Résultats : Partie I

Validation des siRNAs comme outils biologiques performants : évaluation du rôle des RhoGTPases dans les propriétés contractiles et migratoires des fibroblastes humains en relation avec l'organisation du cytosquelette.

Introduction

In vivo, les cellules interagissent avec leur matrice extracellulaire environnante constituée par un réseau tridimensionnel de protéines fibrillaires organisé au sein d'un gel hydraté de protéoglycans et de glycoprotéines. Les cellules adhèrent à cette matrice extracellulaire par l'intermédiaire de récepteurs transmembranaires dont les plus représentés font partie de la famille des intégrines. Suite à leur activation par leur liaison à des séquences consensus des protéines matricielles, les intégrines recrutent au niveau de leur domaine cytoplasmique des protéines structurales et de signalisation formant les adhésions focales. Ces structures permettent l'établissement d'un lien physique entre la matrice extracellulaire et le cytosquelette d'actine. Ce couplage, indispensable à la transmission des forces mécaniques issues de la matrice (« outside-in ») ou générées par le cytosquelette (« inside-out »), est requis pour de nombreuses fonctions cellulaires telles que la migration, la contraction ou l'assemblage et le remodelage des protéines matricielles. Les protéines de la famille des RhoGTPases sont des protéines de signalisation relayant des signaux extracellulaires,

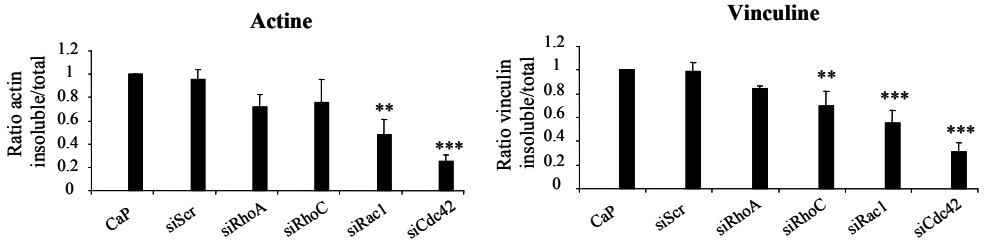

Figure 12 : Modulation de la distribution d'actine et de vinculine par l'extinction de l'expression des RhoGTPases dans des fibroblastes humains de derme. *Analyse par Western blot des fractions insolubles et solubles dans le détergent non-ionique NP-40. Les différences significatives sont montrées par comparaison avec le contrôle siScr en utilisant le test statistique « ANOVA one way » suivie par un test de Tuckey-Kramer ** p ‹ 0.01, *** p ‹ 0.001.*

chimiques et mécaniques, largement impliquées dans la dynamique du cytosquelette et le contrôle de nombreux processus cellulaires associés. A l'aide de siRNA spécifiques, nous avons évalué le rôle individuel de quatre membres apparentés de la famille des RhoGTPases RhoA, RhoC, Rac1 et Cdc42 dans les propriétés contractiles et migratoires des fibroblastes humains. Des doubles transfections ont permis en outre d'inhiber simultanément des couples de RhoGTPases.

Résumé des résultats

- La répression de l'expression de RhoA, RhoC, Rac1 et Cdc42 par transfection de siRNAs s'est révélée spécifique et efficace durant 5 à 7 jours pour au moins deux siRNAs différents ciblant deux séquences distinctes de l'ARNm de chacune des RhoGTPases étudiées. Nous avons noté d'emblée que l'inhibition de RhoC induit une surexpression de RhoA.

- Les changements de morphologie détectables par microscopie en contraste de phase des fibroblastes humains transfectés par les différents siRNA sont liés à des modifications de la distribution de l'actine polymérique (fibres de stress et corticale) et de la vinculine associée aux adhésions focales, comme le montre un marquage par la phalloïdine et un anti-vinculine réalisé trois jours après transfection. L'inhibition de l'expression de Rac1 et Cdc42 semble induire une réduction de la quantité d'actine fibrillaire et de la vinculine associée aux adhésions focales. Ces résultats morphologiques ont été quantifiés par Western blotting réalisés sur des échantillons obtenus par une extraction détersive différentielle permettant de séparer les protéines

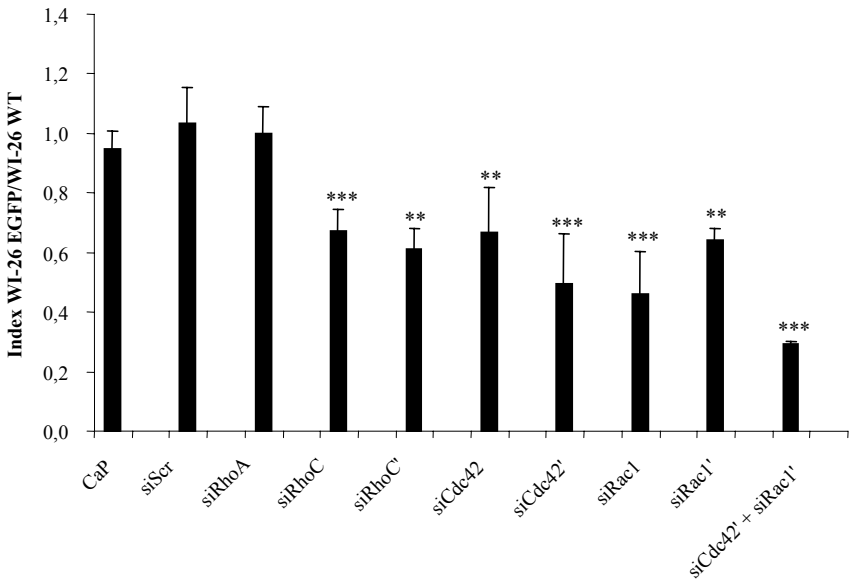

Figure 13 *:* ***Rôle des RhoGTPases dans la migration des fibroblastes humains.*** *Index de migration des WI-26 EGFP transfectées par différents siRNA déterminé 24h après réalisation de la plaie. Les différences significatives sont montrées par comparaison avec le contrôle siScr en utilisant le test statistique « ANOVA one way » suivie par un test de Tuckey-Kramer ** p ‹ 0.01, *** p ‹ 0.001.*

associées au cytosquelette des protéines devenant libres dans le cytoplasme (Figure 12). Ces analyses montrent que l'inhibition de l'expression de Rac1 ou de Cdc42 induit effectivement une réduction de la proportion d'actine polymérique insoluble dans le détergent. Par ailleurs, la répression de RhoC, Rac1 et Cdc42 conduit à une diminution significative du niveau de vinculine associée aux adhésions focales.

