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Rap1GAP regulates renal cell carcinoma invasion

Wan-ju Kim^a, Zachary Gersey^a, and Yehia Daaka^{a,b}

^aDepartment of Urology and Prostate Disease Center, University of Florida College of Medicine, Gainesville, Florida

^bDepartment of Anatomy and Cell Biology, University of Florida College of Medicine, Gainesville, Florida

Abstract

Although patients with localized and regional kidney tumors have a high survival rate, incidence of mortality significantly increases for patients with metastatic disease. It is imperative to decipher the molecular mechanisms of kidney tumor migration and invasion in order to develop effective therapies for patients with advanced cancer. Rap1, a small GTPase protein, has been implicated in cancer cell growth and invasion. Here, we profile migratory and invasive properties of commonly used renal cell carcinoma (RCC) cell lines and correlate that with expression and function of the Rap inactivator Rap1GAP. We report that levels of Rap1GAP inversely correlate with invasion but not migration. We also report that forced over-expression of Rap1GAP decreases invasion of RCC cells but does not impact their rate of proliferation. Low expression levels of Rap1GAP in RCC cells are due, at least in part, to promoter hypermethylation. Rescued expression of Rap1GAP with a demethylating drug, decitabine (5-azadC), decreases the RCC SN12C cell invasion of collagen, fibronectin, and Matrigel matrices. RCC cell lines express distinct levels of cell adhesion proteins and the forced over-expression of Rap1GAP attenuated levels of both cadherins and integrins that are known to regulate the cancer cells invasion. These results demonstrate that targeted restoration of Rap1GAP expression may serve as a potential therapeutic approach to reduce metastasis of kidney cancers.

Indexing terms

renal cell carcinoma; invasion; Rap1GAP; promoter methylation

1. Introduction

According to the American Cancer Society, an estimated 60,920 individuals will be diagnosed with tumors of the kidney and 13,120 will die from the disease in 2011 in the USA, accounting for 3.8% of new cancer cases and 2.3% of cancer related deaths [1]. As

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Corresponding author: Yehia Daaka, Tel: 352-273-8112; Fax: 352-273-8335; ydaaka@ufl.edu.

Author Contributions

W.K. and Y.D. designed the experiments. W.K. and Z.G. performed all experiments in this work. W.K., Z.G. and Y.D. wrote the paper.

Competing Interest Statement

The authors declare no competing financial interests.

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such, kidney cancer is the third most common genitourinary tumor type and the vast majority of these cases are classified as renal cell carcinomas (RCC). The five major subtypes of RCCs are clear cell, papillary, chromophobe, oncocytoma, and angiomyolipoma--all of which are stratified based on histopathologic appearance [2]. Although surgical excision of organ- confined tumors is an effective form of treatment, many patients relapse because of the metastatic ability of this cancer. Patients with metastatic RCC have a relatively low survival rate of less than 10% at 5 years of disease [3]. Hence, discovery of therapeutic methods that decrease metastasis is expected to reduce patients' morbidity and mortality.

No biomarkers for RCC have been approved for routine clinical use, although several molecules have been suggested as a potential solution. For example, mutation of the von Hippel-Lindau tumor suppressor gene was shown to be a trademark of the histologic subtype clear cell RCC. Also, several components of the mTOR pathway have been recognized as potential biomarkers for RCC, such as the expression of pS6K and phosphorylated Akt [4]. In the case of metastatic RCC, only a few biomarkers have been proposed. As an example, reduced expression of carbonic anhydrase IX correlates with the development of the metastatic stage [5]. Rap is a small GTPase protein that is involved in cancer growth, invasion, and metastasis [6; 7].

Rap cycles between active Rap•GTP and inactive Rap•GDP. Inactivation of Rap (i.e. via the hydrolysis of Rap-bound GTP to GDP) is carried out by members of the Rap GTPase-activating protein (GAP) family, which have been documented to regulate integrin-mediated cell adhesion pathways [8]. Available evidence indicates that Rap1GAP is underexpressed in several types of cancer. Rap1GAP down-regulation has been shown to arise as a consequence of proteasomal degradation in thyroid cancer [9], loss of heterozygosity in papillary thyroid and pancreatic cancers [10], promoter methylation in melanoma and thyroid cancer [9; 11], and genomic mutation in breast cancer [12]. Hence, several mechanisms account for the Rap1GAP expression and they vary among cancer types.

