Résultats : Partie III

Analyse des rôles respectifs de RhoA et RhoC dans le phénotype des cellules d'adénocarcinome prostatique

Introduction

Parmi les protéines du sous-groupe Rho, RhoC a été décrit comme marqueur d'invasion et de dissémination métastatique. Dans le même ordre d'idée, les résultats exposés dans le premier chapitre montrent clairement une implication différente pour RhoA et RhoC au cours de la migration de fibroblastes humains. Afin de comparer leurs rôles respectifs dans le phénotype des cellules tumorales, nous avons inhibé spécifiquement leur expression dans des cellules d'adénocarcinome prostatique (PC-3) qui expriment un niveau significatif de chacune d'entre elles. Les conséquences de ces inhibitions spécifiques ont été évaluées par des analyses transcriptomiques et par détermination des propriétés tumorigéniques *in vitro* et *in vivo*.

Résumé des résultats

- La suppression de RhoA ou RhoC nous a permis de conclure que RhoC contribue significativement à la croissance sans ancrage des PC-3, alors que RhoA ne semble pas impliqué dans ce processus (Figure 17).

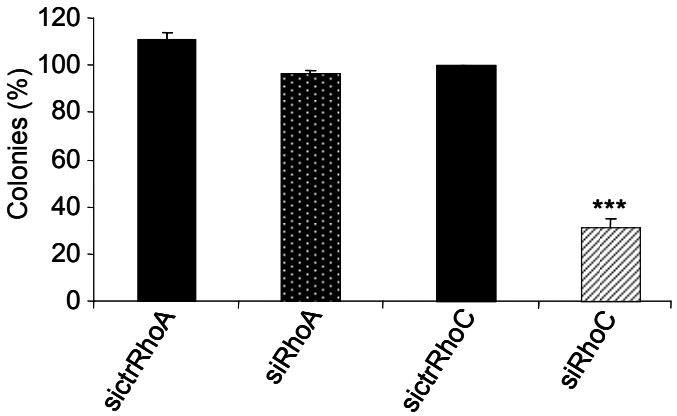

Figure 17 : RhoC et non RhoA est impliqué dans la croissance sans ancrage des PC-3. *Nombre de colonies en agar mou de PC-3 transfectées par les différents siRNA exprimé en pourcentage par rapport au sictrRhoC considéré comme 100%. Les différences significatives sont montrées par comparaison avec le contrôle sictrRhoC par le test statistique « ANOVA one way » suivie par un test de Tuckey-Kramer *** : p<0.001.*

- L'analyse de l'expression génique des cellules PC-3 par microarray montre que la répression de RhoC, mais non de RhoA, induit la surexpression d'un groupe de gènes codant pour des protéines décrites comme suppresseurs de tumeurs tels que NAG-1 (Non steroidal anti-inflammatory drug activated gene-1), p21Cip1 et p8 (Figure 18). RhoC réprime également l'expression de gènes codant pour des protéines impliquées dans les cascades de signalisation induites par le stress au niveau du réticulum endoplasmique cellulaire et susceptibles d'induire l'apoptose. Il s'agit de GADD153 (Growth Arrest and DNA Damage inducible transcription factor 153), GADD34 (Growth Arrest and DNA Damage inducible transcription factor 34), ATF-3 (Activating transcription factor-3), TRIB3 (Tribbles Homolog 3), HERPUD1 (Homocysteine-inducible, Endoplasmic Reticulum stress-inducible, Ubiquitin-like Domain member 1) et CTH (Cystathionase). La réduction de l'expression de protéines matricellulaires contribuant à la tumorigenèse comme SPARC (Secreted protein acidic and rich in cysteine ou ostéonectine) (Chen *et al.* 2007) a été aussi observée par inhibition de RhoC. Ces modulations ont été confirmées par RT-PCR en temps réel et/ou Western blot. Des données de la littérature permettent d'établir des relations entre les produits de certains de ces gènes dans divers modèles de carcinomes, dont l'adénocarcinome prostatique (Jiang *et al.* 2006; Niknejad *et al.* 2007; Papineni *et al.* 2008). Parmi les facteurs différentiellement régulés par RhoA et RhoC, NAG-1 joue un rôle central. En effet, sa répression par co-transfection d'un siNAG-1 avec un siRhoC réverse la plupart des régulations de gènes observées après l'extinction de RhoC (Figure 18).

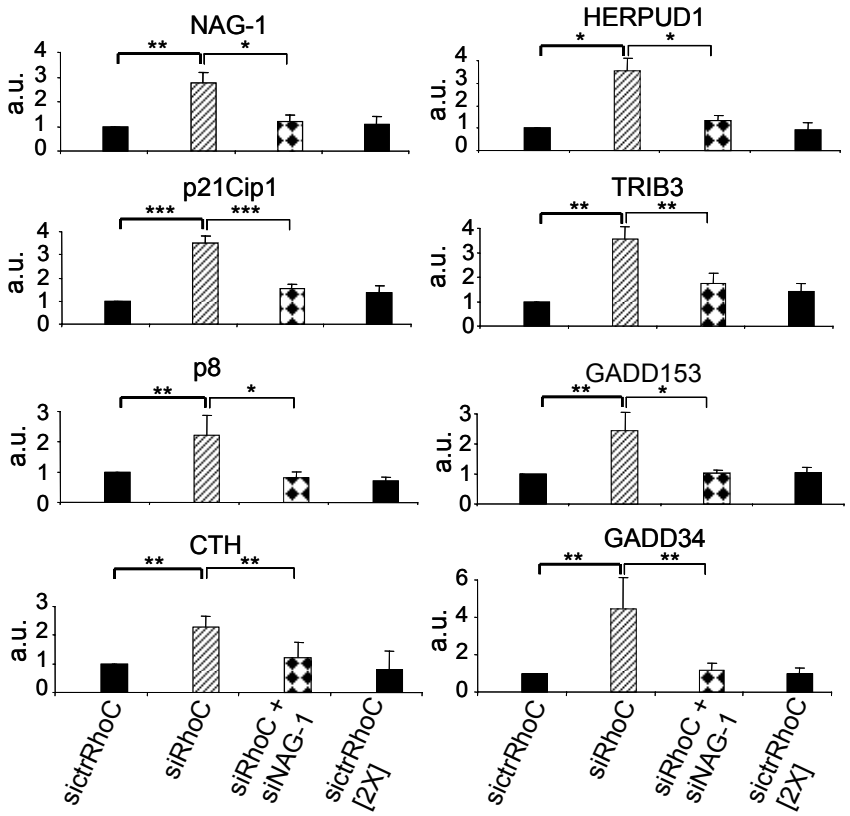

Figure 18 : L'induction de p21Cip1, p8, CTH, TRIB3, HERPUD1, GADD153 et GADD34 dans les PC-3 invalidées pour RhoC est dépendante de NAG-1. *En effet, les surexpressions observées après transfection avec le seul siRhoC sont abolies lors d'une double transfection siRhoC+siNAG-1. PCR en temps réel réalisées à partir d'ARN totaux extraits des PC-3 transfectés par sictrRhoC, siRhoC, siRhoC+siNAG-1. Les résultats sont exprimés en unités arbitraires (a.u.). Les différences significatives sont montrées par comparaison avec sictrRhoC et avec siRhoC+siNAG-1 en utilisant le test statistique « ANOVA one way » suivie par un test de Tukey-Kramer * : p<0.05; **: p<0.01; ***: p<0.001.*

L'inhibition de NAG-1 restaure également les capacités de croissance en agar mou de cellules PC-3 transfectées par siRhoC. Enfin, le rôle de NAG-1 dans le contrôle de la croissance tumorale *in vivo* par RhoC a été confirmée en comparant le développement de tumeurs PC-3 injectées *in situ* chez la souris par siRhoC seul ou siRhoC+siNAG-1 (Figure 19).

L'analyse des voies de signalisation potentiellement impliquées dans la régulation de NAG-1 par RhoC montre qu'elle dépend de GSK3β et de p38 MAPK mais n'implique pas Erk 1,2.

Conclusions

Nos résultats indiquent que la RhoGTPase RhoC, contrairement à RhoA, joue un rôle promoteur dans la tumorigenèse des cellules d'adénocarcinome prostatique *in vitro* et *in vivo*. L'exploration des mécanismes de régulation de ce processus par une analyse transcriptomique a mis en évidence l'implication du suppresseur de tumeurs NAG-1 dont l'expression est réprimée par RhoC par l'intermédiaire de la voie p38 MAPK et GSK3β. Dans notre modèle, NAG-1 régule positivement d'autres suppresseurs de tumeurs tels que p8 et p21Cip1 et module négativement l'expression de protéines contribuant à l'invasion tumorale telles que SPARC.

