex set of signal transduction pathways that include both the Rho-associated coiled-coil-containing protein kinase (ROCK) activation pathway, which is responsible for the actin polymerization required for cell locomotion, and the phosphatidylinositol 3-phosphokinase/protein kinase B (PI3-K/AKT) pathway, thought to be essential for cell survival and expression of genes involved in cell proliferation [5]. In several human cancers, RhoA is overexpressed during tumorigenesis [6]. Fritz *et al.* [7] examined several breast cancers and concluded that the overexpression of RhoA

Abbreviations used: AKT, protein kinase B (or PKB); bFGF, basic fibroblast growth factor; CDS, cell dissociating solution; FAK, focal adhesion kinase (p125^{FAK}); FTI, farnesyl transferase inhibitor; GGTI, geranylgeranyl transferase inhibitor; PI3-K, phosphoinositide 3-kinase; Rho, Ras homologous; ROCK, Rho-associated coiled coil-forming serine/threonine protein kinase (Rho kinase); siRNA, small interfering RNA.

GTPase is involved in human carcinogenesis. Indeed, all breast tumors analyzed contained large amounts of RhoA protein, whereas RhoA was hardly or not at all detectable in adjacent normal tissue. In addition, the progression of breast tumors from WHO grade I to grade III was accompanied by a significant average increase in RhoA protein levels. Moreover, cancer aggressivity is also associated with increased angiogenesis, and RhoA signaling is implicated in this process. Indeed, we previously showed that angiogenic factors induce activation of RhoA in HMEC-1 microvascular endothelial cells and that this activation is required for angiogenesis (proliferation, migration, and capillary tube formation) [8,9]. More recently, RhoC was also shown to be involved in cancer invasion in melanoma [10], inflammatory breast cancer [7,11], and ovarian cancer [12].

The low-molecular-weight GTPases must be prenylated to become biologically active [13]; Ras and Rho prenylations are controlled by the enzymes farnesyl transferase and geranylgeranyl transferase, respectively. Since Ras has been found to be activated in more than 30% of cancers due to mutation, and since RhoA is overexpressed and spontaneously activated in several aggressive cancers [6], farnesyl transferase inhibitors (FTIs) [15,16] and geranylgeranyl transferase inhibitors (GGTIs) [17,18] are currently being tested as anti-cancer agents.

The isoprenoids farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively required for activation of Ras and Rho [6,14], are precursors of cholesterol. It is therefore not surprising that Ras and Rho signaling can also be inhibited by statins (such as cerivastatin), which are inhibitors of hydroxymethylglutaryl coenzyme A reductase [5,8]. Indeed, statins are potent inhibitors of cholesterol biosynthesis that prevent formation of the isoprenoids necessary for activation of these GTPases. Accordingly, recent experimental and clinical evidences indicate that some of the cholesterol-independent or "pleiotropic" effects of the statins result from inhibition of the small GTP-binding proteins Rho, Ras, and Rac, whose membrane localizations and functions are dependent on isoprenylation and which may play important roles in mediating the direct cellular effects of statins [19,20].

We previously reported that, in aggressive breast cancer-derived cell lines such as MDA-MB-231 cells (which have constitutive activation of Ras and RhoA and overexpression of RhoA and RhoC), cerivastatin strongly inhibits *in vitro* their aggressive proliferation and invasiveness [21]. Inhibitory effects of statins on other types of cancer cells have also been reported [22]. Cerivastatin also prevents angiogenesis [8,9]. These effects on cancer and endothelial cells are consequences of the blockage of two pathways [6,9,23–25]: the ROCK pathway, involved in cell spreading and motility, which is activated during tumor invasion, and the

RhoA/FAK/PI3-K/AKT signaling pathway [5]. Interference with the latter pathway might be crucial for cerivastatin's anti-cancer activity, since signaling through this pathway in turn activates three different transcriptional factors involved in the expression of genes implicated in cancer cell proliferation: nuclear factor κ B, activator protein 1, and β -catenin [5]. In addition, the observed inhibitory effects of cerivastatin on cancer and endothelial cells result from blockage of Rho-mediated cell signaling, but not Ras-mediated signaling, since they can be reversed by GGPP but not by FPP [5,8,9].

These observations prompted us to assess the anti-cancer effects of specific RhoA and RhoC inhibitors and to compare them with those of cerivastatin, FTI, and GGTI in MDA-MB-231 breast cancer cells and in HMEC-1 endothelial cells. In an attempt to inhibit RhoA and RhoC specifically, we used chemically synthesized short interfering RNAs (siRNAs), i.e., small double-stranded oligonucleotides that can specifically and effectively direct homology-dependent posttranscriptional gene silencing. Each siRNA triggers the degradation of the endogenous mRNA to which it hybridizes [26,27]; this mechanism is specific and leads to switching-off of synthesis of the target protein. Furthermore, it has been shown that siRNAs are able to enter tumors and penetrate cells both *in vitro* and *in vivo* [28]. In the present study, we determined the *in vitro* effects of anti-RhoA and anti-RhoC siRNAs on proliferation and invasiveness of MDA-MB-231 cells and on capillary tube formation by HMEC-1 cells. We also evaluated the ability of these siRNAs to inhibit the growth of xenografted MDA-MB-231 tumors *in vivo* in a mouse model.