Although down-regulation of Rap1GAP is frequent in human tumors, the biological significance of decreased Rap1GAP expression has only been studied in a few cancer cell lines. In head and neck squamous cell carcinoma, Rap1GAP inhibits tumor growth by delaying the G1/S transition of the cell cycle [13]. Rap1GAP also inhibits activity of extracellular signal-regulated kinase, cell proliferation, survival, and migration of melanoma cells [11]. Evidence has been presented to show that reintroduction of Rap1GAP in cancer cell lines may alleviate some cancerous traits. For example, forced over-expression of Rap1GAP in oropharyngeal squamous [13] and pancreatic [10] carcinoma cell lines blocks tumor formation in animal models. *In vitro*, over-expression of Rap1GAP impairs tumor cell proliferation [10; 11; 13; 14; 15] and enhances apoptosis [10; 11; 14].

Recently, we showed that forced over-expression of the GAP domain of Rap1GAP impairs the pro-inflammatory prostaglandin E2-mediated invasion of RCC7 renal carcinoma cells [6]. We also showed that expression of Rap1GAP protein varies among the commonly used kidney cancer cell lines. These results, along with knowledge of the roles of Rap1GAP in cell adhesion pathways, led us to hypothesize that down-regulation of Rap1GAP may serve to increase the cancer cell migration and invasion. Our findings show epigenetic-regulated Rap1GAP expression: *rap1gap* promoter appears to be hypermethylated and restoration of its expression with the demethylating agent decitabine reduces the RCC cell invasion. These results suggest that the rescued expression of Rap1GAP may serve as a therapeutic approach to decrease Rap1 activity, thereby prompting reduction of kidney cancer cell invasion.

2. Materials and Methods

2.1. Cell culture and reagents

Caki-1, TK10, SN12C and 786-0 RCC cell lines were obtained from the National Cancer Institute (NCI). The cells were maintained in DMEM or MEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Cellgro) in a humidified incubator containing 5% CO₂ at 37°C. Antibodies were obtained as follows: anti-Rap1GAP, anti-HSP70, anti-Stat3, anti-β-catenin, and anti-EGFR from Santa Cruz Biotechnology (Santa Cruz, CA), anti-E-cadherin and anti-N-cadherin from BD Biosciences (Bedford, MA), anti-GAPDH from Sigma (St. Louis, MO), and anti-integrin sampler kit from Cell Signaling Technology (Danvers, MA). Diff-Quick Stain kit was purchased from Siemens Healthcare Diagnostics (Newark, DE).

2.2. Cell transfection

For stable over-expression of Rap1GAP, a full-length cDNA encoding human Rap1GAP gene cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen) was used. Prior to transfection, Rap1GAP low-expressing Caki-1 and SN12C cells [6] were seeded at a density of 2×10^5 cells per well in 6-well tissue culture plates. Cells in serum-free Opti-MEM medium (Invitrogen) were transfected with 4 µg of pcDNA-Rap1GAP or empty vector pcDNA3.1 and 10 µl of Lipofectamine 2000 reagent (Invitrogen). The transfected cells were seeded into 100-mm culture plates, selected with G418 at 600 µg/ml for 3 weeks, and maintained with G418 at 200 µg/ml.

For small interfering RNA (siRNA) transfection, oligonucleotides targeting Rap1GAP were synthesized by Dharmacon (Lafayette, CO). The siRap1GAP sequences used were 5'-CAA UGU GGA UCG GUU CUA U-3', 5'-GAC CGA AUC UGU GUAC UGC-3', 5'-GCA AGG AGC AUU UCA AUU A-3' and 5'-CAG AGG CGC UCA AGG ACU U-3'. Oligonucleotides were transfected into decitabine-treated Caki-1 or SN12C cells using Lipofectamine RNAiMax reagent according to the manufacturer's protocol (Invitrogen). In brief, 50–70% confluent cells were incubated with a mixture of 5 µl Lipofectamine RNAiMAX reagent and 100 nM siRNA for 48 hrs. Cells were then subjected to invasion and Western blot assays, as described.

