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Genomic analysis of metastasis reveals an essential role for RhoC

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The most damaging change during cancer progression is the switch from a locally growing tumour to a metastatic killer. This switch is believed to involve numerous alterations that allow tumour cells to complete the complex series of events needed for metastasis[1]. Relatively few genes have been implicated in these events[2–5]. Here we use an *in vivo* selection scheme to select highly metastatic melanoma cells. By analysing these cells on DNA arrays, we define a pattern of gene expression that correlates with progression to a metastatic phenotype. In particular, we show enhanced expression of several genes involved in extracellular matrix assembly and of a second set of genes that regulate, either directly or indirectly, the actin-based cytoskeleton. One of these, the small GTPase RhoC, enhances metastasis when overexpressed, whereas a dominant-negative Rho inhibits metastasis. Analysis of the phenotype of cells expressing dominant-negative Rho or RhoC indicates that RhoC is important in tumour cell invasion. The genomic approach allows us to identify families of genes involved in a process, not just single genes, and can indicate which molecular and cellular events might be important in complex biological processes such as metastasis.

To provide insight into the pattern of gene expression that allows tumours to metastasize, we compared the gene expression profile of melanoma variants with low or high metastatic potential. As shown in Fig. 1, the system involves the *in vivo* selection of highly metastatic melanoma cells from a population of poorly metastatic tumour cells[6]. When nude mice were injected intravenously with amelanotic human A375P tumour cells, relatively few pulmonary metastases were observed (Fig. 2a). When these rare metastases were dissected free from the lungs and the cells grown in tissue culture, however, the resulting cells showed enhanced metastatic capacity, confirming that highly metastatic cells can be selected from a heterogeneous population of poorly metastatic tumour cells[7]. Furthermore, if successive metastases (designated M1 and M2) were isolated, expanded in tissue culture, and re-introduced into host mice as shown in Fig. 1, significantly more pulmonary metastases were observed (Fig. 2b). When mouse B16F0 melanoma cells were subjected to this same *in vivo* selection scheme, highly metastatic pulmonary tumours (designated F1, F2 and F3) were isolated, as previously described for this cell line[6]. When the poorly metastatic A375P or B16F0 and the *in vivo*-selected metastatic A375 or B16 cells were grown as subcutaneous tumours, there was no observable difference in tumour size (see Supplementary Information), indicating that we had selected for a difference in metastatic, but not tumorigenic, properties of the melanomas. These results support the hypothesis that specific gene products can regulate metastasis without altering the growth properties of a tumour[8]. Therefore, we sought to identify metastasis-specific genes using a functional genomics approach.

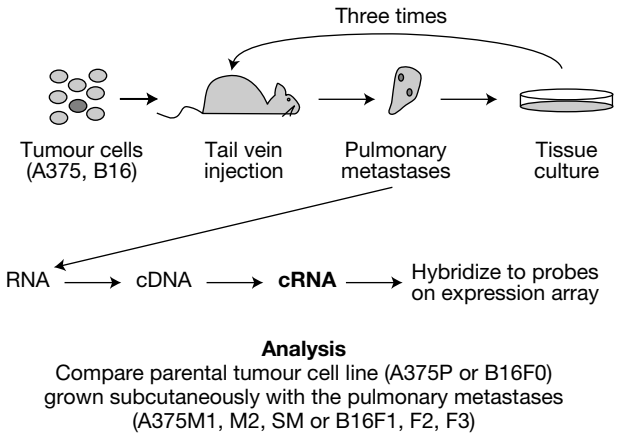

Figure 1 *In vivo* selection scheme. Poorly metastatic melanoma cell lines (human A375P or mouse B16F0) were injected intravenously into the tail veins of host mice and pulmonary metastases were isolated. Either these metastases were minced and grown in tissue culture (to be injected into additional host mice) or RNA was extracted to prepare the labelled cRNA used to hybridize to the oligonucleotide arrays. The procedure to select for highly metastatic tumour cells was repeated two (A375) or three (B16) times. A375SM cells were previously derived in a similar manner[11].