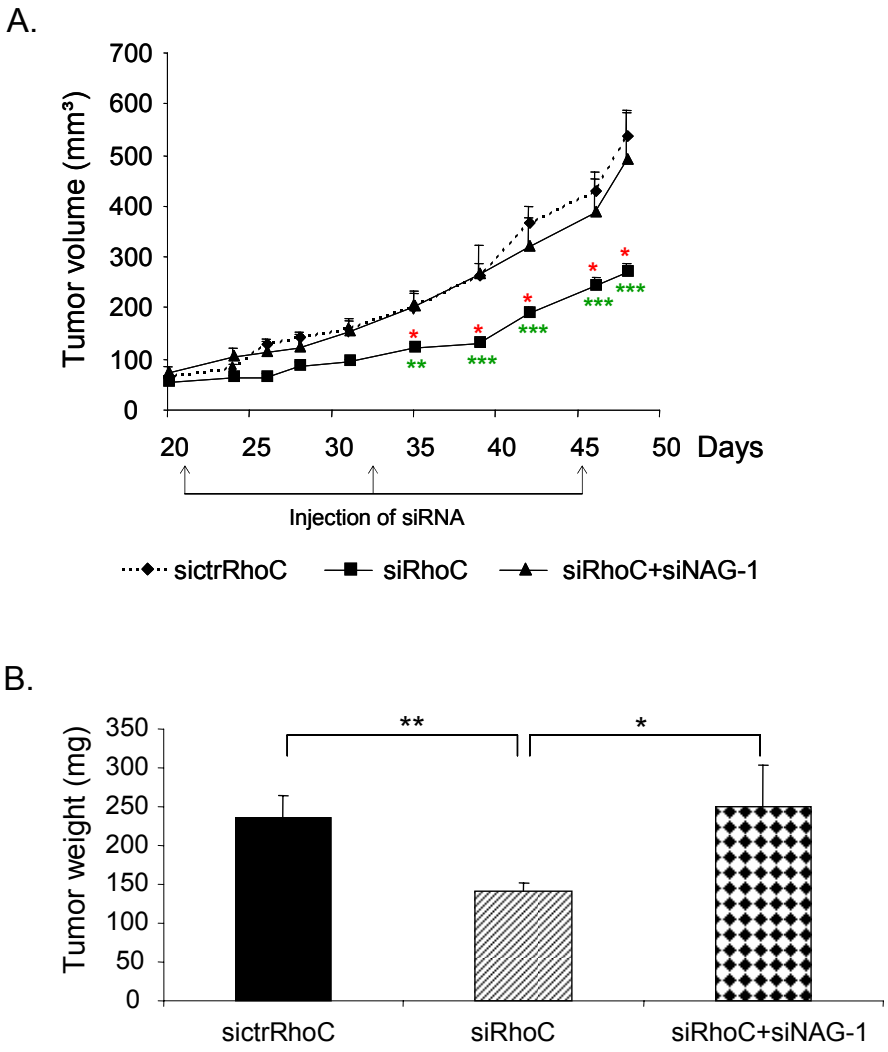

Figure 19 : RhoC est impliqué dans la croissance tumorale in vivo par un mécanisme dépendant de NAG-1. *Les PC-3 sont injectées en sous-cutané dans le flanc de souris athymiques. Après formation des tumeurs (j20), celles-ci sont injectées par sictrRhoC, siRhoC ou siRhoC+siNAG-1 associés à de l'atelocollagène à j21, j33 et j45.* ***A****. Évolution du volume des tumeurs. Les différences significatives sont montrées en utilisant le test statistique « ANOVA one way » suivie par un test de Tukey-Kramer * : p<0.05; **: p<0.01; ***: p<0.001. En vert, comparaison avec sictrRhoC. En rouge, comparaison avec siRhoC+siNAG-1.* ***B****. Après le sacrifice des animaux, les tumeurs sont excisées et pesées. Les différences significatives sont montrées en utilisant le test statistique « ANOVA one way » suivie par un test de Tukey-Kramer * : p<0.05; **: p<0.01.*

RhoC, but not RhoA, is involved in Prostate Cancer Cells Tumorigenesis through GSK3beta-dependent Regulation of NAG-1.

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RUNNING TITLE: RhoC controls tumorigenesis via NAG-1 regulation

ABSTRACT

The small GTPases of the Rho family are key elements in signal transduction pathways. Activated RhoGTPases interact with effectors that trigger a wide variety of cellular responses. Within the RhoA-related sub-class, both RhoA and RhoC, in contrast to RhoB, are reported to contribute to cancer progression. To gain insight into the individual role of RhoC and RhoA in prostate cancer cells (PC-3) phenotype, we used siRNA to specifically knock-down each of them. The silencing of RhoC, and not that of RhoA, significantly decreased the anchorage-independent growth of PC-3 and increased the expression of several genes encoding tumor suppressors such as NAG-1, p21Cip1 and p8. This transcriptomic analysis also highlighted the differential regulation of SPARC, a matricellular protein, by RhoA and RhoC. Interestingly, the transfection of RhoC-silenced PC-3 with a siRNA targeting NAG-1 reversed most of these gene regulations and restored the anchorage-independent growth of PC-3. Tumor growth of PC-3 in nude mice was significantly delayed by intratumoral injection of siRNA targeting RhoC, an inhibitory effect relieved by co-injecting siRNA targeting NAG-1. The up-regulation of NAG-1 following RhoC silencing requires p38MAPK and GSK3β but not Erk1/2. These results suggest that RhoC contributes to tumorigenesis of PC-3 through the repression of NAG-1 expression.

KEYWORDS: RhoC, RhoA, NAG-1, GSK3β, PC-3, tumor

INTRODUCTION

The small GTPases of the Rho family are key intermediates in transducing signals that originate from clustered cell adhesion receptors and drive cystoskeleton organisation and cell migration. They play also a central role in several aspects of cellular physiology including cell proliferation and survival (Jaffe & Hall, 2005). Although this family include 23 members, most of the studies focused on the three founding members: RhoA, Rac1 and Cdc42 (Aspenstrom et al., 2007). However, other members of the RhoGTPases family have critical functions in various physiological and/or pathological processes. For instance, Rac2, but not Rac1, is involved in B cell adhesion and immunological synapse formation (Arana et al., 2008), RhoE functions both as RhoA antagonist (Wennerberg et al., 2003) and keratinocytes differentiation factor (Liebig et al., 2008) and the atypical RhoGTPase RhoBTB2 is involved in protein ubiquitinylation (Wilkins et al., 2004). Among the RhoGTPase family, the Rho sub-class includes RhoA, its closely related homolog RhoC, and RhoB. These three proteins share several properties including the regulation of actin stress fibers formation. However, their roles in cancer progression are clearly not redundant. RhoB is a short-lived protein displaying anti-tumorigenic properties (Liu et al., 2001; Mazieres et al., 2004; Mazieres et al., 2005) whereas RhoA and RhoC are reported as pro-tumorigenic. Despite their high degree of homology, RhoA and RhoC display different affinity for their downstream effectors (Sahai & Marshall, 2002) and exert differential functions in several key steps of cancer progression like epithelial-to-mesenchymal transition (Bellovin

et al., 2006) and invasion of breast carcinoma cells (Simpson et al., 2004). While RhoA is ubiquitously expressed, increasing evidences suggest that RhoC expression is associated with highly aggressive cancers where it promotes metastasis (Clark et al., 2000; Kleer et al., 2002; Shikada et al., 2003; Wang et al., 2008; Wang et al., 2004; Yao et al., 2006). In particular, this was observed in prostate cancer cell lines and tissues where RhoC protein expression was associated with a metastatic phenotype (Iiizumi et al., 2008).

Interestingly, it has been established that small GTPases of the Rho family are also integral components of signalling pathways regulating transcription (Coso et al., 1995; Hill et al., 1995) and potential RhoGTPases responsive genes have been identified. The effect of Rho sub-class protein on the overall cell transcriptome have been first analysed by Teramoto et al. (Teramoto et al., 2003) and Wu et al. (Wu et al., 2004) who investigated the effect of RhoA and RhoC in NIH3T3 and MCF10A cells, respectively. More recently, a comparison of the gene expression profiles in cells transfected by constitutively active RhoA, RhoB or RhoC has been conducted (Berenjeno et al., 2007). However, in these studies, the Rho proteins are largely overexpressed and sometimes constitutively active while their targeting to specific sub-cellular compartments -which is required for some of their functions (Wu et al., 2005) and specified in the 3'untranslated region of their mRNA- is not warranted. Moreover, the cell lines used in these studies could lack components of the specific signalling pathways activated by one of these RhoGTPases. Thus, these models are not entirely satisfactory for determining some of the specific functions of each of these RhoGTPase. In this work the individual contribution of endogenous RhoA and RhoC to the cancer phenotype was evaluated by specifically knocking down their expression in PC-3 cells, a prostate cancer cell line known to express a significant level and activity of these two Rho proteins (Yao et al., 2006). The knockdown of RhoC, but not that of RhoA, inhibited the anchorage-independent growth of PC-3 cells in vitro and stimulated the expression of genes encoding proteins involved in cell survival and apoptosis regulation such as NAG-1 (also called GDF-15 or MIC-1). The oncogenic properties of RhoC via NAG-1 were also demonstrated in vivo by using intratumoral delivery of siRNA. Our results suggest that endogenous RhoC contributes to the tumorigenic phenotype of PC-3 cells by repressing the anti-proliferative factor NAG-1.