2.3. Cell migration and invasion

Cell migration assay was performed using the Boyden chamber containing a membrane with a 8 µm pore size (BD Bioscience). Cell invasion studies were also done using the Boyden chamber but membranes were pre-coated with collagen (200 µg/ml; Roche), fibronectin (100 µg/ml; Sigma) or Matrigel (1 mg/ml; BD Bioscience) matrices. In both cases, cells were seeded in wells at a density of 0.2×10^5 cells/100 µl in DMEM (Caki-1) or MEM (SN12C, 786-0, TK-10) medium containing 0.2% (v/v) FBS in the upper chamber. In the lower chamber, 650 µl of DMEM or MEM media containing 0.2% (i.e. random movement) or 10% (i.e. directed movement) FBS were added. After 22 hrs of incubation at 37°C in a 5% CO₂ incubator, the chamber was removed, fixed, and stained with Diff-Quik. Cells in the upper chamber were removed using a cotton swab. Cells migrating through the membrane and cells invading the matrix were photographed in 4 randomly-selected fields and counted using ImageJ software (NIH).

2.4. Cell proliferation

Cells were seeded at 1,000 cells/well in 96-well plates in DMEM (Caki-1) or MEM (SN12C) medium supplemented with 10% (v/v) FBS. At the indicated time, 20 µl of MTT reagent were added to each well and the plates were incubated at 37°C for 4 hrs, followed by

measurement of absorbance at 570 nm using a microplate spectrophotometer. Each independent experiment represents at least four separate measurements.

2.5. Western blot analysis

Cell monolayers were washed with PBS and harvested in RIPA buffer containing protease and phosphatase inhibitors, as we previously described [16]. Equal amounts of protein were resolved in SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). All antibodies were diluted in 5% skim milk-TBST and were used at the following dilutions: anti-E-cadherin and anti-N-cadherin at 1:500; anti-Rap1GAP, anti- β -catenin, anti-HSP70, anti-EGFR, anti-Stat3, and anti-integrin at 1:1,000; and anti-GAPDH at 1:10,000. Secondary anti-mouse or anti-rabbit antibodies were used at 1:20,000 dilutions and filters were developed using SuperSignal West Pico reagent (Thermo, IL).

2.6. Reverse transcription and real-time PCR

Total RNA was isolated using TRIzol (Invitrogen). The cDNA was synthesized using SuperScript III FIRST Strand synthesis kit (Invitrogen). For quantification of mRNA expression, real-time PCR was performed using the following primer pairs: Rap1GAP, 5'-GCA CTT TCT CGG CAA GGA GCA TTT-3' (forward) and 5'-TGA CAT CAT GGT ATG TCC GGC ACT-3' (reverse); GAPDH, 5'-TCG ACA GTC AGC CGC ATC TTC TTT-3' (forward) and 5'-ACC AAA TCC GTT GAC TCC GAC CTT-3' (reverse). Real-time PCR was performed with the BioRad Thermocycler (iQ5) using SYBR green reagents (BioRad), according to the manufacturer's instructions. The mRNA expression was calculated using the comparative threshold cycle method and normalized against expression of GAPDH [6; 16].

2.7. DNA demethylation

Genomic DNA was extracted from the human kidney cancer cell lines using TRIzol (Invitrogen). DNA bisulfite modification was followed by EZ DNA methylation (Zymo Research; Irvine, CA). Briefly, 500 ng of denatured genomic DNA was treated with sodium bisulfite at 50°C for 16 hrs in a dark water bath. Samples were then transferred to columns, which were washed, desulfonated, followed by DNA elution. A total of 2 μ l of demethylated DNA were used in methylation-specific PCR (MSP) amplification, as described [9]. MSP primers specific for the methylated (M) Rap1GAP gene were the 74A forward primer: 5'-TTT GGG TTC GGT ATT TTG GTG TTC-3' and reverse primer: 5'-ACG CTC CGC GAT CAT ATA ACG T-3'; the 74B forward primer: 5'-CGA GTT TAG TTG CGT TTA TTT TTC-3' and reverse primer: 5'-ATA TCC AAA CTC CCG TCG AC-3'; and the 24 forward primer: 5'-TAG AGA TAA AGT TTA AGA GTC GCG A-3' and reverse primer: 5'-TTC TAA ATC AAA TAA AAA CGT CGA A-3'. MSP primers specific for the unmethylated (U) Rap1GAP gene were the 74A forward primer: 5'-TTT TGG GTT TGG TTT TTG GTG TTT-3' and reverse primer: 5'-AAC ACT CCA CAA TCA TAT AAC ATC CC-3'; the 74B forward primer: 5'-TGA GTT TAG TTG TGT TTA TTT TTT G-3' and reverse primer: 5'-AAA ATA TCC AAA CTC CCA TCA AC-3'; and the 24 forward primer: 5'-TAG AGA TAA AGT TTA AGA GTT GTG A-3' and reverse primer: 5'-TTC TAA ATC AAA TAA AAA CAT CAA A-3'. MSP reactions were performed with primer sets specific to methylated or unmethylated Rap1GAP and *Taq* PreMix (Zymo Research) under the following conditions: 95°C for 10 min, followed by 37 cycles at 95°C for 20 sec, 55°C for 30 sec, and 72°C for 40 sec. The final extension step was performed at 72°C for 7 min. The PCR products were loaded onto 1.5% agarose gel and visualized under UV light.