- La contribution des RhoGTPases dans les propriétés contractiles des fibroblastes a été étudiée dans le modèle des gels flottants de collagène. Dans ces conditions expérimentales, les fibroblastes exercent des forces de traction et compactent les fibres de collagène menant à une réduction progressive du diamètre et du volume de ces gels.
Les fonctions migratoires des fibroblastes ont été étudiées en réalisant des stries décellularisées (« wound assay ») *in vitro* et en analysant le recouvrement de ces zones après 24h. La transfection par siRac1 ou siCdc42 entraîne une réduction des capacités contractiles et migratoires des fibroblastes. Leur inhibition simultanée par une double transfection siRac1+siCdc42 induit un effet inhibiteur plus marqué de type additif (Figure 13).

- Nos résultats montrent également que RhoA n'est requis ni pour les fonctions contractiles ni pour les fonctions migratoires des fibroblastes. En revanche, si RhoC n'intervient pas dans les capacités contractiles des fibroblastes, il contribue par contre à leur migration (Figure 13). L'implication d'un processus de compensation entre RhoA et RhoC dans nos expériences a été écartée en réalisant des expériences de

double transfection siRhoA+siRhoC. Celles-ci ont donné des résultats équivalents à la simple transfection avec siRhoC.

Conclusions

Ces résultats suggèrent que Rac1 et Cdc42 participent à la formation de l'actine polymérique et des complexes d'adhésion. Ils indiquent également que, contrairement aux résultats publiés dans la littérature mais obtenus avec des outils moins spécifiques, RhoC mais non RhoA joue un rôle dans la formation des adhésions focales.

L'extinction de l'expression de Rac1 et Cdc42 induit une inhibition de la migration et des propriétés contractiles des fibroblastes. RhoC ne participe pas aux fonctions contractiles mais module leur migration tandis que RhoA n'est à nouveau pas impliqué.

Les modifications engendrées, notamment au niveau de l'organisation du cytosquelette et des fonctions cellulaires qui en dépendent, par les siRNA ciblant les RhoGTPases des trois classes principales de cette famille supportent l'efficacité et la spécificité de ces outils biologiques et valident leur utilisation dans la suite de nos travaux.

Differential modulation of migratory and contractile behaviour of fibroblasts by silencing RhoA, RhoC, Rac1 and Cdc42

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Abstract

The small GTPases of the Rho family are involved in signalling events initiated by integrins and have been implicated in the contractile and migratory properties of cells. The function of RhoA, RhoC, Rac1 and Cdc42 in fibroblasts was assessed by means of siRNA. With the exception of an up-regulation of RhoA following the repression of RhoC, the silencing of the three other RhoGTPases did not modulate the expression of the non-silenced RhoGTPases. Silencing RhoC, Rac1 or Cdc42 induced cytoskeletal modifications that were quantified by a differential non-ionic detergent extraction. The silencing of Rac1 and Cdc42 induced a highly significant reduction of the non extractable actin and vinculin, associated with the cytoskeleton and the focal adhesions, while the silencing of RhoC decreased only the vinculin associated with the focal adhesions. The contractile properties of the fibroblasts were evaluated in a floating three-dimensional collagen gel assay. The silencing of Cdc42 or Rac1 significantly repressed the fibroblast-mediated collagen gel retraction. By contrast the silencing of either RhoA, RhoC or RhoA and RhoC did not affect the collagen gel retraction. The silencing of RhoC, Rac1 or Cdc42 significantly repressed the migration of fibroblasts tested in an *in vitro* wound assay while the silencing of RhoA had no effect. These results emphasise the key role played by Rac1 and Cdc42 in the contractile and migratory properties of fibroblasts. Our siRNA approach revealed that RhoA was not involved in these cellular functions while RhoC operated only in cell migration.

Keywords: RhoA, RhoC, Rac1, Cdc42, siRNA, cytoskeleton, migration, contraction, collagen-gel, fibroblasts.

Introduction

In vivo, most cells interact with a three-dimensional network of polymeric macromolecules, the extracellular matrix (ECM), through transmembrane receptors of the integrin family. These receptors integrate the biological and mechanical signals issued from the ECM at specific sites of cell-matrix interactions, the focal adhesions. The integrins and the focal adhesions provide a physical continuum between the ECM and the cytoskeleton. This coupling allows the transmission, inside-out and outside-in, of mechanical forces required for cellular functions such as migration, assembly and architectural remodelling of the ECM. Members of the RhoGTPases family and their effectors have been implicated in these cellular events, but their contribution is quite variable depending on the experimental model [1, 2]. The tools used up to now to decipher the individual function of the

closely related RhoGTPases such as bacterial toxins, pharmacological agents are compromised by their lack of specificity. Moreover, the schematic view implying that the GTP-bound form is active and the GDP-bound form inactive is probably too simplistic [3]. Conventional tools mask a potential contribution of the GDP-bound form to intracellular signalling. These limitations can be bypassed thanks to a siRNA-based approach which allows analysis of closely related proteins and reveals the specific contribution of each of them. Moreover, the exquisite flexibility of this technology allows multiple knock-down experiments that can be used to dissect signalling pathways even in primary cells that are refractory to transfection of plasmids [4].

The current study was carried out to evaluate the contribution of 4 members of the small GTPases of the Rho family, i.e. RhoA, RhoC, Rac1 and Cdc42, to the contractile and migratory properties of human fibroblasts. They were analysed respectively in a free floating collagen gel model - where the gel contraction reflects the level of inward-pulling forces within the cells - and in a migration wound assay used as a model of integrated mechanical functions. Our results point to the key role played by Rac1 and Cdc42 in the contractile and migratory properties of fibroblasts. The siRNA approach allowed to analyse separately the contribution of RhoA and RhoC in fibroblasts and revealed that RhoA was not involved in these cellular functions while RhoC was only involved in migration.

Materials and methods

Cell culture

Human primary skin fibroblasts (HSF) were collected by explants outgrowth as described earlier [5]. Cells were regularly passaged by trypsin-EDTA and used between passages 9 and 13. Transformed SV-40 human lung fibroblasts (WI-26 cell line ATCC CCL 95.1) and HSF were grown in Dulbecco's Modified Eagle's Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Cambrex) at 37°C in 5% CO_2. WI-26 cells transformed with a vector allowing expression of Enhanced Green Fluorescent Protein under the control of a CMV promoter (WI-26 EGFP) were cultured in the same culture medium supplemented with puromycin (0.5 µg/ml) [6].

