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14. ABSTRACT Once breast cancer is diagnosed, the most critical question is whether the disease is localized or has it already metastasized to other organs. However, the molecular basis of tumor metastasis is poorly understood as yet. The major goal of this project was to elucidate the function of the tumor metastasis suppressor gene, Drg-1/NDRG1. Toward this goal, we have set three specific aims; (i) to clarify the role of the NDRG1 gene in the progression of breast cancer(Task 1), (ii) to clarify the control mechanism of the NDRG1 gene by PTN (Task 2), and (iii) to evaluate diagnostic/prognostic value of NDRG1 in breast cancer (Task 3). We have successfully accomplished Task 1 and Task 3. Task 1 especially generated exciting data that provide several promising future directions to further study the function of NDRG1. Task 2 is still ongoing and we plan to continue our effort on this task even after completion of this grant.					
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This is the revised final report for our project. We had submitted our original report but it was disapproved because (i) the report was not “comprehensive in providing a complete record of the research findings for the reporting period”, and (ii) “data and list of key research accomplishments are identical to that provided in the third annual report”. In addition, the reviewer pointed out that (iii) “the revised report should include justification for the use of breast cancer research funds to create a stable prostate tumor cell line and for in vivo experiments utilizing that reagent”.

Regarding the first and second points, we misunderstood the instruction in the past years, and we always reported up-to-date results including the previous years’ progress. Because those reports were approved by DOD each year, we thought that our reporting format is acceptable. Regarding the second point, although reviewers pointed out that the final report is identical to the third year’s report, we did include additional results in the final report. In fact, the third year’s report contained 8 figures and 3 tables, while the final report contained 10 figures and 4 tables. Nevertheless, we tried to clarify these problems and be more comprehensive in this revised report. Regarding the third point, we did use several prostate cancer cell lines in some of our experiments to compare the results of both breast and prostate cancers. These cell lines were already generated in our lab using different funding sources. The comparisons of two organ systems are very important in cancer research, which will reveal the generality or specificity of our hypothesis. We do believe these approaches are quite useful to identify specific druggable targets for breast cancer. Again, however, we did not use the breast cancer fund for those experiments. During the funding period of this project, we made significant progress toward the understanding of the mechanism of tumor metastasis in breast cancer, and we are grateful for the generous support by DOD. The following is our revised final report, and we hope it is acceptable now.

INTRODUCTION

Despite significant improvement in the treatment options and the impressive patient survival rate of breast cancer, the current available statistics show that more than 180,000 women in the US are still diagnosed with breast cancer every year. Therefore, a better understanding of the mechanism of oncogenesis in breast cancer is essential to develop a new and more effective anti-cancer drug. Once breast cancer is diagnosed, the most critical question is whether the disease is localized or has it already metastasized to other organs (1). However, the molecular basis of tumor metastasis is poorly understood as yet. The proposed research in this application aims at elucidating the function of the tumor metastasis suppressor gene, Drg-1/NDRG1, in the hope that we can define a specific target for novel and effective therapies to prevent metastatic disease of breast cancer. Toward this goal, we have set three specific aims; (i) to clarify the role of the NDRG1 gene in the progression of breast cancer (**Task 1**), (ii) to clarify the control mechanism of the NDRG1 gene by PTEN (**Task 2**), and (iii) to evaluate diagnostic/prognostic value of NDRG1 in breast cancer (**Task 3**). Our ultimate goal is to develop a novel therapeutic method which mimics the function of the NDRG1 gene. We believe that the knowledge gained from the proposed study will eventually be translated into clinical trials.

BODY

Task 1. To clarify the role of the NDRG1 gene in the progression of breast cancer

We first constructed MDA-MB231 cell line which expressed the luciferase gene using a lentivirus system. We then introduced NDRG1 expression vector into this cell line using a flag-tagged NDRG1 gene and obtained 3 independent clones. These cell lines were confirmed for NDRG1 expression by Western blot. One of the clones (MDA-MB231-NDRG1) and the parental cell line were injected into 5 nude mice/group through i.v. As shown in Fig. 1, the ectopic expression of NDRG1 significantly suppressed the colonization of MDA231 cells as expected, suggesting that NDRG1