RESULTS

Anti-RhoA and Anti-RhoC siRNA Transfections Specifically Down-Regulate the Corresponding mRNA and Protein Levels in MDA-MB-231 and HMEC-1 Cells

We analyzed the efficacy of siRNA-mediated inhibition of RhoA and RhoC synthesis in MDA-MB-231 and HMEC-1 cells by both reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting. Because the results obtained were virtually identical in the two cell types, only one of them is presented here. As shown in Fig. 1, when we transfected MDA-MB-231 cells with the corresponding siRNA, RhoA and RhoC mRNAs (Figs. 1A and 1B) and proteins (Figs. 1D and 1E) were down-regulated 24 h later; two transfections were more effective than one. This down-regulation is specific, because anti-RhoA siRNA did not modify RhoC mRNA expression (Fig. 1A), and anti-RhoC siRNA did not modify RhoA mRNA expression (Fig. 1B). Furthermore, β -actin mRNA levels were not modified by exposure to either of these siRNAs (Figs. 1A and 1B). Finally, an unrelated control siRNA also failed to modify RhoA or

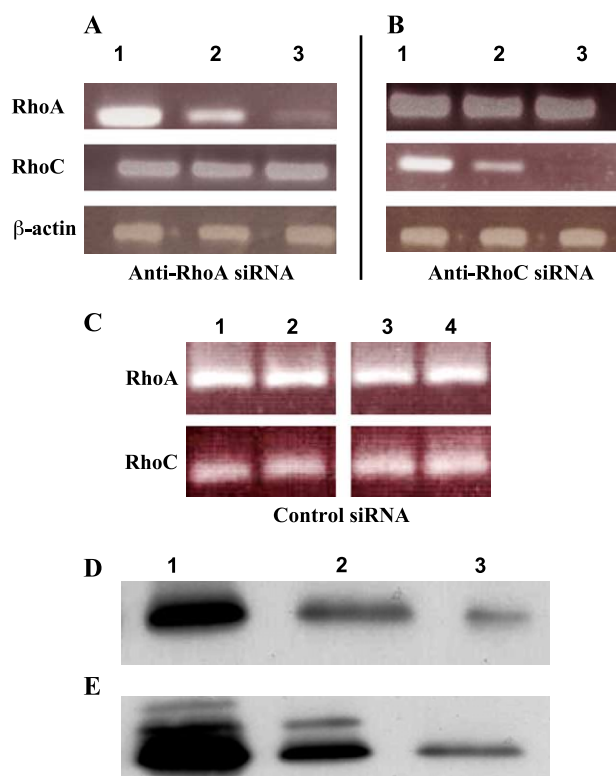


FIG. 1. Anti-RhoA and anti-RhoC siRNAs inhibit RhoA and RhoC mRNA levels and protein synthesis. MDA-MB-231 cells were either untreated (lanes 1) or transfected with 8.5 nM anti-RhoA, anti-RhoC, or unrelated control siRNA once (lanes 2), twice (lanes 3), or three times (lane 4; C only) at 24-h intervals. Assays were performed 24 h after the end of treatment. For mRNA analyses, cells were treated with (A) anti-RhoA siRNA, (B) anti-RhoC siRNA, or (C) control siRNA, and mRNA expression was followed by RT-PCR; β -actin served as an internal control. Western blots were performed to assay RhoA protein expression after (D) anti-RhoA siRNA treatment and (E) RhoC protein expression after anti-RhoC siRNA treatment.

RhoC mRNA expression when transfected two or even three times at 24-h intervals (Fig. 1C). Western blot analysis revealed that when anti-RhoA (Fig. 1D) or anti-RhoC (Fig. 1E) siRNA was transfected twice, expression of the corresponding target proteins was decreased by at least 90%; but when the incubation was prolonged for another 24 h without any further addition of siRNA, expression levels began to increase, mainly because of the short life span of siRNA in these cells (not shown). For this reason, we selected the two-transfection protocol for subsequent studies of the physiological consequences of RhoA and RhoC knockdown.