Reagents

The mouse monoclonal antibody against RhoA (clone Sc-418) and the rabbit polyclonal antibody directed to RhoA and RhoC (Sc-179) were purchased from Santa Cruz Biotechnology, against Cdc42 from Transduction Laboratories and against Rac1 (clone 05-389) from Upstate. The rabbit polyclonal antibody to p42/44 MAPK (M-5670), the mouse monoclonal anti-vinculin (V9131), anti-actin (A4700) and FITC-Phalloidin were purchased from Sigma-Aldrich. The secondary horseradish peroxydase conjugated antibodies (rabbit anti-mouse P0260 and swine anti-rabbit P0217) were from DAKO. The TRITC-coupled anti-mouse was from Molecular Probes and bis-benzimide from Calbiochem.

Design of siRNA and transfection procedures

The sequences of siRNAs targeting RhoA, RhoC, Rac1 and Cdc42 as well as the randomly mixed sequence of the siRNA targeting RhoA used as control (siScr) are detailed in table 1. The 21 nucleotides siRNAs (19 sens and antisens target sequences followed by 2 T) were chemically synthesized, desalted, deprotected and purified by using polyacrylamide-gel electrophoresis (PAGE) (Eurogentec). SiRNA annealing was performed following manufacturer's instruction at a final concentration of 20 µM. Cells attached for 24 h were transfected by siRNAs using calcium phosphate precipitation. A mix containing 285 µl RNase free H_2O, 32.5 µl 2.5 M $CaCl_2$, 8 µl 20 µM siRNA and 325 µl HBSP 2X (NaCl 280 mM, Na_2HPO4 1.5 mM, glucose 12 mM, KCl 10 mM, HEPES 50 mM, pH 7.05) was prepared extemporaneously and immediately added to the culture dishes containing 8 ml of DMEM supplemented with 10% FBS. After 14 to 16 h of incubation at 37°C-5% CO_2, cells were washed twice with PBS before incubation with fresh culture medium for the indicated period of time. The efficiency of transfection was tested by Western blot analysis in all reported experiments.

Cell morphology and immunohistochemistry

SiRNAs transfected human skin fibroblasts were seeded on 4-wells LabTek® chambered coverglasses (Nalge Nunc). After 24 h, cells were washed twice with ice-cold PHEM (PIPES 60 mM, HEPES 25 mM, EGTA 10 mM, $MgCl_2$ 2 mM). The cytosolic components were extracted *in situ* by 15 min incubation with ice-cold extraction buffer (NaCl 10 mM, $MgCl_2$ 3 mM, TRIS-HCl 10 mM, NP-40 0.1%). The remaining detergent insoluble components including polymerized cytoskeleton and adhesion plaques were fixed with 3% formaldehyde in PBS for 30 min at room temperature, washed with PBS, treated with 0.1% Triton X-100 and washed again. For labelling, they were incubated for 1 h with anti-vinculin, then for 1 h with the secondary antibody, for 20 min with FITC-phalloidin and finally for 20 min with bis-benzimide. After washing, cells were mounted and observed under oil immersion with a

Zeiss Axiovert 25 microscope coupled with an Axiocam Zeiss camera allowing analysis of images using a KS Kontron program.

Cell fractionation

The subcellular distribution of actin and vinculin was analysed as previously described [7]. Briefly, siRNAs transfected fibroblasts cultured on 6-wells plates were washed with PHEM buffer and extracted with NP-40 buffer on ice. After 15 min, the soluble fraction was removed and complemented with SDS-PAGE buffer. The insoluble fraction was lysed with an equal volume of the same PHEM-SDS-PAGE mixture. The proteins were separated by SDS-PAGE and actin and vinculin levels were analysed by Western blotting as detailed below.

Western blotting

Cells were lysed in SDS-PAGE lysis buffer and proteins were separated by polyacrylamide gel electrophoresis. 15%, 12.5% and 7.5% acrylamide gel were used for the RhoGTPases, actin and vinculin, respectively. Proteins were transferred to a PVDF Transfer Membrane (NEN Life Science Products). Membranes were blocked for 1 h with 3% dry milk in PBS-0.05% Tween 20 and incubated for 1 h with the diluted primary antibody. Membranes were then washed three times and incubated in the diluted secondary horseradish peroxydase-conjugated antibody for 1 h. After washing, immunoreactivity was 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.

Active GTPase assays

The assay was carried out as previously described [8]. 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 PMSF, 4 µg/ml aprotinin, 1 µg/ml leupeptin and 1 µg/ml pepstatin. Lysates were centrifuged for 8 min at 13000g. Supernatants were immediately frozen in liquid nitrogen and stored at –80°C until used. An aliquot of each supernatant was denatured in SDS-PAGE lysis buffer before freezing to measure the total RhoGTPase content by Western blotting. For pull-down assays, supernatants were incubated for 30 min with 30 µg of GST-PBD protein containing the Cdc42 and Rac binding region of PAK-1B, or GST-RBD protein containing the Rho binding region of rhotekin, affinity linked to glutathione-Sepharose beads [9, 10]. The beads were washed 4 times in lysis buffer and boiled in 60 µl SDS-PAGE lysis buffer.

mRNA measurement by RT-PCR

Total RNA was extracted from cell cultures with the High Pure RNA Isolation kit (Roche Molecular Biochemicals) as described by the manufacturer. Ten ng of RNA were reverse-transcribed and amplified using the GeneAmp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer) and specific pairs of primers (Eurogentec). A known copy number of an original synthetic internal standard was used for 28S rRNA [11]. Primers for RhoA were: forward 5'-TCGATATCTGCCACATAGTTCTCAAA-3', reverse 5'-CATTAGTCCACGGTCTGGTCTTCA-3', for RhoC: forward 5'-TCCAGGCTGTCAGGGCTGTCGA-3', reverse 5'-GACCTGCCTCCTCATCATCGTCTTCA-3', for 28S: forward 5'-GTTCACCCACTAATAGGGAACGTGA-3', reverse 5'-GGATTCTGACTTAGAGGCGTTCAGT-3'. The RT step (70°C for 15 min) was followed, after 2 min incubation at 95°C for denaturation of RNA/DNA heteroduplexes, by PCR amplification for the adequate number of cycles and terminated by a final elongation step of 2 min at 72°C. The PCR conditions for amplification of RhoA (24 cycles), RhoC (24 and 27 cycles) and 28S rRNA (18 cycles) were 94°C for 15 s; 66°C for 20 s and 72°C for 10 s. The PCR products were analysed on a 10% polyacrylamide gel and the signals quantified using Fluor-S MultiImager (BioRad) after staining with GelStar dye (FMC BioProducts).