2.8. Statistical analysis

Statistical analysis was performed using Student's *t*-test. For comparisons between control and treatments, significance was determined using one way ANOVA test followed by Tukey. All values are presented as mean $\pm$ SE. Significance was determined at $P < 0.05$.

3. Results

3.1. Cell migration and invasion

Cancer metastasis may be experimentally modeled using *in vitro* cell migration and invasion assays. Generally, cancer cells with metastatic potential exhibit increased migratory and invasive behavior. We compared the ability of commonly studied kidney cancer cell lines to migrate and invade solid matrices. RCC cells in a serum-free medium were placed on membranes that were either uncoated (migration) or coated (invasion) with collagen, fibronectin, or Matrigel. After 22 hrs of incubation, the cells that transmigrated to the undersurface of the membrane were counted. The results show that the RCC cells were capable of motility onto both uncoated and coated surfaces. The results show that 786-0 cells migrate most efficiently and SN12C cells migrate least efficiently with Caki-1 and TK10 cells migrating intermediately (Fig. 1A and 1B).

While collagen, fibronectin, and Matrigel permit cell invasion, there were significant differences in the cancer cell invasion on the three matrices. The RCC cell invasion on fibronectin-coated transwells showed more cells invading than on collagen- or Matrigel-coated membranes (Fig. 1C and D). Cells that invaded on the fibronectin-coated membranes were highly spread-out and formed confluent monolayers. In contrast, cells that invaded on collagen- or Matrigel-coated membranes were less spread-out but had a more elongated, motile appearance (Fig. 1C). The 786-0, Caki-1, and TK10 cells migrated significantly more than SN12C cells on uncoated surfaces (Fig. 1A). The results also showed that the opposite is true for the invasion of collagen, fibronectin and Matrigel matrices (Fig. 1C); SN12C cells invaded most. Hence, there is a lack of correlation between these RCC cells migration and invasion.

3.2. Decitabine restores Rap1GAP DNA hypomethylation and increases gene expression in RCC cells

The small GTPase Rap is involved in cell migration and invasion [6; 7]. Rap1GAP is an inactivator of Rap, and we recently showed that Rap1GAP expression varies among RCC cell lines [6], albeit by undetermined mechanisms. We studied possible mechanisms involved in the Rap1GAP expression. The promoter of *rap1gap* contains CpG islands [9; 11]. We hypothesized that hypermethylation of these CpG islands leads to the silencing of the *rap1gap* in the RCC cell lines. To confirm this idea, SN12C cells were incubated for 24 hr intervals with 3 μ M of the demethylating agent decitabine (5-aza-2'-deoxycytidine (5-AzacC)) three times for a total of six days. Methylation Specific PCR (MSP) analysis revealed that the *rap1gap* promoter is hypermethylated (Fig. 2A). Treatment with decitabine induced specific hypomethylation of the *rap1gap* promoter and demonstrated that all three CpG islands (74A, 74B, and 24) were methylated in the SN12C cell line (Fig. 2A). However, the methylation level of primer 24 was significantly higher compared to primers 74A and 74B. In the SN12C cells, we could detect both unmethyl- and methyl-specific PCR products using primers 74A and 74B, although the methylation patterns were different (Fig. 2A). Furthermore, quantitative PCR analysis confirmed that the *rap1gap* is methylated in additional kidney cancer cells lines (data not shown), and this gene was significantly demethylated in these cells following treatment with decitabine. Real-time PCR analysis showed that the relative expression level of *rap1gap* gene significantly increased after the decitabine treatment; SN12C cells treated with decitabine exhibited a 2-fold increase in

rap1gap compared to the untreated sample (Fig. 2B). This suggests that *rap1gap* is silenced via promoter methylation in these kidney cancer cells.