MATERIALS AND METHODS

Cells and reagents

U0126 was from Calbiochem (Hull, England) and SB203580 was from Alexis (Zandhoven, Belgium). Mouse anti-RhoA (#sc-418), goat anti-RhoC (#sc-26480), rabbit anti-p21 (#sc-397), rabbit anti-SPARC (#sc-25574), goat anti-NAG-1 (#sc-10603) and donkey anti-goat IgG (#sc-2020) were from Santa-Cruz (Boechout, Belgium). Rabbit anti-ERK1/2 (#M-5670) was from Sigma. The secondary horseradish peroxydase conjugated rabbit anti-mouse IgG (P0260) and swine anti-rabbit IgG (P0217) were from DAKO (Heverlee, Belgium). Atelocollagen is pepsinized bovine skin type I collagen purified as described previously (Delvoye et al., 1991). Human prostate adenocarcinoma cells PC-3 were cultured in F-12 Kaighn's medium (Invitrogen, Merelbeke, Belgium)) supplemented with 7% FBS (Lonza, Verviers, Belgium).

siRNA transfection

21-nucleotides long siRNAs chemically synthesized, desalted, deprotected and PAGE purified were from Eurogentec (Liège, Belgium). The sequences of the siRNA targeting RhoA (siRhoA and siRhoA#2) and RhoC (siRhoC and siRhoC#2), of the irrelevant siRNA used as control (siScr), and of the specific controls for siRhoA and siRhoC (ctrRhoA and ctrRhoC) were described previously (Ho et al., 2008). The siRNA used to silence NAG-1 were described by others (Shim & Eling, 2005). The 5'-GAAUCGAGAGCUCCAGAUCTT-3' and 5'-GAUCUGGAGCUCUCGAUUCTT-3' oligoribonucleotides were used to silence GSK3beta. Each pair of oligoribonucleotides was annealed at a concentration of 20 µM in 50 mM NaCl, 1mM EDTA, 10 mM Tris-HCl pH 7.5. siRNA transfection was carried out as previously described (Deroanne et al., 2003). Briefly, calcium phosphate-mediated transfection was performed overnight (14-16 hours) on subconfluent cells at a final concentration of 20 nM siRNA. Cells were washed twice with PBS and once with complete medium, this last step was defined as time 0 post-transfection. Cells were lysed for Western blot or RT-PCR analysis 48 hours post-transfection.

Microarray analysis

The effect of RhoA and RhoC silencing on the gene expression profile of PC-3 cells was assessed by microarray analysis using the Affymetrix® HG-U133 Plus 2.0 chip containing 22,000 probe sets. Total RNA was isolated from siRNA transfected cells using the High Pure RNA isolation kit (Roche Molecular Biochemical). The integrity of the RNA

was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies). Probe synthesis hybridisation, washing protocols and signals scanning were performed on the Genomics Facility of the GIGA, University of Liège.

Real time quantitative PCR

Total RNA was isolated from siRNA transfected cells using the High Pure RNA isolation kit (Roche, Vilvoorde, Belgium). 1 µg of total RNA was reversed transcribed using SuperScript II Reverse Transcriptase (Invitrogen). Real time PCR was performed in a final volume of 20 µl containing 2 µl of cDNA (corresponding to 10 ng of total RNA for GDF-15, p21Cip1, GADD 153, ATF-3, SPARC and p57Kip2 amplification and corresponding to 0,1 ng of total RNA for GAPDH amplification), 300 nM of each primer and 10 µl of the qPCR MasterMix Plus for SYBR® green (Eurogentec) in the Abi Prism 7000 Sequence Detection system (Applied Biosystems, Halle, Belgium). The results were normalized to the GAPDH transcript. PCR was performed with the primers listed in table 1.

Creation of PC-3 clones expressing SPARC in a tetracycline-dependent way

The entire coding sequence of human SPARC was amplified by RT-PCR and cloned into the pcDNA4/TO (Invitrogen). The cDNA sequence was checked by sequencing. Clones of PC-3 cells expressing a high level of tetracycline repressor (PC-3/TR) isolated as previously described (Ho et al., 2008) were transfected with pcDNA4/TO/SPARC and selected in medium supplemented with 1 µg/ml blasticidin + 200 µg/ml zeocin™. 2 clones expressing SPARC in a tetracycline-dependent way (PC-3/TR/SPARC) were isolated and used in this study.

Western blotting

Cells were lysed in SDS-PAGE lysis buffer and proteins were separated by polyacrylamide gel electrophoresis. Proteins were transferred to a PVDF Transfer Membrane (NEN Life Science Products). Membranes were blocked for 1 hour with 3% dry milk in PBS-0.05% Tween 20 and incubated for 4 hours with the diluted primary antibody. Membranes were then washed three times, incubated in the diluted secondary horseradish peroxydase-conjugated antibody for 1 hour, and revealed by chemoluminescence using the ECL kit (Amersham Biosciences) and X-ray film exposure. The membranes were reprobed with anti-Erk1/2 antibodies to control protein loading.

GTPase activity assay

The assay was carried out as previously described (Deroanne et al., 2003; Sander et al., 1999). Briefly, cells were chilled on ice and lysed in ice-cold buffer containing 1% Triton X-100, 25 mM HEPES pH 7.3, 150 mM NaCl, 4% glycerol, 0.1 mM AEBSF, 4 µg/ml aprotinin. Lysates were centrifuged for 6 minutes at 16000 g. Supernatants were immediately frozen in liquid nitrogen and stored at –80°C until used. An aliquot of each supernatant collected before freezing was denatured in SDS-PAGE lysis buffer to measure the total RhoGTPase content by Western blotting. For pull-down assays, supernatants were incubated for 30 minutes with 30 µg of GST-PBD protein containing the Cdc42 and Rac binding region of PAK-1B affinity linked to glutathione-Sepharose beads. The beads were washed 4 times in lysis buffer and boiled in 60µl SDS-PAGE lysis buffer.

GENE		PRIMER SEQUENCE
p21Cip1	Forward Reverse	5'-TGGAGACTCTCAGGGTCGAAA-3' 5'-GGCGTTTGGAGTGGTAGAAATC-3'
p57Kip2	Forward Reverse	5'-GCGCGGCGATCAAGAA-3' 5'-ACATCGCCCGACGACTTC-3'
GADD153	Forward Reverse	5'-AGAACCAGGAAACGGAAACAGA-3' 5'-TTCATGCGCTGCTTTCCA-3'
ATF-3	Forward Reverse	5'-TCAAGGAAGAGCTGAGGTTTGC-3' 5'-GATTCCAGCGCAGAGGACAT-3'
SPARC	Forward Reverse	5'-CCTGGATCTTCTTTCTCCTTTGC-3' 5'-ATCAGGCAGGGCTTCTTGCT-3'
NAG-1/ GDF-15/ MIC-1	Forward Reverse	5'-TGCCCGCCAGCTACAATC-3' 5'-TCTTTGGCTAACAAGTCATCATAGGT-3'
HERPUD1	Forward Reverse	5'-GACAGTATCCTGAGGATTCCTCAA-3' 5'-GGTGTGTAACCGGAGAAACCAGGA-3'
GADD34	Forward Reverse	5'-TGATGGCATGTATGGTGAGCGAGA-3' 5'- TCAGAAGGCTGGGAGACAGGGGA-3'
CTH	Forward Reverse	5'-GATCTCACTGTCCACGTTCAA-3' 5'- CCAGTGCTGCCACTGCTTTTTCAA-3'
p8	Forward Reverse	5'-GCACGAGAGGAAACTGGTGACCAA-3' 5'-CTCTATTGCTGGGTGTAGTGTCCA-3'
TRIB3	Forward Reverse	5'-CACCTACTGCTCCAGATCGTGCAA-3' 5'-TGGACGGGGTACACCTTGCAGGTA-3'
RhoC	Forward Reverse	5'-TCCAGGCTGTCAGGGCTGTCGA-3' 5'-GACCTGCCTCCTCATCATCGTCTTCA-3'
GAPDH	Forward Reverse	5'-CCTGGCCAAGGTCATCCATGACA-3' 5'-GGGATGACCTTGCCCACAGCCTT-3'

Table 1: Sequences of primers used in Real time quantitative PCR

Soft agar assay

Anchorage-independent growth was determined by soft agar assay (Laboisse et al., 1981). $2x10^3$ siRNA-transfected PC-3 cells were plated in 60 mm dishes in growth medium containing 0.3% agar, on top of a 0.6% agar gel. After 21 days colonies were counted in the whole dishes using an inverted phase-contrast microscope. Each assay was done in triplicate. Results are the means ± sem of three independent experiments.

Tumorigenicity studies in nude mice.

A volume of 200 µl of serum-free medium containing $2X10^6$ PC-3 cells was inoculated into both flanks of 4-week-old male nude (athymic) mice by using a 27-gauge needle (n=6). After 3 weeks, the tumors had reached an average volume of 50-100 mm^3. They were then injected with 50 µl of siRNA (10 µM) mixed with atelocollagen (2.5 mg/ml) in PBS at day 21, 33 and 45. Tumor growth was

assessed by measuring the length and width of tumors and the volume was determined by using the following formula: (length) x (width) ^{2} x 0.4. Tumors were resected and weighted at day 48. Data are presented as means ± sem. This experimental protocol was approved by the Ethical Committee of the University of Liège.

RESULTS

Silencing RhoC, but not RhoA, significantly decreased the anchorage-independent growth of PC-3 cells.