RNAs extracted from these pulmonary metastases and from the parental A375P and B16F0 lines grown as subcutaneous tumours were used to prepare complementary RNAs (cRNAs), which were hybridized to oligonucleotide microarrays (human: 7,070 genes; mouse: 6,347 genes, with around 50% overlap in the genes represented) to determine the array of differentially expressed genes (Fig. 1). The entire data set is available at our web site at http://www.genome.wi.mit.edu/MPR and in Supplementary Information. Table 1 lists those genes expressed at consistently higher levels in pulmonary metastases derived from the A375P line (M1, M2 and SM) and the mouse B16F0 line (F1, F2 and F3). To ensure that the enhanced expression of these genes in the pulmonary metastases was not due solely to the influence of the microenvironment in which the metastatic cells were growing, we also grew metastatic A375SM cells subcutaneously and compared their expression profile with that of subcutaneous A375P tumours. We found that 15 of the 16 genes continued to show enhanced expression when metastatic A375 cells were grown as a subcutaneous tumour (see Supplementary Information), indicating that the expression of these genes is intrinsic to the metastatic cells. Note, however, that the tumour microenvironment may help to regulate the absolute level of gene expression.

As the set of genes represented on the human and mouse arrays partially overlapped, some signals appeared in both species (Table 1). Three genes, fibronectin, RhoC and thymosin β4, were expressed at higher levels (≥2.5-fold) in all three metastases selected from both the human A375 and mouse B16 cell lines. Enhanced expression of these three genes in the pulmonary metastases was

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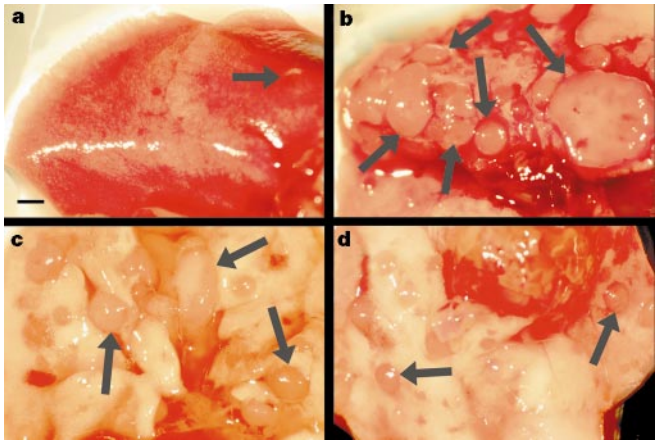

Figure 2 Pulmonary metastases in lungs of mice injected with tumour cell lines. **a**, A375P cells; **b**, A375M; **c**, A375P-RhoC; **d**, A375M-dnRho. Arrows indicate representative metastatic nodules. Scale bar, 1 mm.

confirmed by RNAse protection (see Supplementary Information). Note that this assay will detect only human RNAs (from the tumour cells) and not mouse RNAs from stromal or vascular cells. This confirms that changes in expression of these three genes occur in the tumour cells rather than in host tissue. This is also probably true for all the oligonucleotide array data given the sequence divergence between species (Table 1).

Fibronectin is an extracellular glycoprotein that serves as a ligand for the integrin family of cell adhesion receptors and regulates cytoskeletal organization. Fibronectin expression has been linked with tumorigenesis[9] and metastasis[10], although these studies are only correlative. Peptides that mimic the cell adhesive region of fibronectin are, however, known to inhibit metastasis[11], which may indicate that tumour cells must interact with molecules such as fibronectin to metastasize. RhoC is a member of the Rho GTPase family that can regulate many cellular functions, most notably cytoskeletal organization, in response to extracellular factors[12]. Enhanced expression of RhoC has been reported to correlate with the progression of pancreatic adenocarcinomas to a metastatic phenotype[13]. Thymosin β4 is an actin-sequestering protein that regulates actin polymerization; its expression in renal tumours has been correlated with malignancy[14]. Expression of two other family members, thymosin β10 and thymosin β15, also correlates with metastasis[4,15]. Other regulators of the cytoskeleton also appear on the list, including α-catenin and expressed sequence tags (ESTs) for α-actinin 1 and α-centractin. The altered expression of so many genes whose products regulate the actin cytoskeleton either directly or indirectly indicates that cytoskeletal organization may be important in tumour metastasis.