To examine Rap1GAP protein expression, SN12C cells were cultured in the presence or absence of decitabine. Cell lysates were fractionated on SDS-PAGE, transferred to nitrocellulose filters and immunoblotted with anti-Rap1GAP antibodies. The results (Fig. 2C) show that basal levels of Rap1GAP were detected in the lysate of untreated cells, which is consistent with previous reports [6]. However, SN12C (Fig. 2C) and other RCC cells (data not shown) cultured with decitabine for 3 days showed stronger expression levels of Rap1GAP protein. These results indicate that the RCC cells analyzed in this study display reduced Rap1GAP expression and that this defect can be restored with decitabine treatment.

To link the effect of *rap1gap* promoter methylation to invasiveness of the kidney cancer cells, SN12C cells were treated with decitabine and examined for their ability to invade collagen, fibronectin, and Matrigel matrices. Treatment with decitabine that restored expression of Rap1GAP (Fig. 2C) evidenced a significant reduction in the invasiveness of the SN12C cells (Fig. 2D). SN12C cell invasion of collagen, fibronectin and Matrigel was decreased to about 65% compared to the control cells (Fig. 2D). To directly link actions of decitabine and the levels of Rap1GAP expression, cells were treated with sequentially decitabine and siRNA. Expectedly, the treatment with decitabine restored the expression of Rap1GAP protein which was effectively knocked down with siRap1GAP (Fig. 2E). Remarkably, the decitabine-induced decrease of SN12C (Fig. 2F) or Caki-1 (data not shown) cell invasion of Matrigel was reversed in response to the knockdown of Rap1GAP expression. These results are supportive of the idea that restoration of Rap1GAP expression may impede the cancer cell invasion.

3.3. Forced expression of Rap1GAP decreases invasion but does not affect proliferation of kidney cancer cells

Rap1GAP expression is undetectable in the Caki-1 and SN12C cells. To provide a direct link between Rap1GAP expression and invasiveness, Caki-1 and SN12C cells were transfected with Rap1GAP. Stable clones were selected over a three week period using G418. Several clones were isolated and screened for Rap1GAP expression. The results from representative clones (Caki-1 #2 and Caki-1 #5; SN12C #1, SN12C #4 and SN12C #31) revealed levels of Rap1GAP over-expression (Fig. 3A). Empty-vector expressing cells were used as control and they did not show the Rap1GAP expression.

The effect of Rap1GAP over-expression on cell invasion was investigated using the stably transfected Caki-1 and SN12C cells. Results illustrate a significant invasion decrease in the cells over-expressing Rap1GAP as compared to control cells stably expressing empty vector (Fig. 3B). Specifically, Caki-1-Rap1GAP clones evidenced a 50% decrease and SN12C-Rap1GAP clones a 75% decrease in their invasive ability of collagen, fibronectin and Matrigel matrices, in comparison to the corresponding control cells (Fig. 3B). The over-expression of Rap1GAP had no effect on cell proliferation (Fig. 3C). Hence, it appears that Rap1GAP impacts the Caki-1 and SN12C cells invasion and not proliferation.

3.4. Effect of Rap1GAP on cadherin and integrin expression

Cancer cell migration and invasion is dependent upon, among other things, expression levels of extracellular attachment molecules. We examined the effect of Rap1GAP on expression levels of cadherin and integrin proteins. Predictably, the various RCC cell types expressed distinct levels of cadherin and integrin proteins (Fig. 4A), implying that different proteins mediate the attachment and migration of the distinct RCC cells. Remarkably, the results show that increased expression of Rap1GAP yielded a decrease in the expression of both E-