The specific role played by RhoA and RhoC in PC-3 cells phenotype was evaluated by using a siRNA approach. To reduce concerns about "off-target" effects, experiments were performed using two distinct siRNA targeting RhoA (siRhoA or siRhoA#2) and two other targeting RhoC (siRhoC or siRhoC#2). An irrelevant siRNA (siScr), which is a randomly mixed sequence of siRhoA, was used as control. The effective silencing of RhoA and RhoC was checked 48h after transfection by Western blot analysis with specific antibodies to RhoA, RhoC and Erk1/2 used as total proteins loading control. As illustratcd in figurc 1A, both siRNAs efficiently and specifically repressed their target. The effect of RhoA or RhoC silencing on the in vitro tumorigenic properties of PC-3 cells was tested in an anchorage-independent growth model. Immediately after transfection, PC-3 cells were seeded in soft-agar. After 3 weeks, colonies were counted in the whole culture dishes. The silencing of RhoC with the two different siRNA strongly reduced the number of colonies (Fig. 1B). By contrast, the silencing of RhoA did not affect the number of colonies or even slightly increased it as observed with the siRhoA#2.

To further validate our results, two additional control siRNA were designed by introducing two nucleotide changes from the target sequence (sictrRhoA and sictrRhoC, (Ho et al., 2008)) as recommended in an editorial of Nat. Cell Biol. (2003). These two mutations completely abrogated their silencing activity since the expression level of RhoA or RhoC was not altered by these control siRNA even at concentrations as high as 60 nM (Fig. 2A and 2B). The culture in soft-agar of PC-3 transfected with these control siRNA, with siRhoC or with siRhoA further showed that RhoC contributes to their anchorage-independent growth capacity (Fig.2C).

It was previously reported that inhibiting RhoC activity, by ectopic expression of a dominant-negative mutant, or silencing RhoA, by stable expression of a specific shRNA, modulated Rac1 activity (Simpson et al., 2004; Yao et al., 2006). In PC-3 cells, the silencing of RhoA did not affect the activity of Rac1 and that of RhoC only barely decreased active Rac1 (Supplementary Fig. S1). As shown in Fig.1 and 2, and in agreement with Simpson et al. (Simpson et al., 2004), silencing of RhoA up-regulates RhoC and vice-versa.

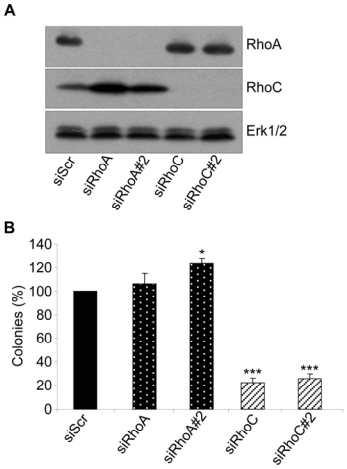

Figure 1: **(A)** RhoA and RhoC are efficiently silenced by their respective siRNA in human prostate adenocarcinoma cells. PC-3 cells were transfected by 20 nM of two different siRNAs targeting RhoA (siRhoA and siRhoA#2), by 20 nM of two different siRNAs targeting RhoC (siRhoC and siRhoC#2) and by an irrelevant siRNA as control (siScr). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to RhoA, RhoC and Erk1/2. **(B)** RhoC but not RhoA is involved in the anchorage-independent growth properties of PC-3 cells. PC-3 cells were transfected by 20 nM of siScr, siRhoA, siRhoA#2, siRhoC and siRhoC#2. 24 hours post-transfection, cells were plated in soft agar as described in material and methods. After three weeks of culture, colonies were counted in the whole dishes. Results are reported as the number of colonies in percentage of the siScr condition taken as 100% and are the mean± sem of three independent experiments. * p< 0.05 and *** p< 0.001 ANOVA followed by Tukey-Kramer analysis.

To assess if the inhibition of anchorage-independent growth of PC-3 cells upon RhoC silencing could be due also to the concomitant RhoA up-regulation, we performed a double silencing of RhoA+RhoC that repressed PC-3 colonies formation in soft-agar similarly to the single silencing of RhoC, ruling out the involvement of this compensatory mechanism (Supplementary Fig. S2).

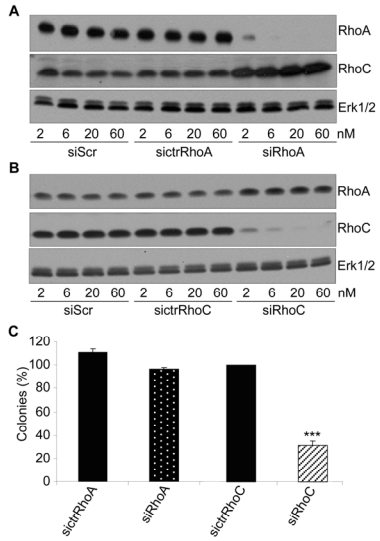

Figure 2: (A) Specific controls for siRhoA and **(B)** for siRhoC do not silence the expression of RhoA and RhoC. PC-3 cells were transfected with the indicated concentrations of an irrelevant siRNA (siScr), of specific controls for siRhoA (sictrRhoA) and siRhoC (sictrRhoC), of the first siRNA targeting RhoA (siRhoA) or of the first siRNA targeting RhoC (siRhoC). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to RhoA, RhoC and Erk1/2. **(C)** The use of specific control for siRhoA and siRhoC confirmed the involvement of RhoC in the anchorage-independent growth properties of PC-3 cells. PC-3 cells were transfected with 20 nM of specific controls for siRhoA (sictrRhoA) and siRhoC (sictrRhoC), of the first siRNA targeting RhoA (siRhoA) or of the first siRNA targeting RhoC (siRhoC). 24 hours post-transfection, cells were plated in soft agar as described in material and methods. After three weeks of culture, colonies were counted in the whole dishes. Results are reported as the number of colonies in percentage of the sictrRhoC condition taken as 100% and are the mean ± sem of three independent experiments. * $p< 0.05$ and *** $p< 0.001$ ANOVA followed by Tukey-Kramer analysis.

Silencing of RhoC regulates genes involved in cell proliferation and survival.

In order to identify mechanisms involved in the PC-3 anchorage-independent growth two independent comparative analyses of the gene expression profiles following silencing of RhoA or RhoC versus siScr were carried out with Affymetrix® microarrays. An additional microarray analysis comparing PC-3 transfected with siRhoC#2 versus siScr was performed to validate the regulations observed with the first siRNA targeting RhoC. The most significant variations are listed in Table 2. Several genes were up-regulated upon RhoC silencing and unchanged or down-regulated in RhoA silenced PC-3. Among them, NAG-1, p21Cip1, p57Kip2, ATF-3, GADD153, GADD34, p8, TRIB3 or HERPUD1 are involved in signaling networks controlling cell growth arrest and apoptosis, and in the endoplasmic reticulum stress response. Microarray results were confirmed by real-time PCR (Fig.3) and, at the protein level, by Western blot analysis (Fig.4 and supplementary Fig.S3). Interestingly, the expression level of SPARC (for secreted protein, acidic, cysteine-rich; also called osteonectin), an extracellular matrix component potentially involved in tumorigenesis (Hooi et al., 2006), was repressed upon RhoC silencing and up-regulated upon RhoA silencing. This antagonistic regulation was confirmed by real-time PCR (Fig.3) and Western blot analysis (Fig.4).

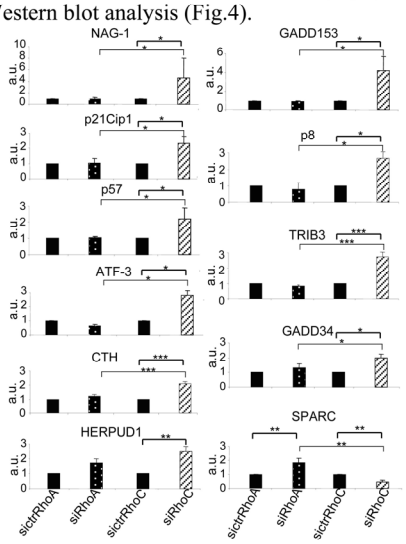

Figure 3: Confirmation of the microarray data by Real Time Q-PCR. Real time quantitative PCR analysis of the expression of the indicated mRNAs was performed with total RNA extracted from PC-3 cells 48 hours after transfection with 20nM of specific controls for siRhoA (sictrRhoA) and siRhoC (sictrRhoC), of the first siRNA targeting RhoA (siRhoA) or of the first siRNA targeting RhoC (siRhoC). Results are the mean ± sem of three independent experiments. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ ANOVA followed by Tukey-Kramer analysis.

Repression of NAG-1 partly reversed the gene regulations driven by RhoC silencing.

NAG-1 was reported to regulate the expression of genes controlling proliferation and apoptosis. To address its role in the modulations of gene expression induced by RhoC silencing, a simultaneous repression of RhoC and NAG-1 was performed. As illustrated in figure 5 and 6A, both molecules were efficiently silenced. The simultaneous silencing of NAG-1 and RhoC

reversed the induction of p21cip1 both at the mRNA level (Fig.5) and at the protein level (Fig.6A). Among the genes stimulated by RhoC silencing, p8, CTH, HERPUD1, TRIB3, GADD153 and GADD34 overexpression was as well reversed by co-silencing NAG-1 (Fig.5). The repression of SPARC induced by RhoC silencing was also dependent on NAG-1 since the co-silencing RhoC+NAG-1 restored SPARC expression (Fig.6B). For some genes however, as ATF-3 and p57kip2, the inhibition of NAG-1 did not significantly repress the regulation induced by silencing RhoC (Fig.5).