Three-dimensional collagen gel retraction assay

The collagen gel retraction assay was performed as previously described [12]. Briefly, purified bovine skin type I collagen (final collagen concentration of 0.6 mg/ml) was mixed with Dulbecco's modified Eagle's medium (Gibco) supplemented with sodium bicarbonate (42 mM), glutamine (3.3 mM), ascorbic acid (0.47 mM), penicillin-streptomycin (100 units/ml) and fetal bovine serum (10% final concentration). All steps were performed on ice. Human skin fibroblasts (5.10^4 cells) were added and the mixture was poured in 60 mm bacteriological dishes (Nalge Nunc International) and maintained at 37°C under 5% CO_2. After polymerization, the collagen gels were detached from the sides and bottom of the dishes. The diameter of the gels was measured daily for 5 days. After 5 days, the collagen gels were washed with PBS and digested with 1 mg/ml of collagenase (C-9891, Sigma-Aldrich) for 15 min at 37°C. Cells were collected by centrifugation and sonicated in 250 µl PBS. The DNA content was measured by a fluorimetric procedure using bis-benzimide [13].

Migration assay

WI-26 EGFP were transfected with the various siRNAs and mixed with WI-26 wild type in the respective proportion of 20% and 80%. The mixed cultures were then seeded on 4-wells Lab-Tek®. After one day cells have formed a confluent monolayer. The monolayer was wounded by a scratch performed with a sterile P1000 pipette tip along the centre of each well. Several representative images of the scratched areas under each condition were photographed after 0, 8, 24, 32 and 48 h in both phase contrast or under UV illumination (488 nm). For EGFP cells, the emitted light had a wavelength of 506 nm.

The migration rate was evaluated 24 h after wounding. A mean wound width was determined at t=0 by averaging the width of three randomly chosen regions of a single wound. This average width was reported on images photographed at t = 24 h and the

number of cells between the lines was counted both in phase contrast and in UV light (siRNAs transfected cells). An index of migration was determined as the mean percentage of migrating siRNAs treated WI-26 EGFP to the mean percentage of WI-26 EGFP at t = 0. An index of 1 means that the siRNA has no effect on cell migration, higher than 1 indicates an increased migration and lower than 1 a reduced migration as compared to non transfected cells.

$$\text{Index of migration} = \frac{(\%\ \text{EGFP cells/total cells})_{t = 24\ \text{in the wound}}}{(\%\ \text{EGFP cells/total cells})_{t = 0}}$$

Results

Silencing of RhoGTPases in human fibroblasts

Inhibition of the expression of RhoGTPases RhoA, RhoC, Rac1 and Cdc42 was performed with specific small interfering RNAs (siRNAs) in HSF and WI-26 fibroblasts. As described recently for HSF [4], an inhibited expression of the GTPases was already observed one day after transfection, maximal three days after and lasted until 7 days after the introduction of the siRNA into cells. The scrambled sequence of the siRNA targeting RhoA was used as a negative control (siScr). Transfection with the siScr as well as with calcium phosphate alone had no effect on the Rho proteins level. The efficiency and the specificity of each siRNA are illustrated in Fig. 1 and Fig. 2.

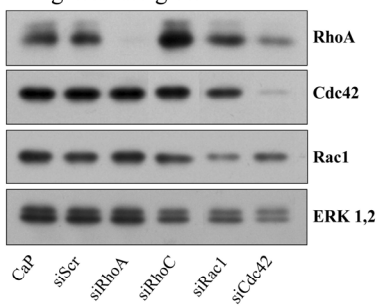

Fig. 1: Silencing of RhoA, Rac1 and Cdc42 by siRNAs in primary human skin fibroblasts. *Cells were transfected with siRNA targeting RhoA (siRhoA), RhoC (siRhoC), Rac1 (siRac1) and Cdc42 (siCdc42). Fibroblasts transfected with siScr or treated with calcium phosphate alone were used as controls. 72 h after transfection, the cells were lysed in SDS-PAGE loading buffer and analysed by immunoblotting using antibodies to RhoA, Cdc42, Rac1 and ERK 1,2.*

Three days after transfection, a reduction of more than 90% of the protein level of RhoA and Cdc42 was observed while the extinction of Rac1 was around 70% (Fig. 1). The mRNA of RhoC was reduced by more than 80% (Fig. 2, top). By using a polyclonal antibody recognizing both RhoA and RhoC, we observed that the double transfection of HSF with siRhoA and siRhoC induced an extinction of the expression of both proteins (Fig. 2, bottom).

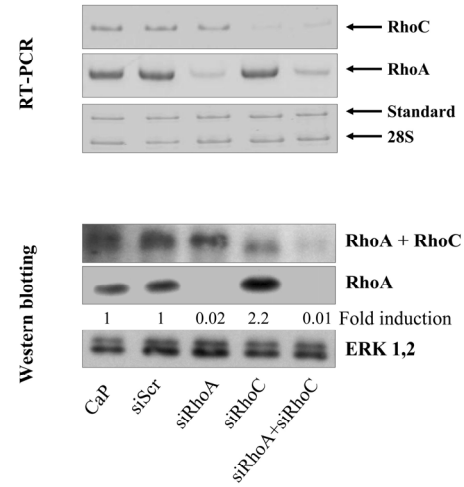

Fig. 2: The silencing of RhoC induced an increase in RhoA protein. *Fibroblasts were transfected with siRhoA, siRhoC or siRhoA and siRhoC (siRhoA+siRhoC). Fibroblasts transfected with siScr or treated with calcium phosphate alone were used as controls. Cells were lysed 72 h after transfection.* ***A:*** *Representative RT-PCR analysis of RhoA and RhoC mRNA levels, and 28S rRNA levels in RNA extracted from transfected fibroblasts.* ***B:*** *Representative western-blot analysis of whole cell lysates using antibodies recognizing RhoA or RhoA and RhoC (RhoA+RhoC), or antibodies to ERK1,2. The fold induction is calculated from densitometric measurements of RhoA signal in lysates of siRNA-transfected fibroblast normalized to RhoA signal in calcium phosphate-treated fibroblast.*

No modification of the level of the non-targeted RhoGTPases was observed in the cells transfected with the siRhoA, siRac1 and siCdc42. By contrast, the siRhoC transfected cells presented a two-fold increase of the RhoA at the protein level (Fig. 1 and 2). Pull-down experiments confirmed that a two-fold increase of GTP-bound RhoA was observed in RhoC-silenced cells while the active forms of

Rac1 and Cdc42 were not modified (not shown).