cadherin and N-cadherin (Fig. 4B), and similar results were obtained in response to the treatment of cells with decitabine (Fig. 4C). Moreover, a significant increase in the levels of integrin $\alpha 5$ was seen in both Caki-1 and SN12C cells with over-expression of Rap1GAP compared to the control cells (Fig. 4D). In Caki-1 cells, a decrease in integrins αV , $\beta 1$, $\beta 3$, and $\beta 4$ was shown in the Rap1GAP-expressing cells (Fig. 4D). In SN12C cells, integrin $\beta 1$ levels increased in Rap1GAP-expressing cells, and integrins αV , $\beta 3$, and $\beta 4$ levels were undetectable in control and Rap1GAP-expressing cells (Fig. 4D). Treatment with decitabine also impacted expression of the integrins (Fig. 4E) and, in general, there was a concordant effect of forced over-expression of Rap1GAP and treatment with decitabine (Fig. 4D, Fig. 4E) on the expression levels of the integrin proteins.

We also determined the effect of Rap1GAP over-expression on the levels of other molecules involved in cancer cell attachment and invasion. In Caki-1 and SN12C cells, β -catenin and Stat3 expression levels decreased, whereas EGFR levels remained relatively unchanged in the Rap1GAP-expressing cells in comparison to control cells (Fig. 4F), and consistent results were observed in cells treated with decitabine (Fig. 4G). These results suggest that Rap1GAP may impact the RCC cell migration and invasion by affecting the expression levels of multiple regulatory proteins.

4. Discussion

Advances in methodologies used to detect solid tumors evidence increased incidence of kidney cancer. Although organ-confined kidney tumors can be successfully resected with partial or total nephrectomy, management options of locally advanced or metastatic disease are few and not curative. Hence, there is an urgent need to identify mechanisms involved in the local invasion and consequent dissemination of the cancer cells to distal organs as a prerequisite to discover effective therapies. Here, we have evaluated the ability of commonly-used human RCC cell lines to migrate and invade solid matrices, and investigated involved mechanisms. The major finding of this study is that abilities of individual RCC cell lines to migrate and invade are distinct and not overlapping. Also, Rap1GAP whose expression is down-regulated in RCC cells impedes the cancer cells' ability to invade solid matrices. Restoration of Rap1GAP expression with the demethylating agent decitabine impairs the directed RCC cell invasion, suggesting possible utility of decitabine as a therapeutic option to treat patients diagnosed with advanced kidney cancer.

Cell migration and cell invasion assays are commonly used in basic science research in an interchangeable manner. In the case of RCC, our results show that each cell line has a distinct potential to migrate and invade supporting matrices; 786-0 cells migrate most efficiently, Caki-1 and TK10 cells migrate intermediately, and SN12C cells migrate least efficiently. The four RCC cell lines exhibited different invasion efficiency on collagen, fibronectin, and Matrigel matrices. The RCC cell lines invaded more efficiently in fibronectin than in collagen or Matrigel. Also, SN12C cells invaded the most in the fibronectin matrix in comparison to the other RCC cells. These results point to a distinct migratory and invasive characteristic of the individual RCC cell lines that may arise from having different expression patterns of cell attachment proteins.

Rap1GAP is widely down-regulated in RCC cell lines [6]. Metastasis of cancer cells is dependent upon invasive properties and we investigated whether Rap1GAP over-expression in RCC cell lines impacts their invasion behavior. The results show that forced over-expression of Rap1GAP significantly reduces the cell invasion of collagen, fibronectin and Matrigel, three commonly used matrices. Rap1GAP expression is down-regulated in several human tumors, such as thyroid carcinoma and oral cancer [9; 11; 13]. The expression level of Rap1GAP is significantly less in invasive colorectal lesions compared to benign lesions

[15]. In Papillary thyroid cancer cells, over-expression of Rap1GAP impaired not only the invasion, but also the cell proliferation [9]. Remarkably, the over-expression of Rap1GAP in SN12C and Caki-1 RCC cell lines showed no significant effect on their proliferative rates, suggesting Rap1GAP may exert cell type- and context-dependent functions.