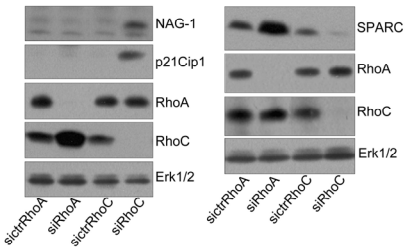

Figure 4: The siRhoC-induced regulations of NAG-1, p21Cip1 and SPARC are expressed at the protein level. PC-3 cells were transfected with 20nM of specific controls for siRhoA (sictrRhoA) and siRhoC (sictrRhoC), of the first siRNA targeting RhoA (siRhoA) or of the first siRNA targeting RhoC (siRhoC). 48 hours post-transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to NAG-1, p21Cip1, SPARC and Erk1/2. Representative blots of three independent experiments are shown.

The up-regulation of NAG-1 after RhoC silencing requires p38MAPK and GSK3β but not Erk1/2.

The role of GSK3β and p38MAPK, two potential regulators of NAG-1 expression (Shim & Eling, 2008; Yamaguchi et al., 2004), and of Erk1/2 was evaluated in RhoC-silenced cells obtained by co-transfecting a siRNA targeting GSK3β or by mean of pharmacological inhibitors, SB203580 or U0126. The increased expression of NAG-1 observed in RhoC-silenced PC3 cells was effectively repressed by co-silencing of GSK3β to a level close to that observed in control transfected cells (Fig. 7A). It should be noted that GSK3β silencing alone did not alter significantly the basal expression level of NAG-1. The inhibition of p38MAPK also repressed the expression of NAG-1 but in both RhoC-silenced and control-transfected PC3 cells suggesting that this pathway, by contrast to GSK3β, is required also for the basal expression of NAG-1 (Supplementary Fig. S4). Erk1/2 inhibition slightly increased the expression of NAG-1 in control-transfected and RhoC-silenced cells (Fig.7B).

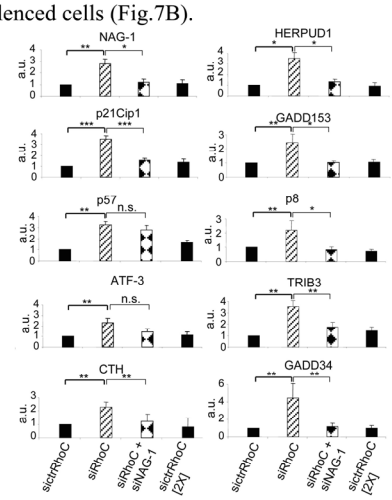

Figure 5: The induction of p21Cip1, p8, CTH, HERPUD1, TRIB3, GADD153 and GADD34 following RhoC silencing are dependent on NAG-1. Real time quantitative PCR analysis of the expression of the indicated mRNA was performed with total RNA extracted from PC-3 cells 48 hours after transfection with 20 nM (or 40 nM) of specific controls for siRhoC (sictrRhoC or sictrRhoC[2X]), with 20nM of the first siRNA targeting RhoC (siRhoC) or co-transfection with 20 nM of the first siRNA targeting RhoC + 20 nM of the siRNA targeting NAG-1 (siRhoC+siNAG1). Results are the mean ± sem of three independent experiments. n.s.: not significant; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$ ANOVA followed by Tukey-Kramer analysis

The repression of NAG-1 restored the anchorage-independent growth of RhoC-silenced PC-3 cells.

To address the role of NAG-1 in the repression of the anchorage-independent growth upon RhoC silencing, PC-3 cells were transfected with siRhoC alone or with siRhoC+siNAG-1 and tested immediately after transfection for colonies formation in soft-agar. After 3 weeks, the colonies were counted in the whole culture dishes. As illustrated in figure 8, the silencing of RhoC significantly reduced the number of colonies as compared to sictrRhoC-transfected cells as previously described. By contrast, cells co-transfected with siRhoC and siNAG-1 had the same ability to form colonies as the sictrRhoC-transfected cells. It should be noted that increasing the concentration of sictrRhoC up to 40 nM did not alter the ability of PC-3 cells to form colonies in soft-agar as compared to cells transfected with 20 nM of sictrRhoC,

suggesting that the restoration of the ability of PC-3 cells to form colonies upon the double silencing RhoC+NAG-1 is not an unspecific effect due to the increased concentration of siRNA.

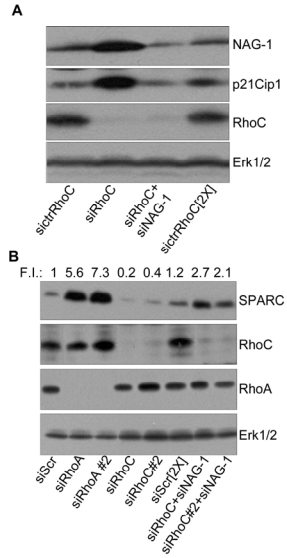

Figure 6: The regulation of p21Cip1 and SPARC protein level following RhoC silencing are dependent on NAG1. (**A**) PC-3 cells were transfected with 20 nM (or 40 nM) of specific controls for siRhoC (sictrRhoC or sictrRhoC[2X]), with 20 nM of the first siRNA targeting RhoC (siRhoC) or co-transfected with 20 nM of the first siRNA targeting RhoC + 20 nM of the siRNA targeting NAG-1 (siRhoC+siNAG1). (**B**) PC-3 cells were transfected with 20 nM (or 40 nM) of an irrelevant siRNA (siScr or siScr [2X]) with 20 nM of two different siRNAs targeting RhoA (siRhoA and siRhoA#2), of two different siRNAs targeting RhoC (siRhoC and siRhoC#2) or with 20 nM of siRhoC + 20 nM of siNAG-1 (siRhoC+siNAG-1) or with 20 nM of siRhoC#2 + 20 nM siNAG-1. 48 hours post-transfection, cells were lysed and analyzed by immunoblotting with specific antibodies to NAG-1, p21Cip1, SPARC and Erk1/2. The fold induction (F.I.) was calculated from densitometric measurements of SPARC normalized to Erk1/2 in each condition compared with the siScr condition taken as 1.0.

The re-expression of SPARC in RhoC-silenced PC-3 is sufficient to restore their anchorage-independent growth properties.

SPARC has been implicated in tumorigenesis and we observed that it was antagonistically regulated by RhoA and RhoC. To test if its repression upon RhoC-silencing was involved in the growth inhibition of PC-3 in soft-agar, we generated clones expressing SPARC in a tetracycline-dependent way (PC-3/TR/SPARC). These clones were transfected with 20 nM of control siRNA (sictrRhoC) or with 20 nM of siRhoC. Immediately after transfection, the cells were processed for culture in soft-agar either in absence or in presence of 1 ng/ml of tetracycline. As assessed by Western blot analysis, this concentration of tetracycline was sufficient to induce the expression of exogenous SPARC (Fig.9). The overexpression of SPARC did not modulate the number of colonies formed by PC-3 cells transfected with the control siRNA but significantly increased the number of colonies form by RhoC-silenced PC-3 cells suggesting that SPARC is involved, at least partly, in the in vitro tumorigenic properties of PC-3 cells (Fig.9).

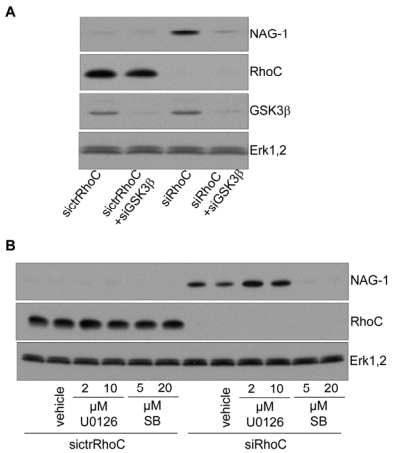

Figure 7: The up-regulation of NAG-1 following RhoC silencing is dependent on GSK3beta and p38MAPKinase. **(A)** PC-3 cells were transfected with 20 nM of a specific control for siRhoC (sictrRhoC), with 20 nM of a specific control for siRhoC + 20nM of the siRNA targeting GSK3beta (sictrRhoC+siGSK3β), with 20 nM of the first siRNA targeting RhoC (siRhoC) or with 20 nM of the first siRNA targeting RhoC + 20 nM of the siRNA targeting GSK3beta (siRhoC+siGSK3β). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to NAG-1, RhoC, GSK3β and Erk1/2. Representative blots of three independent experiments are shown. **(B)** PC-3 cells were transfected with 20 nM of a specific control for siRhoC (sictrRhoC) or with 20 nM of the first siRNA targeting RhoC (siRhoC) and cultured between day 1 and 2 after transfection with the indicated concentrations of MEK-kinase inhibitor U0126 and of the p38MAPKinase inhibitor SB203580.

Intratumoral injection of siRNA targeting RhoC delayed PC-3 tumor growth, a process reversed by co-injecting siRNA targeting NAG-1.