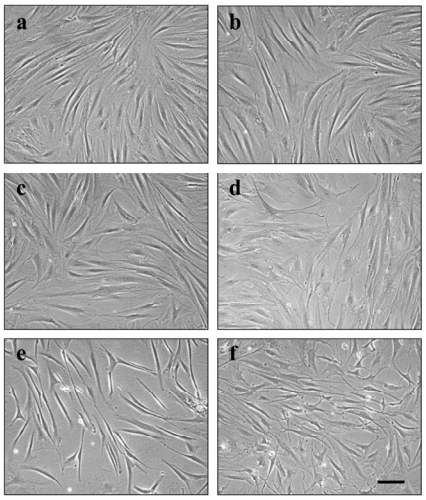

Fig. 3: Morphological effects of RhoA, RhoC, Rac1 or Cdc42 silencing. *Representative phase-contrast images of human fibroblasts 72 h after treatment with calcium phosphate alone (**a**), or transfected with siScr (**b**), siRhoA (**c**), siRhoC (**d**), siRac1 (**e**) or siCdc42 (**f**). Bar = 100 µm.*

Effects of RhoGTPases silencing on fibroblasts morphology

The inhibition of the RhoGTPases affected the morphology of HSF as seen by phase contrast microscopy (Fig. 3), the silencing of RhoC lead to a more spread shape and increased cell surface, Rac1-silenced fibroblasts were smaller and thinner with less lamellipodia while the Cdc42-silenced fibroblasts were also thinner but presented more cytoplasmic projections. By contrast, the suppression of RhoA did not induce any significant shape alteration. These observations suggested the existence of cytoskeletal alterations in fibroblasts transfected with the various siRNAs. The fibrillar actin and the focal adhesions were labelled respectively with phalloidin-FITC and an anti-vinculin antibody.

The silencing of RhoA did not alter the general shape and the cytoskeleton of the cell while the silencing of RhoC induced a more spread shape with multidirectional stress fibres. The inhibition of Cdc42 altered the general shape of the cell and the actin cytoskeleton, the actin stress fibres were less abundant and the focal complexes reduced and mostly observed at the cell edges. The suppression of Rac1 resulted in reduced focal complexes at the edges of the cells and more diffuse and rarified actin stress fibres (Fig. 4).

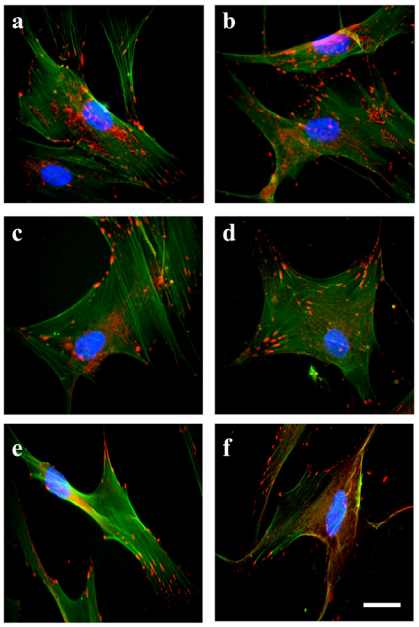

Fig. 4: Alteration of the actin-cytoskeleton and of the focal adhesion following silencing of RhoA, RhoC, Rac1 or Cdc42. *Human fibroblasts were fixed 72 h after transfection with calcium phosphate alone (**a**), siScr (**b**), siRhoA (**c**), siRhoC (**d**), siRac1 (**e**) or siCdc42 (**f**) and analysed by immunofluorescence labelling with bisbenzimide (nucleus-blue), phalloidin-FITC (actin-green) and anti-vinculin antibody (red) after NP-40 extraction. Bar = 25 µm.*

Analysis of the subcellular distribution of actin and vinculin in RhoGTPases-silenced HSF

To quantify the modifications of actin and vinculin observed by immunohistochemistry, HSF tranfected with the various siRNAs for three days were treated with NP-40 buffer to extract the cytoplasmic fraction of the cells. The soluble and insoluble fractions in NP-40 were analysed by Western blotting (Fig. 5A and B). In the control conditions, i.e. calcium phosphate-treated or siScr-transfected fibroblasts, actin distributed equally between both fractions (Fig. 5A) while the majority of vinculin (80%) was in the soluble fraction (Fig. 5B).

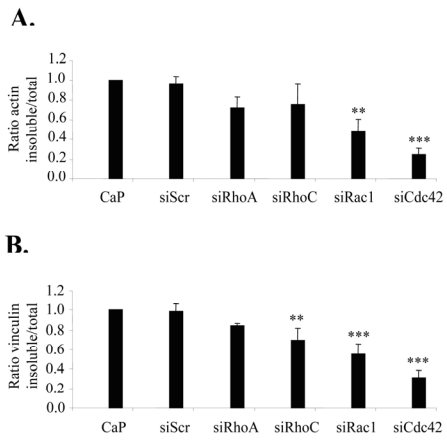

***Fig. 5**: **Effect of the silencing of the RhoGTPases on the distribution of the actin and of the vinculin in HSF**. The NP-40 insoluble and NP-40 soluble fractions of siRNAs transfected fibroblasts were analysed by Western blotting with the anti-actin (**A**) and anti-vinculin (**B**) antibodies. The ratio insoluble/total was calculated from densitometric measurements of vinculin or actin signals. Data are expressed as the mean ± SD of three independent experiments. Significant differences by comparison to siScr are shown for ** p<0.01 and *** p<0.001 by statistical test ANOVA one way followed by Tukey-Kramer test.*

The distribution of vinculin and of actin was not significantly modified in RhoA-silenced cells as compared to control conditions. The silencing of RhoC induced a reduction of vinculin in the insoluble fraction but did not modify significantly the actin distribution. In Rac1 and Cdc42-silenced cells the amount of actin and vinculin in the insoluble fraction was dramatically reduced.