Anti-RhoA and Anti-RhoC siRNA Inhibit MDA-MB-231 and HMEC-1 Cell Proliferation more Effectively Than GGTI, FTI, or Cerivastatin

After a single transfection with anti-RhoA or -RhoC siRNA, MDA-MB-231 cell counts rose by only about 33% in the ensuing 24 h, contrasting with the 74%

increase seen in untreated or control-siRNA-treated cells during this same period of time (Fig. 2A). After the second transfection, proliferation was totally blocked in the anti-RhoA- or -RhoC-treated groups, whereas a 260% increase was seen in the controls. This represents an inhibition of nearly 85% over the 48-h period. The potent and selective inhibitor GGTI-298 (used at 10 μ M) was less effective than these siRNAs, and the selective inhibitor FTI-277 (used at 10 μ M) had no significant effect on MDA-MB-231 cell proliferation. Cerivastatin (25 ng/ml) inhibited MDA-MB-231 cell proliferation to the same extent as GGTI-298 (not shown).

Similarly, two transfections with anti-RhoA or anti-RhoC siRNA inhibited HMEC-1 cell proliferation by more than 80% (not shown).

Transfection with Anti-RhoA or Anti-RhoC siRNA Inhibits MDA-MB-231 Cell Invasion through Matrigel

As shown in Fig. 2B, two transfections of MDA-MB-231 cells with anti-RhoA siRNA inhibited cell invasion through Matrigel by more than 90%, and transfection with anti-RhoC siRNA resulted in nearly 70% inhibition, whereas the control siRNA had no effect. In comparison, GGTI-298 decreased MDA-MB-231 cell invasion by nearly 45%, and FTI-277 was totally ineffective.

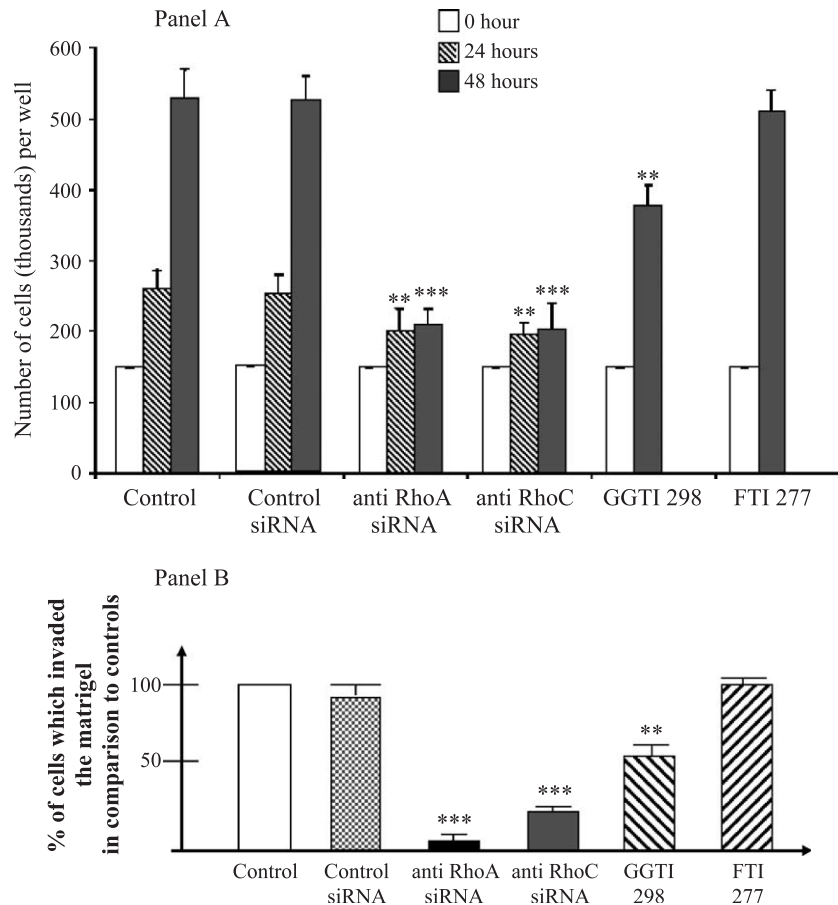
Transfection with Anti-RhoA or Anti-RhoC siRNA Inhibits Capillary Tube Formation by HMEC-1 Cells

The photos presented in Fig. 3 are representative of results obtained in three independent experiments. Basic fibroblast growth factor (bFGF) stimulation of HMEC-1 cells (control) for 9 h triggered capillary tube formation and sprouting of new capillaries; note the closed polygon and complex mesh-like structures with several shunts and clearly formed branches. siRNA transfection abrogated this capillary tube formation, with cells retaining their normal morphology when transfected by anti-RhoC siRNA but becoming rounded after transfection with anti-RhoA siRNA.