The atelocollagen-mediated siRNA transfer as described by Minakuchi et al. (2004) was used to perform in vivo experiments. PC-3 cells were

subcutaneously inoculated in each flank of nude mice. After 3 weeks, when the tumors reached a volume of 50-100 mm^3, a mixture of atelocollagen and siRNA was injected in each tumors. This procedure was repeated 12 and 24 days later. The injection of siRhoC significantly slowed down the growth of PC-3 tumors. By contrast, the simultaneous injection of siRhoC and siNAG-1 restored to PC-3 tumors the same growth capacity as that of sictrRhoC-injected tumors (Fig. 10A). The mice were sacrificed at day 48 to collect the tumors. The weight of the tumors subjected to siRhoC treatment was significantly lower than that of tumors treated with the control siRNA (sictrRhoC) or with siRhoC+siNAG-1 (Fig.10B). These results strongly support a role for NAG-1 in the mechanisms involved in the in vivo anti-tumoral effect of siRhoC.

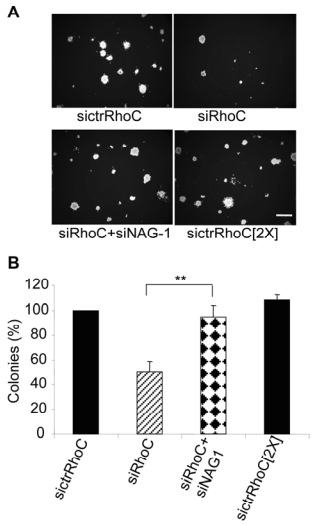

Figure 8: RhoC is involved in tumorigenesis of PC-3 cells by a mechanism dependent on NAG-1. PC-3 cells were transfected with 20 nM (or 40 nM) of specific controls for siRhoC (sictrRhoC or sictrRhoC[2X]), with 20 nM of the first siRNA targeting RhoC (siRhoC) or with 20 nM of the first siRNA targeting RhoC + 20 nM of the siRNA targeting NAG-1 (siRhoC+siNAG1). 24 hours post-transfection, cells were plated in soft agar as described in Material and Methods. After three weeks of culture, colonies were counted in the whole dishes.
(A) Representative fields of colonies growing in soft agar. Bar = 1 mm. **(B)** Results are reported as the number of colonies in percentage of the sictrRhoC condition taken as 100% and are the mean ± sem of three independent experiments. ** p< 0.01 ANOVA followed by Tukey-Kramer analysis.

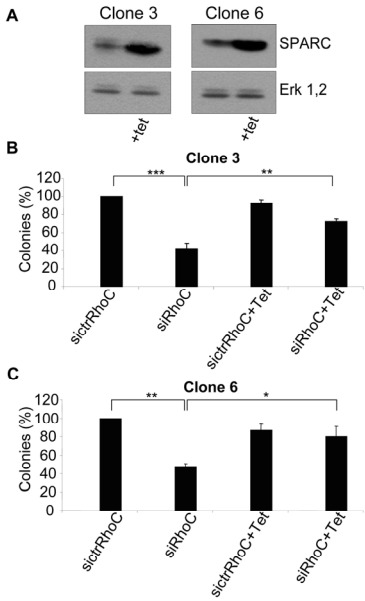

Figure 9: The re-expression of SPARC is sufficient to restore the anchorage-independent growth properties of RhoC-silenced PC-3 cells. Two clones (#3 and #6) of PC-3 cells were selected for their ability to express SPARC in a tetracycline-dependent way (+tet) as illustrated in the representative western blots shown in **(A)**. These clones were transfected with 20 nM of a specific control for siRhoC (sictrRhoC) or with 20 nM of the first siRNA targeting RhoC (siRhoC). 24 hours post-transfection, cells were plated in soft agar as described in Material and Methods. Cells were weekly supplemented (+tet) or not with renewed tetracycline (1 ng/ml). After three weeks of culture, colonies were counted in the whole dishes. Results are reported as the number of colonies in percentage of the sictrRhoC condition taken as 100% and are the mean ± sem of three independent experiments carried out with clone 3 (**B**) or clone 6 (**C**). * p< 0.05 ** p< 0.01 and *** p< 0.001 ANOVA followed by Tukey-Kramer analysis.

DISCUSSION

The three members of the Rho sub-class, RhoA, RhoB and RhoC, play various roles in cancer progression. RhoA and RhoC are reported to contribute to the cancer phenotype while RhoB displays properties that might participate in tumor suppression (Liu et al., 2001; Mazieres et al., 2004; Mazieres et al., 2005). Even if RhoA and RhoC are close homologs sharing downstream effectors and regulating similar biological processes, their implication in key steps of cancer progression like breast carcinoma cell invasion or epithelial-to-mesenchymal transition is clearly distinct (Bellovin et al., 2006; Simpson et al., 2004).

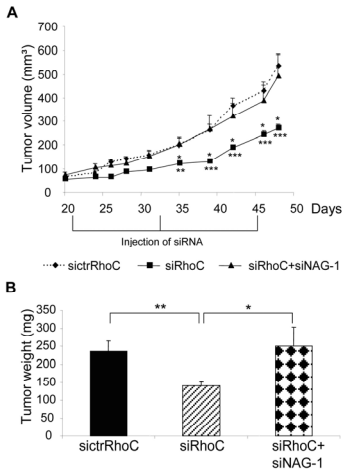

Figure 10: The in vivo anti-tumorigenic effect of the siRNA targeting RhoC is inhibited by co-administration of a siRNA targeting NAG-1. **(A)** Tumor growth curves. After induction of tumor formation by subcutaneous injection of $2X10^6$ PC-3 cells, 50µl of a solution containing 10 µM of a specific control for siRhoC (sictrRhoC), 10 µM of the first siRNA targeting RhoC (siRhoC) or 10 µM of the first siRNA targeting RhoC + 10 µM of the siRNA targeting NAG-1 (siRhoC+siNAG1) mixed with atelocollagen as described in Materials and Methods were injected into the tumor region on day 21, 33 and 45. Tumor volume was calculated from tumor biaxial diameter measurement at regular interval up to day 48. Results represent the means ± sem (n = 6 tumors). * $p< 0.05$ ** $p< 0.01$ and *** $p< 0.001$ ANOVA followed by Tukey-Kramer analysis. **(B)** Weight of the tumor at day 48. Results represent the means ± sem.

Moreover, RhoC expression is preferentially associated with highly aggressive cancers (Clark et al., 2000; Iiizumi et al., 2008; Kleer et al., 2005; Kleer et al., 2002). The specific contribution of RhoA and RhoC to cancer progression could be related to the biological program elicited by their effects on the cytoskeleton dynamics but also to their activity on the overall cell transcriptome. RhoGTPases are indeed involved in intracellular signalling pathways leading to alterations in gene expression. For instance, the serum responsive factor (SRF) can be activated through Rho-mediated cytoskeletal changes (Miralles et al., 2003). By contrast, RhoGTPases are also able to regulate gene transcription through signal transduction pathways independent of their effect on cytoskeleton (Marinissen et al., 2004). To date, three microarray studies using cells transfected by RhoA and/or RhoC oncoproteins are available (Berenjeno et al., 2007; Teramoto et al., 2003; Wu et al., 2004). However, in the most recent of these studies, a similar cellular transformation program was elicited by constitutively active RhoA, RhoB or RhoC overexpression suggesting that these experimental strategies are not relevant to the biological program activated by the physiological endogenous Rho proteins. The excessive expression and/or activation level of the Rho proteins, the potential depletion of upstream or downstream effectors required for specific parts of the Rho-driven biological program in the cell types used and the absence in the expression vector of untranslated regions of the Rho mRNA which can convey cues required for some Rho-mediated cellular events (Wu et al., 2005) weaken the significance of these models. In our work, the specific effect of RhoA and RhoC on the cellular transcriptome was investigated by an inversed approach: the specific silencing of RhoA and RhoC in a cellular model expressing significant amount of both RhoGTPases (Yao et al., 2006). Our results were validated by silencing each target with two different siRNAs and by using general and specific controls. These investigations were completed by assessing the relevance of the gene regulations to the in vitro and in vivo tumorigenic phenotype, i.e. growth in soft-agar and tumor growth in nude mice, respectively.

Our results clearly show that the anchorage-independent growth of PC-3 cells is dependent on RhoC but not on RhoA. In other cellular models, RhoA was however reported to positively contribute to proliferation (Pille et al., 2005). These discrepancies could be related to the variation of the expression level of each of these RhoGTPases from one cell type to another but also to the repertoire of proteins available in each cell type that can trigger the selection of downstream targets for RhoGTPases to achieve a particular response. The direct involvement of RhoC was assessed by several experiments designed to rule out potential compensatory mechanisms. Previous studies suggested that inhibition of RhoC, by expressing a dominant-negative RhoC mutant, or of RhoA, by shRNA, affected the activation level of Rac1(Simpson et al., 2004; Yao et al., 2006). This was not observed in our model suggesting that such indirect effect does not contribute to the regulations observed in our study. This discrepancy could be related to the selection process required in their studies to isolate clones during which counter selection mechanisms could take place.