Modulation of the contractile properties of the fibroblasts by RhoGTPases silencing

The effect of each RhoGTPase on the contractile properties of the HSF was evaluated in freely-retracting collagen gels populated with siRNA-transfected fibroblasts. In this model, the tractional forces exerted by fibroblasts are reflected by the decrease over time of the diameter of the gels. Preliminary experiments showed that 10^4 fibroblasts/ml and 0.6 mg/ml (final concentration) of type I collagen allows the analysis of gel contraction for up to five days.

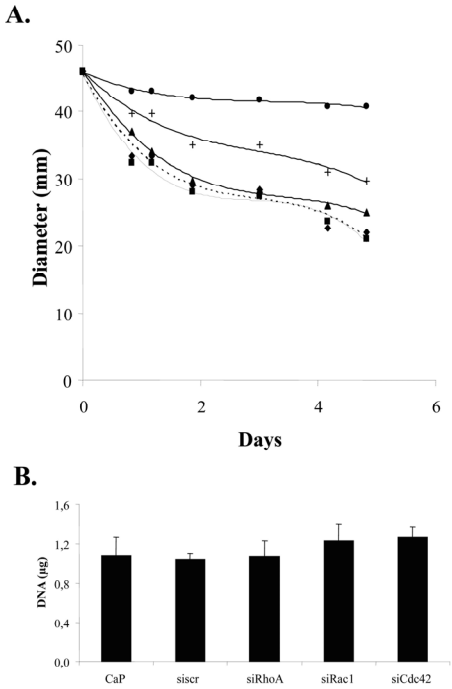

***Fig. 6**: **Rac1 and Cdc42, but not RhoA, are involved in HSF mediated collagen gel retraction**. **A:** SiRNAs-transfected HSF were embedded in collagen gels. **A:** The diameter was measured daily for 5 days. Cell density is controlled as described in materials and methods after digestion of the gels with collagenase. HSF were transfected with siRhoA (▲), siRac1 (✚) or siCdc42 (●). HSF treated with CaP alone (■) or transfected with siScr (◆) were used as controls. Standard deviation was lower than the size of the icons. One representative experiment out of three is shown. **B:** The DNA content in the gels was determined after 5 days of culture as described in Materials and methods.*

CaP and siScr treated cells induced a similar contraction of the collagen gels which was rapid during the first 24 h and slowed-down between day 1 and day 5. In both conditions, the gels reached a similar final diameter. The extinction of Cdc42 induced a strong inhibition of the fibroblasts-mediated collagen gel retraction. This inhibition was already observed after 24 h and lasted until the end of the experiment at day 5. The silencing of Rac1 also reduced significantly the contraction of the collagen gels, but its effect was less pronounced than silencing Cdc42 (Fig. 6). The inhibition of collagen gel contraction, although slightly less pronounced was

confirmed by using a second siRNA targeting Cdc42 or Rac1 (not shown).
By contrast, the suppression of RhoA did not significantly influence the contraction rate of the 3-D collagen gels. In order to investigate the role of RhoC in gel contraction and to determine if RhoA could compensate for RhoC silencing, since silencing RhoC induced a two-fold up-regulation of RhoA at the protein and activity level (Fig. 2), single transfection of siRhoC, siRhoA and double transfection of siRhoA and siRhoC were carried out in parallel (Fig. 7). Neither the individual silencing nor the double silencing RhoA+RhoC significantly altered the rate of gel retraction as compared to control conditions (Fig. 7).

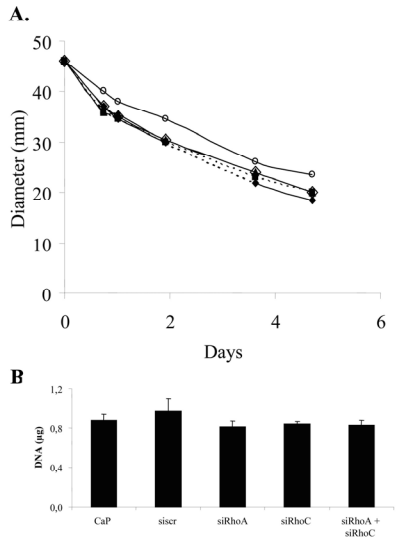

Fig. 7: Neither RhoA nor RhoC are involved in HSF mediated collagen gel retraction*. SiRNAs-transfected HSF were embedded in 3-D collagen gels. The diameter was measured daily for 5 days. Cell density is controlled as described in materials and methods after digestion of the gels with collagenase. HSF were transfected with siRhoA (▲), siRhoC (○) and doubled-transfected siRhoA and siRhoC (◇). HSF treated with CaP alone (■) or transfected with siScr (◆) were used as controls. Standard deviation was lower than the size of the icons. One representative experiment out of three is shown.* ***B:*** *The DNA content in the collagen gels was determined after 5 days of culture as described in Materials and methods*

The up-regulation of RhoA and the lack of effect on gel retraction were confirmed using a second siRNA sequence targeting RhoC (not shown). These data demonstrate that neither RhoA nor RhoC are required for fibroblast-mediated collagen gel retraction. It should be noted that a similar DNA content was measured in each condition at the end of the experiments demonstrating that differences in gel retraction were not caused by variations in cell density (Fig. 6B and Fig. 7B).

RhoA is not required for fibroblast migration.

Fibroblast migration was analysed in an *in vitro* wound assay. Human fibroblasts (WI-26) stably expressing Enhanced Green Fluorescent Protein (EGFP) were transfected with the various siRNAs. These WI-26 EGFP transfected cells were mixed with wild type WI-26, used as internal control, at a ratio of 20:80. This cell mixture was seeded in 4-wells LabTek®.
After 24 h, the cells were confluent. A scrape wound of approximately 600 µm width was made in the cell monolayer. The phase-contrast images allowed to quantify the total cell population whereas the same field photographed under UV-light showed only the siRNAs transfected cells expressing EGFP (Fig. 8). Migrating cells exhibited a polarized shape with ruffling lamellipodia at the leading edge. After 8 h, cells began to fill the gap and wound closure was completed after 48 h. We choose 24 h as the optimal time to analyse cell motility (Fig. 8A). Neither the expression of EGFP, nor the transfection reagent nor the transfection with the control siRNA affected cell migration (Fig. 8 and data not shown).
The silencing of Cdc42 as well as the silencing of Rac1 reduced significantly the migration rate. An inhibition of migration (30%) was observed with the first siCdc42 while the inhibition reached 50% with the second siCdc42. The silencing of Rac1 induced a more dramatic inhibition of WI-26 EGFP migration. An inhibition of 60% was observed with the first siRNA targeting Rac1 and of 40% with the second siRac1. The simultaneous suppression of Cdc42 and Rac1 in the EGFP cells reduced even

more the migration rate (near 70%) (Fig. 8B).