Transfection with Anti-RhoA or Anti-RhoC siRNA Decreases Nuclear Localization of β -Catenin

As mentioned previously, signaling through the RhoA/FAK/PI3-K/AKT pathway leads to β -catenin activation and nuclear translocation [5]. High β -catenin activity in the nucleus of primary breast cancer cells has been shown to correlate with poor prognosis [35], and we previously showed that MDA-MB-231 cells indeed have high levels of nuclear β -catenin [21]. For these reasons, we tested the effects of RhoA and RhoC knockdown on β -catenin levels. Results presented in Fig. 4 show that transfection of MDA-MB-231 cells with anti-RhoA or anti-RhoC siRNA resulted in markedly decreased concentrations of β -catenin in the

FIG. 2. Anti-RhoA and anti-RhoC siRNAs inhibit MDA-MB-231 breast cancer cell proliferation and invasiveness more effectively than GGTI 298 and FTI 277. (A) 1.5×10^5 MDA-MB-231 cells were seeded at t_0 , with or without agents to be tested, and counted in a Coulter counter after 24 or 48 h of culture in medium containing 2% FCS. Agents were added at the following (final) concentrations: 8.5 nM control, anti-RhoA, or anti-RhoC siRNA; 10 μ M FTI-277 or GGTI-298. SiRNAs were added twice, at t_0 and 24 h later. The control was run in medium alone, after verifying that the concentration of cytofectin used for transfection had no effect on cell proliferation (not shown). Mean numbers of cells/well \pm SEM, based on three independent experiments, are presented. (B) MDA-MB-231 cells were preincubated for 24 h with the above concentrations of siRNA, FTI-277, or GGTI-298 before addition to the Transwell chamber; controls for FTI and GGTI were run in medium alone, and siRNA controls, in the presence of cytofectin at the concentration used for siRNA transfection. Cells were then detached, resuspended in the presence of the same agents at the same concentrations, and seeded into the Matrigel-coated upper chamber of a Transwell. The number of cells that had crossed the 8- μ m-pore filter 24 h later in response to bFGF stimulation were counted by light microscopy after May-Grünwald-Giemsa staining. Percentages of cells (means \pm SEM) invading the Matrigel compared to the appropriate control, based on three independent experiments, are presented. Results with HMEC-1 cells were similar (not shown). ** $p < 0.01$, *** $p < 0.001$; Mann-Whitney test.



nucleus, with maximal inhibition being obtained after three transfections.

Intratumoral Injection of Anti-RhoA or Anti-RhoC siRNA Inhibits Tumor Growth and Angiogenesis

To investigate the effects of anti-RhoA or anti-RhoC siRNA transfection on tumor growth, we injected 100 μ l siRNA (85 nM) every 3 days into preestablished human MDA-MB-231 breast carcinoma tumors (approximately 20 mm³) grown in nude mice. As shown in Fig. 5A, the anti-RhoA siRNA-injected group achieved sustained

and significant arrest of tumor growth (85% decrease in mean tumor volume on day 20), with complete inhibition in three mice, whereas the growth of tumors injected with anti-RhoC siRNA was less significantly inhibited (53% decrease). We sacrificed the mice on day 20 after beginning intratumoral injections and removed the tumors for analysis of vascularization (Fig. 5B). Immunolabeling with platelet-endothelial cell adhesion molecule 1 (PECAM-1) was much weaker in tumors excised from anti-RhoA siRNA-injected mice. The angiogenesis indexes were 30.5 ± 4.12 for

FIG. 3. Anti-RhoA and anti-RhoC siRNAs inhibit capillary tube formation by HMEC-1 cells on Matrigel. HMEC-1 cells were either transfected with 8.5 nM anti-RhoA or anti-RhoC siRNA as indicated or incubated in medium containing the same concentration of cytofectin as that used for transfection (Control). 24 h later, cells were resuspended in media containing the same agents at the same concentrations, and 4×10^4 cells were seeded on Matrigel. 9 h later, these preparations were photographed under an inverted light microscope at 40 \times magnification.



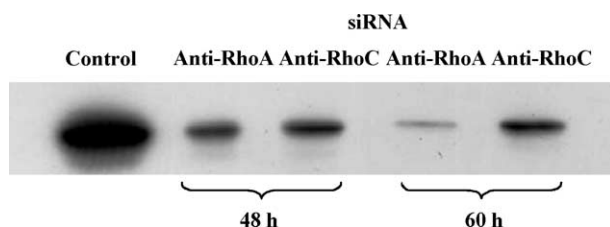


FIG. 4. Transfection of MDA-MB-231 cells with anti-RhoA or anti-RhoC siRNA decreases nuclear β -catenin levels. Cells were either untreated (control) or transfected two or three times at a 24-h interval with 8.5 nM anti-RhoA or anti-RhoC siRNA. After the indicated total incubation times (48 h for two transfections; 60 h for three transfections), nuclear extracts were subjected to SDS-PAGE and Western blot analysis using an anti- β catenin monoclonal antibody.

controls, 8.75 ± 3.30 in the anti-RhoA siRNA-treated group, and 22.5 ± 3.32 in the anti-RhoC siRNA-treated group.