		Fold change				
		siRhoC vs siScr		siRhoC#2 vs siScr	siRhoA vs siScr	
		microarray 1	microarray 2		microarray 1	microarray 2
ras homolog gene family, member C	RHOC	-7	-4.6	-3	1.2	1
ras homolog gene family, member A	RHOA	1	-1.1	-1.1	-5.2	-4.9
downregulated						
secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	-3	-5.7	-2.1	2.3	2.3
parathyroid hormone-like hormone	PTHLH	-1.9	-1.6	-2	1.3	1.3
interleukin 1, beta	IL1B	-4.3	-2.3	-2.3	-1.1	-1.1
angiopoietin-like 4	ANGPTL4	-2.5	-4.3	-2.8	1.1	1.1
chromosome 1 open reading frame 165	C1orf165	-3	-2.1	-2.3	1.1	-1.1
plasminogen activator tissue	PLAT	-2.5	-2	-1.9	1	-1.1
COBL-like 1	COBLL1	-2.3	-2	-1.9	-1.9	-1.2
very low density lipoprotein receptor	VLDLR	-2.3	-2.1	-2	1.1	-1.1
opsin 3 (encephalopsin panopsin)	OPN3	-2.3	-2	-1,6	1	-1.2
retinol binding protein 4 plasma	RBP4	-2.3	-2.8	-1.9	1.1	-1.1
urotensin 2	UTS2	-2	-3,2	-1,7	1.9	1.2
solute carrier family 12 (sodium/potassium/chloride transporters), member 2	SLC12A2	-2.1	-2.8	-2.1	1	1
phosphofructokinase, platelet	PFKP	-2.1	-1.9	-2.1	-1.4	-1.2
T cell receptor beta constant 1	TRBC1	-2.5	-2	-2.1	-1.5	-1.1
solute carrier family 2 (facilitated glucose transporter)	SLC2A1	-1.5	-2.1	-2.5	1	1
procollagen-proline 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase) alpha polypeptide II	P4HA2	-1.6	-2	-2	-1.3	-1.4
upregulated						
NSAID-activated gene 1/ growth differentiation factor 15	NAG-1/GDF15	2.5	2.5	2.6	-1.1	-1.3
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	p21Cip1/CDKN1A	3.2	3	1.1	1.1	1
Growth Arrest and DNA Damage gene 153/DNA-damage-inducible transcript 3	GADD153/DDIT3	4	4.6	2.8	-1.4	-1.3
Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	p57Kip2/CDKN1C	2.1	1.7	1.2	1.3	1.6
activating transcription factor 3	ATF3	2.1	2.3	1.9	-1.2	-1.4
cystathionase (cystathionine gamma-lyase)	CTH	2.1	2.8	1.6	-1.3	-1.1
homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	HERPUD1	2.3	1.9	1.9	-1.1	1
Growth Arrest and DNA Damage gene 34/protein phosphatase 1 regulatory (inhibitor) subunit 15A	GADD34/PPP1R15A	3.5	3.2	1.5	1.1	1.1
Tribbles homolog 3	TRIB3	2	1.7	1.7	-1.9	-1.7
asparagine synthetase	ASNS	2	1.5	1.9	-1.1	-1.2
CCAAT/enhancer-binding protein B	CEBPB	2.8	1.9	1.7	-1.3	-1.2
p8 protein (candidate of metastasis 1)	p8	2.8	2.3	2.6	-1.4	-1.6
sequestosome 1	SQSTM1	2.6	3	3	1.1	1.3
ATPase H+ transporting lysosomal 42kDa V1 subunit C1	ATP6V1C1	2	2	1.7	-1.2	1.4
UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 3 (GalNAc-T3)	GALNT3	2.5	3	1.9	-1.1	1
arginase type II	ARG2	4.3	3.7	1.5	1.2	1
S100 calcium binding protein P	S100P	2.6	2.8	1.5	-2,8	-2.6
GTP binding protein overexpressed in skeletal muscle	GEM	2.1	2.8	1.9	-1.1	-1.1
tripeptidyl peptidase I	TPP1	2.5	2.5	1.6	1.1	1.1
brain expressed X-linked-like 1	BEXL1	2.5	2	1.9	-1.2	-1.4
HSPB (heat shock 27kDa) associated protein 1	HSPBAP1	3	2.1	1.5	1.2	-1.1
cell cycle progression 1	CCPG1	2	2.1	1.5	1.3	1.1
syntaxin 3	STX3	2.1	1.6	2.1	1.1	-1.1
neutrophil cytosolic factor 2 (65kDa, chronic granulomatous disease, autosomal 2)	NCF2	4	7	4	-2	-1.4
metallothionein 1F (functional)	MT1F	1.9	2.5	2	-1.4	-1.4
metallothionein 1X	MT1X	1.9	3.5	1.9	-1.3	1.3
DnaJ (Hsp40) homolog subfamily C member 12	DNAJC12	1.9	2.1	1.7	1.1	1

Table 2: Selected genes regulated in PC-3 cells following RhoA or RhoC silencing

By contrast, our investigations were always carried out on transiently transfected cells avoiding therefore such mechanisms. We and others recently reported cross-regulations within the Rho-subclass (Ho et al., 2008; Simpson et al., 2004; Steffan et al., 2009; Tillement et al., 2008). Notably, an increased expression of RhoA is induced upon RhoC silencing as we also observed in this study suggesting that the inhibition of anchorage-

independent growth by RhoC silencing might be due to either the repression of RhoC or the induction of RhoA. The latter possibility was ruled out by performing a double silencing RhoC+RhoA which resulted in a repression of anchorage-independent growth similar to the silencing of RhoC alone. In the other hand, RhoB protein level is also regulated via cross-talks within the Rho sub-class. As its expression is repressed by RhoA and RhoC, it is likely not involved in their differential effect on the PC-3 cells phenotype (Ho et al., 2008).

Among the genes specifically regulated by RhoC silencing, a cluster of genes, including NAG-1, p21Cip1, p57Kip2, ATF-3, GADD153, GADD34, p8, TRIB3, CTH and HERPUD1, were most interesting since they are involved in cell growth arrest and reported to be cross-regulated (Carracedo et al., 2006; Clark et al., 2008; Jiang et al., 2004; Kim et al., 2005; Zu et al., 2006). NAG-1 (also called GDF-15 or MIC-1) seems to play a central role in this network by controlling the expression of most of the others. This transforming growth factor-beta superfamily cytokine has been generally considered as part of the antitumoral repertoire of the cells. However, its secretion, processing and sequestration within the extracellular matrix, suggest a complex role in cancer progression (Bauskin et al., 2006). In our model, it clearly contributes to the repression of the tumorigenic properties of PC-3 cells upon RhoC silencing not only through the regulation of genes involved in cell growth arrest but also via the inhibition of the expression of SPARC (or osteonectin). This component of the extracellular matrix has been shown to display pro-tumorigenic properties in prostate cancer (Chen et al., 2007; Hooi et al., 2006). The contribution of SPARC to the tumorigenic phenotype of PC-3 cells was demonstrated by restoring the anchorage-independent growth properties of RhoC-silenced PC-3 cells through its re-expression.

Our results emphasized the involvement of the p38MAPKinase pathways in the basal expression of NAG-1, as it was inhibited by SB203580 in RhoC-silenced as well as in control-transfected PC-3 cells. By contrast, GSK3β controls the inducible expression of NAG-1 following RhoC-silencing. These data are in agreement with those of the group of Ehling and Baek who reported NAG-1 induction in cancer cell lines by various anti-tumorigenic agents through these pathways (Shim & Eling, 2008; Yamaguchi et al., 2004). The activity of GSK3β can be modulated by several intracellular signalling cascades potentially regulated by RhoC such as the AKT or Wnt pathways (Iiizumi et al., 2008; Qiang et al., 2003; Ruth et al., 2006). We did not observe any modulation of its phosphorylation status (data not shown) suggesting that the regulation of its activity might be independent of its phosphorylation or this modification could be too transient to be observed. Nevertheless, our siRNA experiments demonstrated clearly the involvement of GSK3β in the regulation of NAG-1. The Erk1/2 pathway seems to be also implicated in the regulation of NAG-1 as suggested by the increased expression of NAG-1 in RhoC-silenced PC-3 cells treated with the MEK1/2 inhibitor U0126. Antagonistic regulations of gene expression by the p38MAPKinase and the Erk1/2 pathways have already been reported (Brauchle et al., 2000; Deroanne et al., 2005; Reunanen et al., 2002). They are likely related to the potential inhibition of MEK1,2 activity by the p38MAPKinase pathway (Westermarck et al., 2001).