DNA promoter methylation analysis of the *rap1gap* gene in Caki-1 and SN12C cells revealed that the DNA promoter is hypermethylated, providing an explanation for reduced Rap1GAP expression. In support of this conclusion, treatment with decitabine restored the expression of Rap1GAP in the kidney cancer cells. Our results show that treatment with decitabine significantly increases Rap1GAP expression at both the gene and protein levels. Concordantly, the treatment with decitabine led to a substantial decrease in the RCC cells invasion of collagen, fibronectin, and Matrigel matrices. These results support the conclusion that Rap1GAP plays a critical role in RCC cell invasion and suggest use of decitabine as a therapeutic to treat patients with advanced kidney cancer.

Most human tumors are derived from epithelial tissues that often lose attachment molecules as they progress towards malignancy. E-cadherin is important in maintaining cell-cell contact [17], and loss of E-cadherin expression has been proposed to be an initial phase in the invasive and metastatic cascade of cancer cells [18; 19; 20]. Our results show that E-cadherin is expressed at relatively low levels in RCC cells. On the other hand, expression levels of the related cadherin molecule N-cadherin associate with cancer cell invasion and are used as a marker for RCC [21]. Our results show that Rap1GAP controls the expression of both E-cadherin and N-cadherin; the over-expression of Rap1GAP in SN12C and Caki-1 RCC cells decreases levels of both cadherins. This is distinct from the epithelial to mesenchymal transition results that we expected, where E-cadherin would decrease and N-cadherin would increase. Nonetheless, lack of concordance between E-cadherin and N-cadherin expression levels and cancer cell invasion has been reported for other cancer types. The data also show that over-expression of Rap1GAP affects levels of several cell-adhesion integrins. Previous results show that integrin $\alpha 5$ is a growth-suppressing integrin and integrin αV increases growth potential of cells [22]. Our results show that cells over-expressing Rap1GAP have higher expression levels of integrin $\alpha 5$ and lower expression levels of integrin αV compared to control cells, implying a tumor suppressor role of Rap1GAP. Also, the mechanism by which increased expression of Rap1GAP affects integrins $\beta 1$, $\beta 3$, and $\beta 4$ expression and the role these integrins play in metastasis may prove to be an interesting area of future investigation.

In addition to impacting the expression levels of structural/attachment proteins, the modulation of Rap1GAP expression levels affected expression of signaling proteins, including β -catenin and signal transducer and activator of transcription 3 (Stat3). Specifically, the over-expression of Rap1GAP decreased expression of β -catenin and Stat3 proteins, but had little effect on the expression of epidermal growth factor receptor or heat shock protein 70, implying the specific cellular response. Recent studies suggest that increased β -catenin and Stat3 levels correlate with tumor progression [23; 24; 25], thereby providing additional support to the idea that Rap1GAP exerts tumor suppressor effects.

In conclusion, our results show that human RCC cell lines exhibit distinct and non-overlapping migratory and invasive characteristics. Previous work from our laboratory has shown that RCC cell invasion is linked to the expression levels of Rap1GAP. In this study we show that the reduced Rap1GAP expression in RCC cells is due to promoter hypermethylation and that treatment with a demethylating agent, decitabine, rescues Rap1GAP expression and decreases the RCC cell invasion. Collectively, these results give support to the idea of using demethylating agents, including decitabine as drugs to treat patients with metastatic kidney cancer.

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Fig. 1.

Migration and invasion characteristics of human RCC cells. Representative photomicrograph (A) and quantitative cell number (B) of RCC cells migrated onto transwell. Cells were grown in media containing 0.2% FBS and allowed to migrate through an 8 μ m pore size membrane towards 0.2% FBS containing media. Representative photomicrograph (C) and quantitative cell number (D) of RCC cells invading through an 8 μ m pore size membrane precoated with collagen, fibronectin, or Matrigel. Cells were grown in media containing 0.2% FBS and allowed to invade towards 0.2% FBS containing media. After 24 hr, cells on the bottom side of membranes were stained with Diff-Quick, washed and photographed through a Leica DMI 4000B inverted microscope. For results shown in panels B and D, each point represents the mean $\pm$ S.E.M. of values obtained from at least three independent experiments performed in duplicate.

Fig. 2.