DISCUSSION

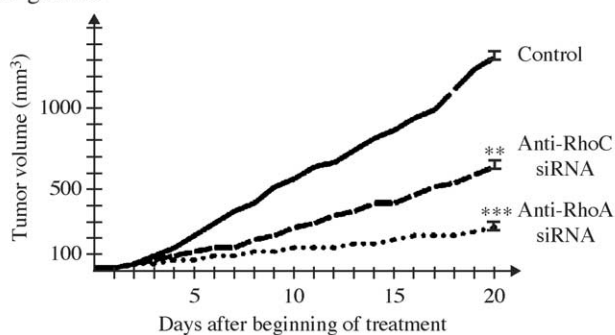
The results of numerous studies indicate that RhoA is overexpressed in cancers and suggest that Rho-protein-dependent cell signaling may be important for malignant transformation [6,21] and angiogenesis [8,9]; it therefore might be anticipated that inhibition of Rho-protein synthesis could provide an effective means for inhibiting cancer cell proliferation and invasiveness.

To inhibit specifically RhoA and RhoC, both known to be involved in cancer aggressivity, we used chemically synthesized siRNAs to achieve direct homology-dependent posttranscriptional gene silencing. After verifying the specificity and efficacy of anti-RhoA and anti-RhoC siRNAs for knocking down RhoA and RhoC protein expression, respectively, we tested their capacity to suppress the proliferation and migration of MDA-MB-231 cells derived from an aggressive human breast cancer characterized by an oncogenic Ras mutation, constitutive activation of RhoA and overexpression of RhoA [5] and RhoC. The effects of two successive transfections of these siRNAs (optimal conditions) were compared to those of cerivastatin, which totally prevents Rho transport to the cell membrane by inhibiting isoprenoid synthesis [5], and of two prenyl-transferase inhibitors, FTI-277 and GGTI-298.

Of all the agents tested, the anti-RhoA and anti-RhoC siRNAs most strongly inhibited the proliferation and invasiveness of MDA-MB-231 cells. Furthermore, translocation of β -catenin into the nucleus of MDA-MB-231 cells was prevented by transfection of these specific siRNAs, which is important because nuclear β -catenin can act as an oncogene [35]. Pertinently, the specific siRNAs also blocked proliferation and capillary tube formation by HMEC-1 cells in response to stimulation by angiogenic factors.

Taken together, our data demonstrate that anti-RhoA and anti-RhoC siRNAs are more effective than the

Panel A: Tumor growth



Panel B: Intratumoral vascularization

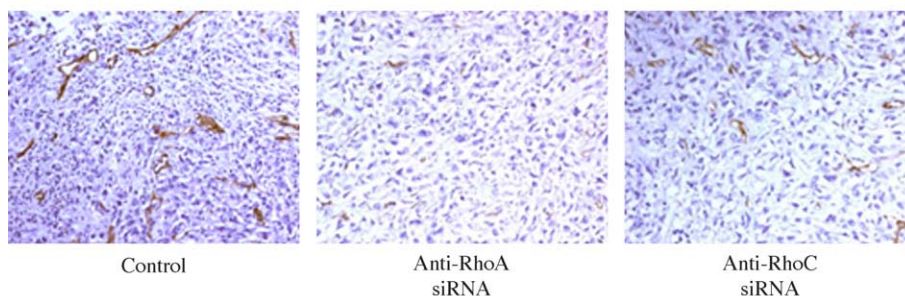


FIG. 5. Effects of intratumoral injections of anti-RhoA or anti-RhoC siRNA on the growth and vascularization of MDA-MB-231 cells xenografted into mice. 4×10^6 MDA-MB-231 cells were injected sc into the upper hind limb of athymic nude mice and allowed to grow until the tumor reached 20 mm³. Mice then received intratumoral injections of anti-RhoA or anti-RhoC siRNA (100 μ l at 85 nM), or cytofectin alone (control), every 3 days. (A) Tumor growth was assessed daily until day 20 of treatment by measuring two perpendicular diameters and calculating the volume in mm³. Means \pm SEM, eight mice per group. ** $P < 0.01$, *** $P < 0.001$ (Mann-Whitney test on day 20). (B) Intratumoral vascularization was assessed by PECAM-1-immunolabeling on paraffin-embedded MDA-MB-231 tumor sections (400 \times original magnification).

classical Rho-dependent signaling inhibitors at blocking cancer cell aggressivity. Indeed, FTI was ineffective on MDA-MB-231 cells, as previously demonstrated [5]. The mechanism responsible for this resistance to FTI is not yet known, but can perhaps be explained by the fact that the product of the *Ki-ras* proto-oncogene remains prenylated in FTI-treated but not in GGTI-treated cells [18]. The weaker inhibitory effect of GGTI (or cerivastatin, not shown) on MDA-MB-231 cell proliferation and invasiveness, compared to anti-RhoA or anti-RhoC siRNA, probably reflects the former's lack of specificity, since GGTI also inhibits the prenylation of several other proteins, including RhoB, known to antagonize malignant transformation [36,37]. Indeed, prenylated RhoB is known to inhibit both anchorage-dependent and anchorage-independent growth, to induce apoptosis, to inhibit constitutive activation of Erk and insulin-like growth factor-1 stimulation of AKT, and to suppress tumor growth in nude mice [36].