Studies by Buchsbaum et al. (Buchsbaum et al., 2002; Buchsbaum et al., 2003) and by Jaffe et al. (Jaffe et al., 2005) suggested that the panel of biological responses elicited by RhoGTPases is exquisitely orchestrated by the selected GEFs and the ability of these GEFs to interact with scaffold proteins which finally dictate the choice of the downstream effectors. Although RhoA and RhoC display common activators and effectors, their respective affinity for these proteins might differ as it was reported for ROCK (Sahai & Marshall, 2002). The relative affinity of RhoA and RhoC for GEFs and/or for their targets, including those who act as scaffold proteins, could explain their differential effect on the phenotype of PC-3 cells and the differential regulation of the transcriptome of these cells by RhoA and RhoC reported here. We are currently investigating the relative affinity of RhoA and RhoC for various activators and effectors to decipher potential points of divergence in their downstream signalling.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

SUPPLEMENTARY INFORMATIONS

Supplementary informations are available at Oncogene's web site

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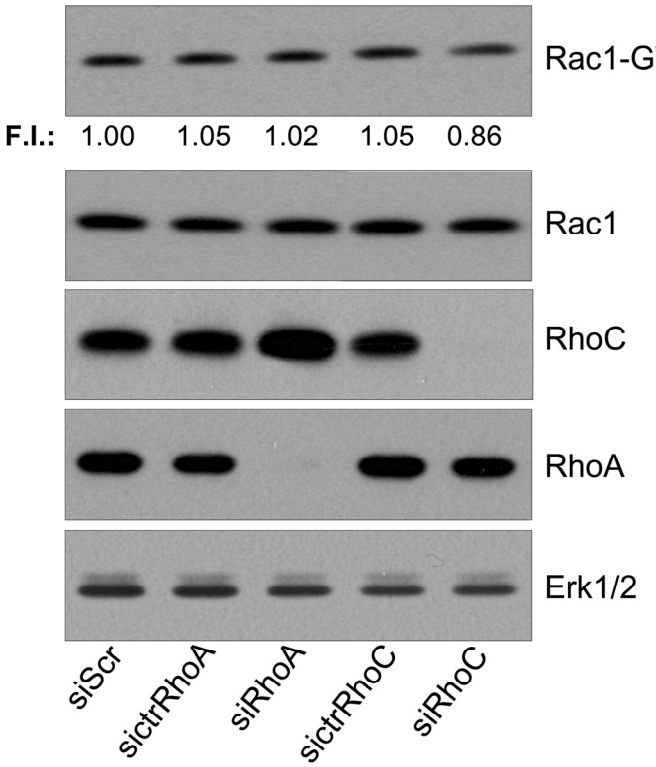
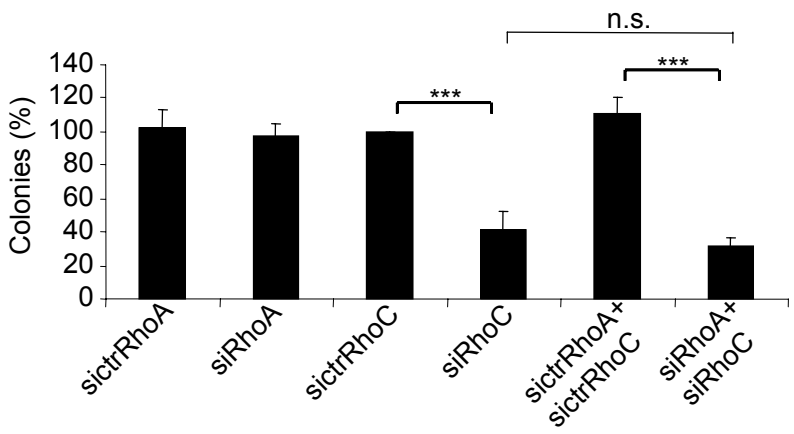
Ho_Supplementary Figure S1



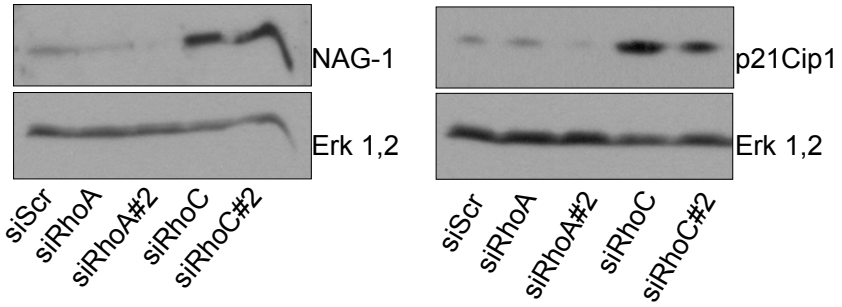
Neither RhoA-silencing nor RhoC-silencing affected the Rac1 expression and activation level. PC-3 cells were transfected with 20 nM of the indicated siRNA. 48h after transfection, cells were harvested and processed for Western blot and pull-down experiments. An aliquot of each lysate was denatured in SDS-PAGE loading buffer to analyse the concentration of total Rac1, RhoA, RhoC and Erk1,2 with specific antibodies. Rac1 activity was determined as the amount of GST-PBD-bound Rac1 (Rac1-GTP) normalized to total Rac1 in whole cell lysates. The fold induction (F.I.) is calculated from densitometric measurements of Rac1-GTP normalized to total Rac1 in each transfection conditions compared to the siScr condition taken as 1.0.

Ho_Supplementary Figure S2



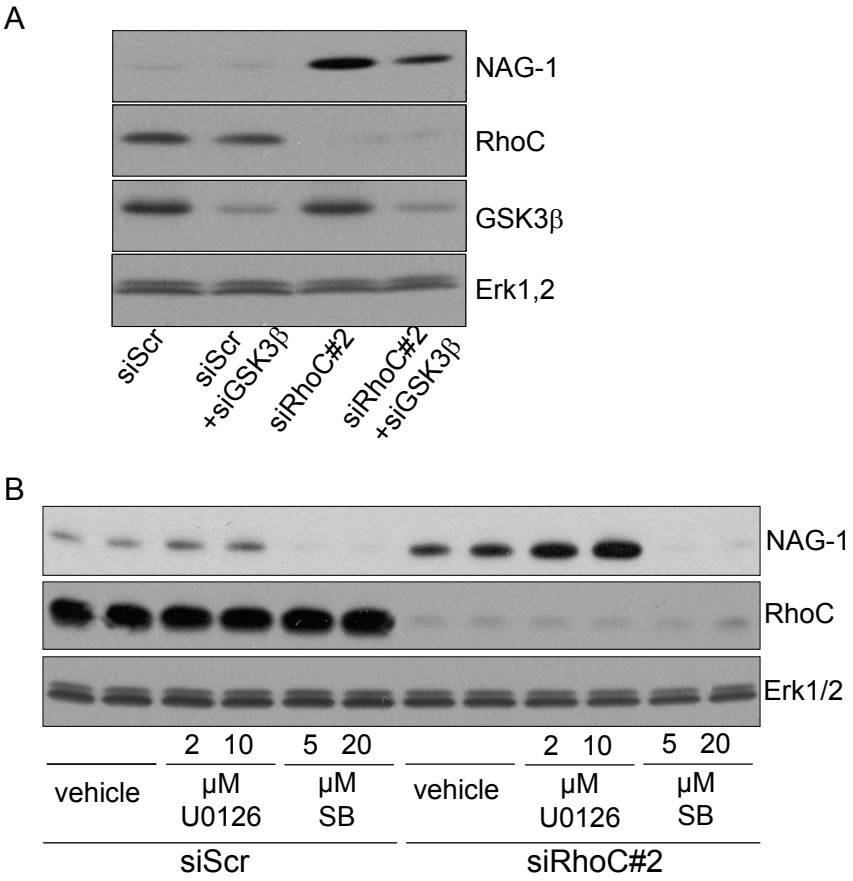
The double silencing RhoA+RhoC was as efficient as the single silencing of RhoC in inhibiting the anchorage-independent growth properties of PC-3 cells. PC-3 cells were transfected by 20 nM of sictrRhoA, siRhoA, sictrRhoC, siRhoC, 20 nM sictrRhoA + 20 nM sictrRhoC (sictrRhoA+C) or 20 nM siRhoA + 20 nM siRhoC (siRhoA+C) . 24 hours post-transfection, cells were plated in soft agar as described in material and methods. After three weeks of culture, colonies were counted in the whole dishes. Results are reported on the sictrRhoC condition and are the mean± sem of triplicate experiments. Two independent experiments were performed with similar results. n.s.: not significant *** $p < 0.001$ ANOVA followed by Tukey-Kramer analysis.

Ho_Supplementary Figure S3



The up-regulation of NAG-1 and p21Cip1 protein level following RhoC-silencing were confirmed with a second siRNA targeting RhoC (siRhoC#2).
PC-3 cells were transfected by 20nM of two different siRNAs targeting RhoA (siRhoA and siRhoA#2), by 20nM of two different siRNAs targeting RhoC (siRhoC and siRhoC#2) and by an irrelevant siRNA as control (siScr). 48 hours post transfection, cells were lysed and analyzed by immunoblotting using specific antibodies to NAG-1, p21Cip1and Erk 1,2. Representative blots of three independent experiments are shown.

Ho_Supplementary Figure S4



Regulation of NAG-1 expression. (A) NAG-1 up-regulation following RhoC silencing with the 2nd siRNA targeting RhoC is also dependent on GSK-3β. Representative Western blot analysis with specific antibodies to NAG-1, GSK3β, RhoC and Erk1/2 of lysates of PC-3 harvested 48h after transfection with 20 nM of the mentioned siRNA. **(B)** p38 MAPK, but not Erk1/2, regulated the expression of NAG-1. Representative Western blot analysis with specific antibodies to NAG-1, RhoC and Erk1/2 of lysates of PC-3 harvested 48h after transfection with 20nM of the mentioned siRNA. To inhibit Erk1/2, cells were cultured with 2 or 10 μM of the MEK inhibitor U0126. To inhibit p38MAPK, cells were treated with 5 or 20 μM of SB203580.