Also prominent on the list in Table 1 are several genes that encode extracellular matrix (ECM) proteins, as well as molecules that regulate their assembly. In addition to fibronectin, two collagen subunits, α2(I) and α1(III), the matrix Gla protein, fibromodulin and biglycan also are expressed at higher levels in the metastatic melanomas. Previous studies of matrix Gla protein have shown that it is overexpressed in breast carcinoma cell lines relative to normal breast epithelial cells[16] and collagen expression has been correlated with the invasive potential of ocular melanomas[10], but expression of the small interstitial proteoglycans biglycan and fibromodulin (which regulate collagen fibril formation[17,18]) has yet to be linked to tumour progression. These findings support hypotheses that enhanced expression of ECM proteins may promote tumour cell survival or angiogenesis[19].

Table 1 Enhanced gene expression in metastatic melanomas

	human A375						mouse B16					
Gene name	Human acc. number	Ch. no.	P	M1	M2	SM	Mouse acc. number	F0	F1	F2	F3	Nuc. ident.
Fibronectin	X02761	2	1	10.1	3.2	4.0	M18194	A	2.8	2.8	2.8	93%
RhoC	L25081	1	A	4.7	3.1	2.8	X80638	A	2.9	4.9	2.5	91%
Thymosin β4	M17733	X	1	3.3	3.6	3.5	W41883	1	4.1	3.5	3.5	92%
t-PA	K03021	8	A	5.2	9.6	5.2	J03520	A	A	A	A	81%
Angiopoietin1	D13628	8	1	4.3	9.4	3.3	U83509				*	
IEX-1/Glu96	S81914	6	1	9.1	3.3	4.5	X67644	1	0.4	0.6	0.5	83%
RTP/NDR1	D87953	8	1	8.6	5.4	4.7	U60593	1	A	0.7	1.5	86%
Fibromodulin	U05291	1	A	8.3	4.7	8.2	X94998	1	2.0	2.0	1.1	80%
Hsp70	M11717	1	1	7.8	4.2	5.0	M20567	1	2.1	1.8	1.8	80%
IL13 Rec., α2	U70981	X	1	7.6	2.9	3.1	U65747				*	
Sec61β	L25085	9	1	3.8	4.7	3.3					†	
snRNP, poly.pep. C	HG1322-	9	1	3.8	5.3	3.2					†	
Collagen Iα2	Z74616	7	A	2.5	3.6	3.6	X58251	A	3.1	2.3	3.7	86%
UBE21	U45328	16	1	3.6	3.4	3.4					†	
KIAA0156	D63879	16	1	3.6	3.4	3.4					†	
TGFβ superfamily	AB000584	19	1	3.4	3.4	3.0					†	
Surfactant protein C	J03890			*			M38314	A	32	12	16	
Lysozyme M				†			M21050	A	20	10	22	
Matrix Gla prot	X53331	12	1	3.2	4.4	1.1	D00613	1	12	11	5.4	81%
Tsa-1				†			U47737	A	9.7	6.1	7.2	
Collagen IIIα1	X06700	2	A	A	A	A	X52046	A	8.2	5.6	5.5	89%
Biglycan	J04599	X	A	A	3.7	A	L20276	A	3.8	4.4	6.9	87%
α-catenin	U03100	5	1.0	1.3	1.0	1.9	X59990	1	3.4	3.0	5.7	91%
Valosin-cont. prot.	AC004472			*			Z14044	1	3.0	3.9	5.9	
ERK-1	X60188	16	A	A	A	A	Z14249	1	2.6	2.6	3.0	85%
α-actinin 1							AA068062	1	3.6	3.3	7.3	
calmodulin							AA103356	A	4.8	6.7	5.5	
EIF4γ							AA002277	A	4.7	3.2	2.6	
α-centractin							W48490	1	2.9	3.8	3.6	
IQGAP1							AA118739	A	3.6	3.5	3.2	
cathepsin s							W13263	A	2.8	2.8	3.1	
EF2							W90866	1	2.6	2.5	2.9	

Genes whose expression is consistently enhanced >2.5-fold in pulmonary metastases (M1, M2, SM, F1, F2 or F3) compared to poorly metastatic cells (P or F0) grown as subcutaneous tumours. The values for P and F0 are the average of two experiments performed with subcutaneous tumours from two mice injected with A375P or B16F0 cells. Data are presented as fold expression compared with the poorly metastatic tumours. When expression was below baseline, the expression was marked as absent (A) and was arbitrarily set at 20. Mouse expressed sequence tags (ESTs) are noted in italics and are named according to the gene to which they show the greatest sequence similarity. Ch. no., human chromosome where the gene resides. Nuc. ident., percentage of nucleotides identical between the human and mouse homologues, as determined by BLAST search. The accession number is the GenBank entry from which the oligonucleotide probe sequences were drawn.
* Mouse or human gene homologue exists in the UNIGENE database but was not part of the oligonucleotide probe set.
† No gene homologue was found in the UNIGENE database.