Effect of decitabine on promoter methylation and expression of Rap1GAP. SN12C cells were treated with decitabine (5 μ M) on 3 alternate days for a total of 6 days. Genomic DNA, total RNA and total protein were isolated, as described. (A) Methylation specific PCR analysis of CpG island methylation in the *rap1gap* promoter region. M, methylated; U, unmethylated. (B) Real-time RT-PCR analysis of *rap1gap* mRNA levels. *gapdh* was used as an internal control. (C) Western blot analysis of Rap1GAP protein. Filters were stripped of antibodies and reprobed using anti-GAPDH antibody to demonstrate equal protein loading among the samples. (D) Effect of decitabine treatment on SN12C cell invasion. Cells were treated with decitabine as indicated above for A-C and allowed to invade through an 8 μ m pore size membranes precoated with collagen, fibronectin, or Matrigel towards a 10% FBS containing media. Following 24 hr incubation, invading cells were stained with Diff-Quick, washed and photographed. (E) Knockdown of Rap1GAP expression. SN12C cells were treated with decitabine as above then transiently transfected with control (siCon) or Rap1GAP (siRap1GAP) siRNAs. Western blot analyses were performed 2 days after transfection. (F) Knockdown of Rap1GAP restores invasive ability of SN12C cells treated with decitabine. For results shown in panels B, D and F, each point represents the mean $\pm$ S.E.M. of values obtained from at least three independent experiments performed in duplicate. * $P < 0.05$ versus corresponding control not-treated samples.

Fig. 3.

Effects of Rap1GAP over-expression on RCC cell invasion and growth. (A) Rap1GAP expression levels. Caki-1 and SN12C cells were transfected with control pcDNA3.1 vector (EV) or pcDNA-Rap1GAP and treated for 3 weeks with G418 (400 μ g/ml). Individual clones were selected and analyzed by Western blot for expression of Rap1GAP. Filter was stripped of antibodies and re-probed with anti-GAPDH antibody to demonstrate the equal protein loading. (B) Invasion assay of Rap1GAP-expressing Caki-1 and SN12C clones of collagen, fibronectin and Matrigel. Cells were allowed to invade through an 8 μ m pore size membrane precoated with collagen, fibronectin, or Matrigel towards 10% FBS-containing media. After 24 hr incubation, invading cells were stained with Diff-Quick, washed and photographed. Each point represents the mean $\pm$ S.E.M. of values obtained from at least three independent experiments performed in duplicate. * $P < 0.05$ and ** $P < 0.01$ versus corresponding control empty vector (EV) transfected cells. (C) Growth rate of Caki-1 and SN12C cells expressing empty vector (EV) or Rap1GAP. Cells (1,000 cells/well) were allowed to grow in medium containing 10% FBS for 1, 2, 3, 4, and 5 days. Cell growth was analyzed using MTT assay, and each point represents the mean $\pm$ S.E.M. of values obtained from at least three independent experiments performed in quadruplicate.

Fig. 4.

Effect of Rap1GAP on protein expression. (A) Expression of attachment proteins in RCC cells. Cell lysates were subjected to Western blot analysis using the indicated antibodies. (B) Expression of Cadherin in Caki-1 (EV), Caki-1 #5, SN12C (EV) and SN12C #31. Cell lysates were used to estimate effect of Rap1GAP over-expression on E-Cadherin and N-Cadherin proteins by Western blot. (C) Effect of decitabine treatment on the expression of Cadherin in Caki-1 and SN12C cells. Cells were treated with decitabine (5 μ M) on 3 alternate days for a total of 6 days. Cell lysates were subjected to Western blot analysis to document expression of N- and E-Cadherin proteins. (D) Expression of integrin in Caki-1 (EV), Caki-1 #5, SN12C (EV) and SN12C #31. Cell lysates were used to estimate effect of Rap1GAP over-expression on α 5, α V, β 1, β 3, and β 4 proteins. (E) Effect of decitabine treatment on the expression of integrin proteins in Caki-1 and SN12C cells. Cells were treated with decitabine as above and subjected to Western blot analysis to show expression of the integrin proteins. (F) Expression of select signaling proteins in Caki-1 (EV), Caki-1 #5, SN12C (EV) and SN12C #31. Cell lysates were used to estimate effect of Rap1GAP over-expression on β -catenin, EGFR, HSP70 and Stat3 proteins. (G) Effect of decitabine treatment on expression of select signaling proteins. Caki-1 and SN12C cells were treated with decitabine as above and subjected to Western blot analysis using the indicated antibodies. For all panels, β Actin or GAPDH expression was used as a loading control.