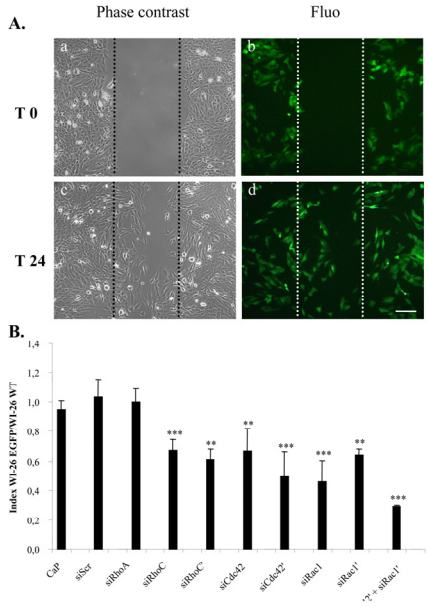

Fig. 8: RhoA is not involved in fibroblast motility. ***A.*** *Representative gap closure in scratch assay just after (T0) or 24 h after (T24) wounding the fibroblast monolayer. a, c: phase contrast microscopy b, d: fluorescent microscopy showing the siScr transfected WI-26 EGFP. Dashed lines show initial gap width. Bar = 100 µm.* ***B.*** *The index of migration of WI-26 EGFP cells transfected with either CaP alone, siScr, siRhoA, siRhoC, siRac1, siCdc42 or siRac1+siCdc42 was determined at t = 24 h as described in Materials and Methods. Data are expressed as the mean ± SD of three independent experiments. Significant differences by comparison to siScr are shown for * p<0.05, ** p<0.01 and *** p<0.001 by statistical test ANOVA one way followed by Tukey-Kramer test.*

Interestingly, fibroblast migration was not affected by RhoA silencing. By contrast, the silencing of RhoC using two different siRNAs reduced the migration rate of the transfected cells by 30% to 40%. These results support the hypothesis that RhoC is involved in fibroblast migration while RhoA does not seem to play a role in this mechanical function. However, the inhibition of migration following RhoC silencing could be due to RhoA up-regulation. This is not the case since the inhibition of migration observed following double silencing RhoA+RhoC is equivalent to the inhibition of migration observed by silencing of RhoC alone (Fig. 9).

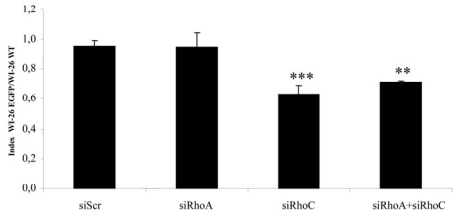

Fig. 9: Inhibition of fibroblast migration by siRhoC is not mediated by RhoA up-regulation. *The index of migration of WI-26 EGFP cells transfected with either Cap alone, siScr, siRhoA, siRhoC, siRac1, siCdc42 or siRac1+siCdc42 was determined at t = 24 h as described in Materials and Methods. Data are expressed as the mean ± SD of three independent experiments. Significant differences by comparison to siScr are shown for * p<0.05, ** p<0.01 and *** p<0.001 by statistical test ANOVA one way followed by Tukey-Kramer test.*

Discussion

RhoGTPases are molecular switches shuttling between an inactive GDP-bound state and an active GTP-bound state. This dogmatic view implies that signalling is mediated solely by the GTP-bound form. However, the GDP-bound state is probably also involved in the regulation of intracellular signalling. Indeed, Cdc42GDP was reported to repress the Erk1,2 pathway [3]. The use of small interfering RNAs to assess the functions of the RhoGTPases RhoA, RhoC, Rac1 and Cdc42 will give a more global picture of their implication in the regulation of the phenotype of human fibroblasts by ablation of both GDP- and GTP-bound forms and open the way to uncover original regulatory pathways. This approach allowed us to highlight the negative regulation of MMP-1, MCP-1 and IL-8 expression by Cdc42 [4]. Further, it allows to discriminate between closely related proteins such as RhoA and RhoC. Non gene-specific effects have been reported by Bridge et al. [14] and Sledz et al. [15] who showed that an interferon response was activated with the 21-nucleotides duplex in contrast to previous studies [16]. No such side effect was observed in our cells [4]. The repression of RhoA, Rac1 and Cdc42 in HSF did not induce any compensatory regulation of the non-silenced RhoGTPases by contrast to previous studies using overexpression of mutated dominant negative RhoGTPases [10]. This could be related to our model but

also to the exquisite specificity of the siRNA. By contrast, the up-regulation of RhoA observed in siRhoC-transfected cells suggests that RhoC is a negative regulator of RhoA. Our results are consistent with the recent study of Simpson et al. [17] where RhoA was also up-regulated at the protein level in RhoC-silenced breast carcinoma cells. Together, these results suggest a general compensatory effect rather than a cell-type restricted process.

Analysis of the distribution of vinculin and actin between detergent-resistant and detergent-soluble fractions as well as immunofluorescence morphological investigation of the actin stress fibres and of the vinculin-containing adhesions indicate that the inhibition of Rac1 and Cdc42 affects both the actin cytoskeleton and the focal adhesions. Polymeric actin and stress fibres as well as insoluble vinculin and the number of focal complexes are reduced after silencing of Rac1 or Cdc42. This agrees with the observations of Aspenstrom et al. [18] who demonstrated an increase of stress fibres and focal complexes in cells expressing constitutively activated Rac1 and Cdc42. These cytoskeletal modifications should be related to the activation by Rac1 and Cdc42 of WASP family members that stimulate Arp2/3 to nucleate actin, induce its polymerization and drive the formation of lamellipodia and filopodia. Rho-dependent activation of ROCK was reported to mediate the formation of the acto-myosin stress fibres and focal adhesions [19]. The approach of that study can however not discriminate between the contribution of RhoA and RhoC. These RhoGTPases have more than 90% homology in amino acids sequence and share the same downstream effector ROCK. The silencing of RhoA alone did not modify the proportion of actin in the stress fibres, of vinculin in focal adhesions and did not alter cell morphology. By contrast, RhoC silencing decreased the proportion of vinculin in the focal adhesions and induced a more spread morphology. A similar phenotype was reported in fibroblasts treated with the ROCK inhibitor Y-27632 [20]. This suggests that RhoC is the main ROCK activator in fibroblasts as already proposed by others [21].