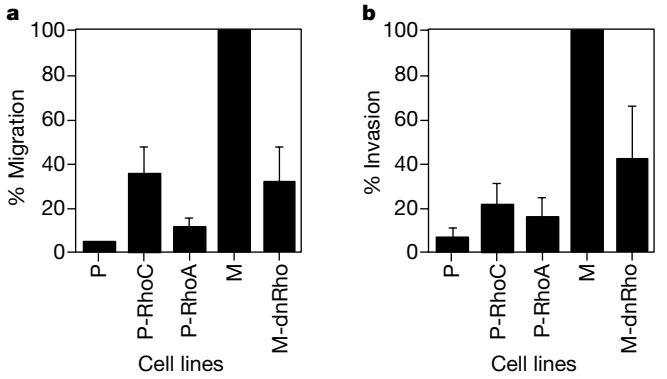

Figure 3 RhoC regulates melanoma cell chemotaxis and invasion. **a**, Poorly (P) or highly metastatic (M) A375 cells expressing rhoC, rhoA or dominant-negative (dn) rho were induced to migrate towards serum for 16 h. Over 25% of the plated A375M cells migrated within this time. Each bar represents the mean ± s.e.m. of four experiments done in duplicate. **b**, The cell lines described above were induced to invade matrigel-coated membranes for 48 h. Over 30% of the plated A375M cells invaded within this time. Each bar represents the mean ± s.e.m. of three experiments done in duplicate.

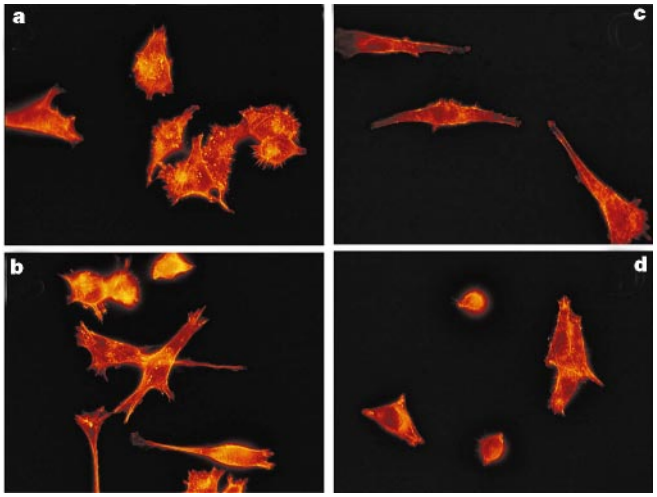

Figure 4 Metastatic capacity of A375 melanoma cells correlates with cell morphology. Poorly (**a**) or highly metastatic (**c**) A375 cells expressing rhoC (**b**) or dominant-negative rho (**d**) were plated on glass coverslips for 16 h at 37 °C, then fixed and stained with phalloidin to detect F-actin.

Several genes identified in other studies are conspicuous by their absence from our list. Metastasis suppressor genes, such as nm23, KiSS1 and CD82, can inhibit tumour metastasis[2]. In our study all three of these genes were absent in both the parental A375 tumours and in the metastases (see Supplementary Information), indicating that, although expression of these genes may inhibit metastasis, lack of their expression is not sufficient for metastasis. Other genes not found in Table 1 but whose expression correlated with melanoma metastasis in previous studies include the Met tyrosine kinase receptor, matrix metalloproteinases (MMPs) such as MMP2, and the β3-integrin subunit[20–22]. In the B16 tumours, Met expression was higher in two of the three metastases but its expression was not detected in any of the A375 tumours, indicating that its expression may not be essential for these tumours to metastasize. Expression of MMP2 and of the β3-integrin subunit was not significantly higher in any of the three metastases (see Supplementary Information), but their expression in both the parental and metastatic tumours may be sufficient to allow the tumour cells to metastasize.