To evaluate the *in vivo* effects of anti-RhoA and anti-RhoC siRNAs, MDA-MB-231 tumor cells were xenografted subcutaneously (sc) into nude mice. Because our *in vitro* results indicated that siRNA efficacy is short-lived and must be repeated to maintain inhibition, intratumoral injections of specific siRNA were administered every 3 days for 20 days. This treatment regimen resulted in an 85% decrease in mean tumor volume in anti-RhoA siRNA-treated mice and 53% in anti-RhoC siRNA-treated mice. This difference in efficacy was not seen in the *in vitro* tests and may reflect the species specificities of the siRNA sequences chosen. Indeed, the anti-RhoA siRNA target is identical in mouse and human, whereas the anti-RhoC sequence is specific to the human gene. As a result, the anti-RhoA siRNA should be able to inhibit both proliferation of the grafted tumor cells, which are of human origin, and angiogenesis, derived from host endothelial cells. In contrast, the anti-RhoC siRNA should interfere only with proliferation of the grafted cells. This hypothesis is supported by the fact that the angiogenic index was much lower in anti-RhoA siRNA-treated than in anti-RhoC siRNA-treated mice. Furthermore, in the latter group, inhibition of tumor growth by the anti-RhoC siRNA would be expected to result in decreased secretion of angiogenic factors, leading to reduced angiogenesis; this is indeed what we observed: the angiogenic index for the anti-RhoC siRNA-treated mice was lower than that seen in the controls.

It has been fortuitously observed that patients with high cholesterolemia who are treated with statins seem to develop fewer cancers [38]. Unfortunately, intratumoral injection of high concentrations of statins in xenografted mice generated only local necroses (not shown). The remarkable tumor growth inhibition we have obtained with intratumoral injection of anti-RhoA and anti-RhoC siRNAs can be explained by the excellent penetration of siRNA into the tumors when cytofectin is used to deliver

the siRNA [28], and it appears that this approach may indeed prove to be applicable in human and thus deserves further study.

In summary, our results indicate that anti-RhoA and anti-RhoC siRNAs represent powerful tools for inhibiting cancer cell proliferation, invasiveness, and angiogenesis both *in vitro* and *in vivo*, with potential therapeutic utility for the treatment of aggressive breast cancers.

MATERIAL AND METHODS

Cell cultures. MDA-MB-231, an aggressive human breast carcinoma cell line, was grown in RPMI 1640 medium (Eurobio, Les Ulis, France) supplemented with 10% heat-inactivated fetal calf serum (FCS; Costar, Brumath, France), 2 mM L-glutamine (Gibco BRL Life Technologies, Grand Island, NY, USA), and 100 IU/ml penicillin/streptomycin (Sarbach, Suresnes, France/Diamant, Puteaux, France). Cells were cultured at 37°C in a humidified 5% CO₂ atmosphere.

The human microvascular endothelial cell-1 (HMEC-1) line was kindly provided by E. W. Ades (Centers for Disease Control and Prevention, Atlanta, GA, USA), who established this cell line by transfecting human dermal endothelial cells with the SV40 A gene product and large T antigen [29]. These cells have properties similar to those of the primary microvascular endothelial cells [30]. Moreover, HMEC-1 proliferation, migration, and capillary tube formation are greatly increased by angiogenic factors, such as bFGF and vascular endothelial growth factor [8,9]. HMEC-1 cells were cultured in complete MCDB131 medium (Sigma, Paris, France) supplemented with 15% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml epidermal growth factor (Euromedex, Souffelweyersheim, France), and 1 µg/ml hydrocortisone (Pharmacia-Upjohn, St-Quentin-en-Yvelines, France). HMEC-1 cells were used before the 15th passage, because after 25 passages the endothelial cells undergo morphological and functional changes that make them unsuitable for angiogenesis assessment.

siRNA treatment. Specific siRNAs directed against human RhoA or RhoC were designed using the criteria established by Tuschl [27]. The RhoA and RhoC coding sequences were scanned to identify AA(n19)TT sequences. Candidate sequences were compared with RhoA and RhoC cDNA sequences and their specificity was verified in the nonredundant human DNA database using a BLAST algorithm (accession through NCBI). A control siRNA was also tested. It was selected because it exhibited no cellular toxicity.

The sequences selected for the sense and antisense strands are for anti-RhoA siRNA, sense 5'-GACAUGCUUGCUCUAUGUCTT-3', antisense 3'-TTCUGUACGACGAGUAUCAG-5'; for anti RhoC siRNA, sense 5'-GACCUGCCUCCUCAUCGUUCTT-3', antisense 3'-TTCUGGACGGAGGAGUAGCAG-5'; for the control siRNA, sense 5'-CAGUCAGGAGGAUC-CAAAGTG-3', antisense 3'-TTGUCAGUCCUCCUAGGUUUC-5'.