Cell contractility and migratory capacities are essential in many processes as embryonic development, wound healing, fibrosis, tumor invasion and metastasis. The contractile properties of the cells have been investigated *in vitro* using cell-populated collagen gels [12]. The contraction of gels is due to the traction forces exerted by the fibroblasts during attachment, spreading and migration on the collagen fibres [22]. Silencing Rac1 or Cdc42 induced a significant inhibition of gel contraction. Similar data were obtained with two siRNA targeting different sequences of the RhoGTPase mRNA. A similar cell density was measured in the retracted collagen gels populated with control, calcium phosphate-treated and siRNA transfected fibroblasts ruling out a modification of cells number by alteration of survival or proliferation. The difference of gel retraction could also be related to a modified regulation of MMP-1 as reported recently in Cdc42-silenced fibroblast cultured in monolayer on plastic [4]. However, in our model of free-floating collagen gels most of the metalloproteinases are largely up-regulated [22, 23] and no difference in the expression of a large panel of MMPs was observed upon treatment with the various siRNA (Ho et al., unpublished data). Several, non mutually exclusive hypotheses could explain the reduced retraction rate by silencing Rac1 and Cdc42. First, Rac1 and Cdc42 trigger the formation by fibroblasts seeded in collagen matrices of cytoplasmic extensions shown to be involved in the contractile forces [24]. Second, the deregulation of actin and vinculin organisation following Rac1 and Cdc42 silencing could affect the attachment and the spreading of the cells on the collagen fibres. Third, the inhibition of gel contraction by Cdc42 silencing may be due to decreased contractility of the acto-myosin machinery as suggested by alterations of the organization of actin stress fibres.

Previous studies reported a role for Rho and its effector ROCK in the contractile forces exerted by fibroblasts on two-dimensional surfaces and on collagen matrices by using bacterial toxins targeting Rho or by chemical inhibitors of ROCK [25-27]. Rho Kinase is involved in cell contractility by

phosphorylating the myosin phosphatase and the light chain of myosin II. These two events activate the motor protein myosin II leading to contraction of the acto-myosin stress fibres [28]. However, neither silencing RhoA alone nor RhoC alone nor both RhoA and RhoC significantly affected fibroblast-mediated collagen gel contraction. Our results are consistent with those of Lee et al. [29] who demonstrated that LPA-stimulated fibroblast contraction of free-floating collagen gels does not require Rho Kinase activity and become ROCK-dependent only when the gels are allowed to put under mechanical tension by the cells for at least two hours before detachment. Moreover, Wozniak et al. [30] have shown that in free-floating collagen gels in which the mechanical tension is low, there exists a feed-back mechanism that inhibits Rho activity, further supporting the idea that neither RhoA nor RhoC are required for fibroblast-mediated collagen gel contraction.

Cell migration is a complex dynamic process requiring coordinate temporal and spatial remodeling of the actin cytoskeleton. It involves cell polarization, formation of membrane ruffling in the direction of migration and formation of new adhesions, contraction of the rear of the cell and detachment from the substrate at the trailing edge [31]. The role of the RhoGTPases in this process is variable and depends on the model used. In most cell types, the activation of Rac1 increases migration except in epithelial cells [1]. RhoA activity positively or negatively affects migration depending on the mode of tumor cells invasion [2]. Divergent effects could also depend on the extracellular matrix surrounding the cells [32]. Fibroblast migration has been studied in an *in vitro* wound healing assay designed to prevent artefactional information. The influence of cell multiplication and modification of cell-cell interaction was reduced by analysing the migration of a subconfluent mixed culture of 80% unlabelled WI-26 wild type with 20% siRNA treated WI-26 EGFP and measuring the proportion of EGFP cells versus unlabelled in the wound. The silencing of Rac1 and Cdc42 inhibited fibroblast migration. This is likely related to the role of Rac in the formation of the meshwork of actin filaments at the level of lamellipodia at the leading edge of the cell, the first step in migration [33]. Rac1 is also involved in the recruitment of high affinity integrins to lamellipodia [34]. Cdc42 also controls several important cellular events required for cell migration like filopodia formation but also cell polarity by redirecting the Golgi apparatus in the direction of movement [35]. The double silencing of Rac1 and Cdc42 impaired more extensively fibroblasts migration suggesting that Rac1 and Cdc42 have additive effects on cell migration.

RhoA and RhoC displayed distinct effects in fibroblasts migration. Silencing RhoC induced a reduction of the migration rate while silencing RhoA had no such effect. The inhibition of migration following RhoC silencing could be due to the observed up-regulation of RhoA. Indeed, Simpson et al. [17] have recently reported a negative role for RhoA in tumor cell migration. However, the double silencing RhoA-RhoC inhibits fibroblast migration as efficiently as silencing of RhoC alone, demonstrating that RhoA is actually not involved in fibroblast migration. RhoC is required for cell motility probably via activation of ROCK which allows the rear of the cell to retract and move forward. These results are consistent with those of Tkach et al. [36] who found that the dominant negative form of RhoA had no effect on cell motility or cell morphology. By contrast inhibition of ROCK induced a partial inhibition of fibroblast migration. The overexpression of RhoC in many cancers also support its significant role in cell migration [37].

In conclusion, Rac1 and Cdc42 are obviously involved in the contractile properties of fibroblasts and play a key role in their motility probably by their role in the formation of polymeric actin structures and focal complexes. In contrast to previous studies using the dominant negative forms of the GTPases or inhibitors, our data did not support a role for RhoA in these cellular functions whereas RhoC was found necessary for cell migration and the formation of focal adhesions but not for fibroblast-mediated retraction of free floating collagen gels. The two processes are known to rely on different mechanisms.

The repression of RhoA expression by RhoC deserves to be further investigated. Work is now in progress to decipher the intracellular pathways mediating these regulations.

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