Having uncovered 32 genes and ESTs whose expression patterns correlate with metastasis, we wished to investigate the function of one of these genes in this process. Because of its elevated expression in metastases derived from both tumour cell lines, RhoC was chosen to test the hypothesis that these expression studies identify genes essential for metastasis. The full-length human RhoC gene was cloned, subcloned into a retroviral vector and introduced into a retroviral packaging cell line. We used retroviral particles to infect the poorly metastatic A375P cells, and selected cells expressing high levels of RhoC by fluorescence-activated cell sorting (FACS). These cells, designated A375P-RhoC (expressing RhoC at 20 times the level expressed in A375M cells), were subjected to the experimental metastasis assay. As seen in Fig. 2c and Table 2, overexpression of RhoC markedly enhanced metastasis in this system.

Next we tested whether we could inhibit metastasis by expressing a known dominant-inhibitory Rho mutant (N19Rho)[23] in the highly metastatic A375M cells (a pool of M1, M2 and SM cells). This mutant is analogous to the N17Ras mutant that blocks Ras signalling[24]. Ras dominant-negatives are actually antagonists of the guanine-nucleotide exchange factors (GNEFs) for Ras, rather than of Ras itself[23] and it is believed that dominant-negative RhoA antagonizes Rho GNEFs, thereby inhibiting RhoC. Expression of N19RhoA in the A375M cells markedly suppressed the generation of metastases when these cells were subjected to the experimental metastasis assay (Fig. 2d and Table 2), indicating that Rho activity may be necessary, and RhoC sufficient, for metastasis in these melanoma lines.

Having established a causal role for RhoC in metastasis, we set out to investigate how RhoC might regulate the ability of tumour cells to metastasize. Tumour cells must complete a complex series of steps to metastasize, one of the most basic of which is cell growth. Rho GTPases affect several aspects of growth control[12], so it was possible that RhoC might control tumour metastasis by regulating cell proliferation. We assayed the A375P, A375P-RhoC, A375M and A375M-dnRho cells for *in vitro* proliferation and *in vivo* tumorigenesis (see Supplementary Information). Proliferation in either assay was not significantly changed by altering RhoC expression or Rho activity, indicating that RhoC may regulate metastasis by a mechanism other than controlling cell proliferation.

Another function of Rho-family GTPases is to control cytoskeletal organization in response to extracellular factors[12]. Cytoskeletal proteins are known effectors for events essential for cell motility[25]. Therefore, RhoC may control metastasis by regulating cell motility. Metastatic A375M cells were more migratory (Fig. 3a) and more invasive (Fig. 3b) than the poorly metastatic A375P cells. Furthermore, RhoC could enhance the migratory and invasive capacity of the A375P cells, whereas dnRho inhibited motility and invasion of the A375M cells, indicating that RhoC may regulate metastasis by controlling cytoskeletal events essential for motility. We also observed that RhoC could induce in A375P cells an elongated morphology similar to that of A375M cells, while dnRho expression suppressed this morphology (Fig. 4). Another morphological difference noted in the A375M cells, the serum-induced formation of filopodia, also correlated with the metastatic capacity of these cells. However, filopodial protrusions (which are regulated by the Rho subfamily member Cdc42 (ref. 12) were not altered by expression of RhoC or dnRho (data not shown), indicating that regulation of these actin-based structures may occur upstream (or independently) of Rho.

We have identified RhoC as essential for tumour metastasis. Compared with RhoA, the canonical family member, little is

Table 2 Pulmonary metastases

Cell line	No. of metastases	No. of mice
A375P	0,0,0,0,0,1,5,10	8
A375P-RhoC	56,70,>100,>100	4
A375M	all >100	8
A375M-dnRho	13,24,29,32	4

Numbers of pulmonary metastases identified on the surface of the lungs of mice injected with A375P, A375P-RhoC, A375M or A375M-dnRho cells.

known about RhoC. RhoA and RhoC are highly homologous, with only six non-conservative amino-acid substitutions, all in the carboxy-terminal end of the molecules. It might be thought that RhoA should be able to enhance tumour metastasis. However, RhoA is expressed at equivalent levels in both the poorly and highly metastatic tumours (see Supplementary Information), indicating that the level of RhoA expression in the A375 cells is not sufficient for metastasis. Furthermore, when expressed at equivalent levels, RhoC was a better motogen than was RhoA (Fig. 3). These results indicate that there may be a functional difference between Rho subfamily members that requires further investigation. Finally, the observation that expression of a single gene is sufficient to induce metastasis is perhaps surprising, given that metastasis is such a complex process. We suspect, however, that many cells within the heterogeneous tumorigenic A375P population may be genetically primed for metastasis so that introduction of a single gene (such as RhoC) which affects a process essential for metastasis is sufficient for metastasis. We are currently examining whether RhoC is capable of inducing metastasis in other tumorigenic cells. □

Methods

Cell lines

The A375 (ATCC#CRL-1619) and B16 (ATCC#CRL-6322) cell lines were maintained as described[7]. Cells were harvested by trypsinization, washed in PBS and diluted to 2.5×10^6 cells per ml for A375 cells and 2.5×10^5 cells per ml for B16 cells.