Synthetic oligonucleotides prepared by Eurogentech (Liege, Belgium) were annealed to form a short double-stranded RNA with a 3'-dithymidine overhang. Hybridization was performed in a buffer containing 2 mM sodium acetate, 100 mM potassium acetate, and 30 mM Hepes buffer, pH 7.4.

The siRNAs were introduced into MDA-MB-231 or HMEC-1 cells by cytofectin-mediated transfection (Ozyme, Paris, France) using the method described by Bertrand *et al.* [28]. Concentration of siRNA used was that previously described by Bertrand *et al.* [28]. Cells were cultured in six-well plates in 200 µl of serum-enriched medium. When 50% confluence was reached, 20 µl of 85 nM siRNA in cytofectin (diluted 1:100) was added dropwise to the cell cultures, and incubation was continued for the indicated times.

RNA extraction and RT-PCR. Briefly, after siRNA transfection, cells were detached with a nonenzymatic cell dissociation solution (CDS; Sigma) and washed twice in phosphate-buffered saline (PBS). Total RNA was

extracted using the SV Total RNA Isolation System (Promega, Charbonnières-les-Bains, France) according to the manufacturer's instructions.

Primers were chosen using a biomolecular sequence database (GenBank) and oligonucleotides used as primers were synthesized by GenSet (Paris, France): for RhoA, forward primer 5'-AGAGGTGTATGTGCCCA-CAGT-3' (position 93–113 bp), reverse primer 5'-CTTCGGATGATGAG-CACAC-3' (position 361–380 bp); for RhoC, forward primer 5'-GGAGGTCTACGTCCTACTGT-3' (position 93–113 bp), reverse primer 5'-TACCCGGACACTGATGTCATC-3' (position 220–240 bp); for β -actin, forward primer 5'-ATCTGGCACACACCTTCTACAATGAGCTGCG-3' (position 253–284 bp), reverse primer 5'-CGTCAACTCTGCTTGTGATC-CACATCTGC-3' (position 1049–1080 bp).

The predicted sizes for RhoA, RhoC, and actin PCR products were, respectively, 287, 147, and 838 bp.

Reverse transcription was performed at 48°C for 45 min. The reaction products were then subjected to 30 cycles of PCR for RhoA and RhoC and 35 cycles for actin. An amplification cycle consisted of 30 s at 94°C for denaturation, 30 s at 60°C, and 30 s at 68°C. Finally, an extension step at 68°C for 7 min improved the quality of the final product by extending truncated products to full length.

Evaluation of protein expression by Western blotting. Western blotting was performed 24 h after two or three transfections with siRNA. Extracts of MDA-MB-231 cells were prepared by lysing cells with hypotonic buffer (5 mM Hepes, pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1 mM aprotinin). Protein concentrations were determined using the Bradford technique [31] (Bio-Rad protein assay; Hercules, CA, USA). Equal amounts of protein extracts (20 μ g) were subjected to 10% polyacrylamide gel electrophoresis. Proteins were electrotransferred onto polyvinylidene difluoride membranes (Amersham, Saclay, France) using a semidry system (Schleicher & Schuell, Dassel, Germany). Membranes were immunoblotted overnight with mouse anti-RhoA monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-RhoC polyclonal antibody (Santa Cruz Biotechnology) diluted 1:500. Primary antibody binding was detected by enhanced chemiluminescence (Amersham) using horseradish peroxidase-coupled anti-mouse or anti-goat antibody (1:10,000; Dako, Glostrup, Denmark) for 40 min at room temperature.

Inhibition of Rho cell signaling. Adherent cells were incubated for 18 h with 10 μ M GGTI-298, 10 μ M FTI-277, or 25 ng/ml cerivastatin (concentration known to translocate RhoA totally from membrane to cytosol) [5].

Cell proliferation assay. To ensure cell viability, minimal concentrations of FCS were added to the medium: 2% for MDA-MB-231 and 7.5% for HMEC-1 cells. Briefly, 1.5×10^5 trypsinized cells were seeded into each well of a 24-well plate (Costar, Cambridge, MA, USA). The agent to be tested was next added at the appropriate concentration. For the siRNAs, two transfections were carried out (at t_0 and again at 24 h) as described above. Cells were detached with CDS and counted in a Coulter counter (Z1; Coultronics, Paris, France) at 24 and 48 h.