Experimental metastasis assay

A375 cells were injected either intravenously (0.2 ml) into the lateral tail vein or subcutaneously (0.1 ml) into the dorsal flank of nude mice, and B16 cells were injected into syngeneic C57BL/6 mice. Three (for B16) to eight (for A375) weeks after injection the mice were killed; the lungs were removed and washed and the pulmonary metastases on the lung surface were counted under a dissecting microscope. Metastatic nodules were removed aseptically, minced and grown *in vitro*, or snap-frozen in liquid nitrogen to purify RNA.

Tumours and tumour-derived cell lines

A375M1, M2 and SM lines were selected using the experimental metastasis assay for their enhanced ability to form experimental pulmonary metastases[6]. Line M1 was derived from metastases isolated from mice injected intravenously with the A375P cells, line M2 from mice injected with A375M1 cells, and line SM was a gift from I. Fidler and was derived by an identical selection procedure[7]. B16 lines were derived in an identical manner, with F1 cells derived from B16F0 cells, F2 from B16F1 cells and F3 from B16F2 cells. The A375M cell line is a pool of cells from A375M1, M2 and SM cells. A375P and A375M cells used in retroviral gene transfer studies were transfected with a plasmid containing the ecotropic receptor (a gift from H. Lodish) and selected for neomycin-resistance. Mock-infected or uninfected cells were used as negative controls in the metastasis assays.

Array hybridization

Total RNA was prepared with a Qiagen RNeasy mini-kit according to the manufacturer's instructions. We prepared cRNA for hybridization essentially as described[26]. Oligonucleotide arrays (GeneChip, Affymetrix) composed of 7,070 human (HUM 6.8K) or 6,347 mouse (MUR 6K) genes and ESTs were used for hybridization according to the manufacturer's instructions. Arrays were scanned using an Affymetrix confocal scanner and analysed using GeneChip 3.0 software (Affymetrix). Intensity values were scaled so that the overall fluorescence intensity of each chip of the same type was equivalent. For a gene to be selected as induced, it has to be expressed in all three metastatic samples at least 2.5 times higher than in the poorly metastatic sample, with experiments done in duplicate. Where expression in the poorly metastatic sample was below baseline (set at 20, the point below which changes in expression could be determined with high confidence), it was determined to be absent and was set to 20.

Retroviral gene transfer

An *Eco*R1 fragment of pCR-BluntII-RhoC containing the entire coding region of human RhoC (see Supplementary Information) was inserted into the *Eco*R1 site of the retroviral bicistronic expression vector pMX-IRES-GFP (pMIG; ref. 27) containing enhanced green fluorescent protein (GFP) as an expression marker. An *Eco*R1 fragment of pEXV-RhoA or pEXV-N19RhoA (a dominant-negative Rho mutant, dnRho) was inserted into the *Eco*R1 site of pMIG. We transfected pMIG-RhoC, pMIG-RhoA and pMIG-dnRho into 293T cell-derived retroviral producer lines (Phoenix cells) as described (see www.stanford.edu/group/nolan/) and A375P or M cells were infected. Cells were sorted by FACStar (Becton-Dickinson) according to their GFP levels. RNAse protection assays showed that RhoC was expressed at 5- to 50-fold higher levels than in the A375M cells, whereas RhoC and RhoA were expressed at similar levels in the A375P-RhoC and A375P-RhoA cells (as determined by GFP expression).

Chemotaxis and invasion assays

Cell migration and invasion assays were performed using 8.0-μm pore size Transwell inserts (Costar Corporation) or Biocoat Matrigel invasion chambers (Becton-Dickinson), respectively[28]. Each data point represents the average of 3 or 4 individual experiments, done in duplicate, and error bars represent the standard error of the mean.

Immunofluorescence

Adherent cells were fixed, permeabilized and stained as described[29].

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