Cell invasion of Matrigel. As previously described [21], a Transwell with an 8- μ m-diameter pore membrane (Dutscher, Brumath, France) was coated with 500 μ l of Matrigel at 100 μ g/ml (Becton–Dickinson Europe, Meylan, France). MDA-MB-231 cells were left untreated (incubated in medium alone), treated with FTI-277 (10 μ M) or GGTI-298 (10 μ M), transfected with siRNA as described above, or treated with cytofectin alone. After 24 h of incubation, the cells were detached with CDS, washed twice with PBS, and resuspended in RPMI 1640 containing 0.2 mg/ml bovine serum albumin (BSA; Sigma) plus the agent being tested (at the same concentration as that used for the previous incubation); in the case of the siRNAs, this corresponds to a second transfection 24 h after the first. In each case, 2×10^5 cells were seeded into the upper, Matrigel-coated chamber of the Transwell. The lower chamber was filled with 1 ml of RPMI 1640 supplemented with 2 mg/ml BSA and 20 ng/ml bFGF (R&D Systems, Minneapolis, MN, USA) to induce chemotaxis. After 24 h of incubation at 37°C, the nonmigrated cells in the upper chamber were gently detached by scraping, and the adherent cells present on the lower

surface of each insert were stained by May–Grünwald–Giemsa; 10 fields were counted by light microscopy at 200 \times magnification. Results were calculated with reference to control values observed after incubation in medium alone (untreated control, for FTI-277 and GGTI-298 treatments) or cytofectin-containing medium (control for all siRNA treatments), arbitrarily set at 100%. Similar experiments were carried out with HMEC-1 cells, except that the detached cells were resuspended in MCDB-131 medium containing BSA (0.2 mg/ml).

Capillary tube formation on Matrigel matrix. HMEC-1 cells were transfected with 8.5 nM anti-RhoA or anti-RhoC siRNA or else incubated in medium containing cytofectin alone. Twenty-four hours later, cells were prepared for layering onto gels by resuspending them (8×10^4 cells/ml) in fresh medium containing the same agents at the same concentrations (i.e., they were transfected a second time). Matrigel was kept on ice for 24 h before being distributed (200 μ l per well) into 24-well culture plates. After gelation at 37°C for 30 min, the gels were overlaid with 500 μ l of cell suspension and 25 ng/ml bFGF as angiogenic agent. Capillary tube formation was observed at different time points over the next 24 h using an inverted light microscope at 40 \times magnification.

β -Catenin expression in the nuclear fraction. Cytosolic and nuclear extracts of MDA-MB-231 cells were prepared at different times after two or three siRNA transfections, using a modification of the method of Dignam *et al.* [32] as previously described [5]. Briefly, MDA-MB-231 cells were washed with cold PBS, lysed in ice-cold lysis buffer containing phosphatase and protease inhibitors, and centrifuged at 100,000g for 30 min at 4°C; the supernatant collected at this step is referred to as the cytosolic fraction. Pellets were rehomogenized in the same lysis buffer containing 2% Triton X-114 (Sigma) and centrifuged at 800g for 10 min at 4°C; the supernatant collected at this step is the nuclear fraction. Protein concentrations were determined as above [31]. To detect β -catenin, 20 μ g of protein extract was subjected to Western blotting using mouse anti- β -catenin monoclonal antibody (Santa Cruz), diluted 1:500.

In vivo tumorigenicity assay and immunohistochemical analysis. Female athymic nude (*nu/nu*) mice (Iffa Credo, L'Arbresle, France), 6 weeks of age, were housed in a temperature-controlled sterile room where humidity and light were carefully monitored. MDA-MB-231 cells (4×10^6 cells in a volume of 250 μ l) were injected sc into the upper portion of the right hind limb. When tumors reached a volume of 20 mm³ (within approximately 2 weeks), the mice were arbitrarily assigned to different groups ($n = 8$ each) to receive intratumoral injections of 100 μ l of anti-RhoA or anti-RhoC siRNA (85 nM) or else cytofectin-containing excipient (Control). Intratumoral injections were repeated every 3 days for a total of 20 days. Tumors were measured (perpendicular diameters) every day and their volumes calculated. On day 20, the mice were killed and their tumors removed for immunohistochemical analysis, to assess the extent of intratumoral vascularization. Immunohistochemical staining was performed as previously described [33]. The tumors were fixed overnight in absolute ethyl alcohol and embedded in paraffin; then 5- μ m-thick sections were cut. For vessel staining, tumor sections were dewaxed in toluene, rehydrated, permeabilized in citrate buffer (pH 6.0), quenched by 3% H₂O₂ for 5 min to eliminate endogenous peroxidase activity, washed in PBS by microwave treatment, and finally incubated with rat anti-mouse PECAM-1 (PharMingen, Paris, France) antibody for 1 h, followed by biotinylated goat anti-rat IgG antibody for 15 min. After being washed, sections were incubated with streptavidin-peroxidase, and the vessels were revealed by diaminobenzidine. Meyer's hematoxylin was used for counterstaining. The extent of vascularization (angiogenesis index) was evaluated as described by Weidner *et al.* [34] and is presented as the mean number of microvessels per microscopic field (200 \times magnification).

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