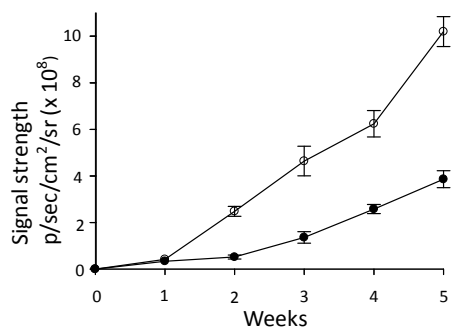


Fig. 1. Breast cancer cells, MDA-MB231-luc, and MDA-MB231-luc-NDRG1, were injected into 4-weeks old female nude mice (n=5) via i.v. The luciferase activities were measured for each mouse once a week for 5 weeks using IVIS® Imaging System (Xenogen).

blocks survival or extravasation of the breast tumor cells. This result is consistent with our previous observation that NDRG1 was able to suppress the metastatic growth of breast cancer cells without affecting primary tumor growth. Therefore, NDRG1 appears to function as a tumor metastasis suppressor in both breast and prostate cancers.

We have also constructed MCF7 cell lines that expressed NDRG1. Because MMP2 and MMP9 genes are known to be most commonly disregulated in metastatic cells, we examined the effect of NDRG1 on these genes (Fig. 2). However, our results indicate that NDRG1 did not activate these metalloproteases, suggesting that the metastasis suppressor activity of NDRG1 is not due to MMP2 or MMP9. Next, we have established tetracycline-inducible expression of NDRG1 in a tumor cell and performed a microarray analysis using the Affymetrix human gene array. The results of our microarray analyses indicated that the ATF3 gene, a member of ATF/CREB transcription factor family (2, 3), was most significantly suppressed by induction of the NDRG1 gene. To verify the result of the microarray data, NDRG1 expression plasmid

(pcDNA3/NDRG1) or the empty pcDNA3 vector was transiently transfected into breast cancer (MCF-7 and MDA-435) as well as prostate (PC3 and ALVA) cell lines and the level of ATF3 protein was examined by Western blot (Fig. 3 A). We found that NDRG1 indeed attenuated the ATF3 expression in a dose-dependent manner in all these cell lines, while the empty vector did not have any notable effect. In a complementary approach, we introduced NDRG1 siRNA or GFP siRNA in the

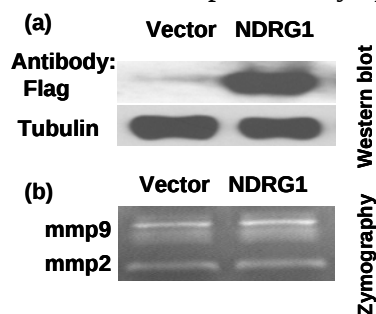


Fig. 2. Expression of NDRG1 does not affect MMP2 or MMP9. (a) NDRG1 was ectopically expressed in MCF7 and cell lysates were prepared. The lysates were then subjected to Western blot (a) and Zymography assays.

cancer cells and found that the NDRG1 siRNA specifically abrogated the expression of the NDRG1 gene which led to concomitant up-regulation of the ATF3 expression in these cells (Fig. 3B). These data strongly suggest that NDRG1 plays a crucial role in the regulation of the ATF3 gene, and down regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To further examine whether down-regulation of ATF3 expression by NDRG1 is mediated at the transcriptional level, tumor cells were co-transfected with NDRG1 expression vector (pcDNA3/NDRG1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was performed. We found that the ATF3-CAT reporter activity was significantly attenuated by NDRG1; thereby strongly suggesting that NDRG1 negatively controls expression of the ATF3 gene at the transcriptional level (Fig. 3C).

To corroborate the above in vitro results, we established prostate tumor cell lines that expressed the ATF3 gene and they were then injected into SCID mice. The growth of primary tumor was measured for a period of 3 weeks and mice were then sacrificed to examine metastatic lesions in

the lungs. We found that the growth rate of primary tumor did not change notably between the tumors with and without expressing ATF3. However, the number of metastatic lesions in the lungs were significantly increased in the mice that received tumor cells over-expressing ATF3, suggesting that ATF3 indeed is capable of promoting tumor metastases.

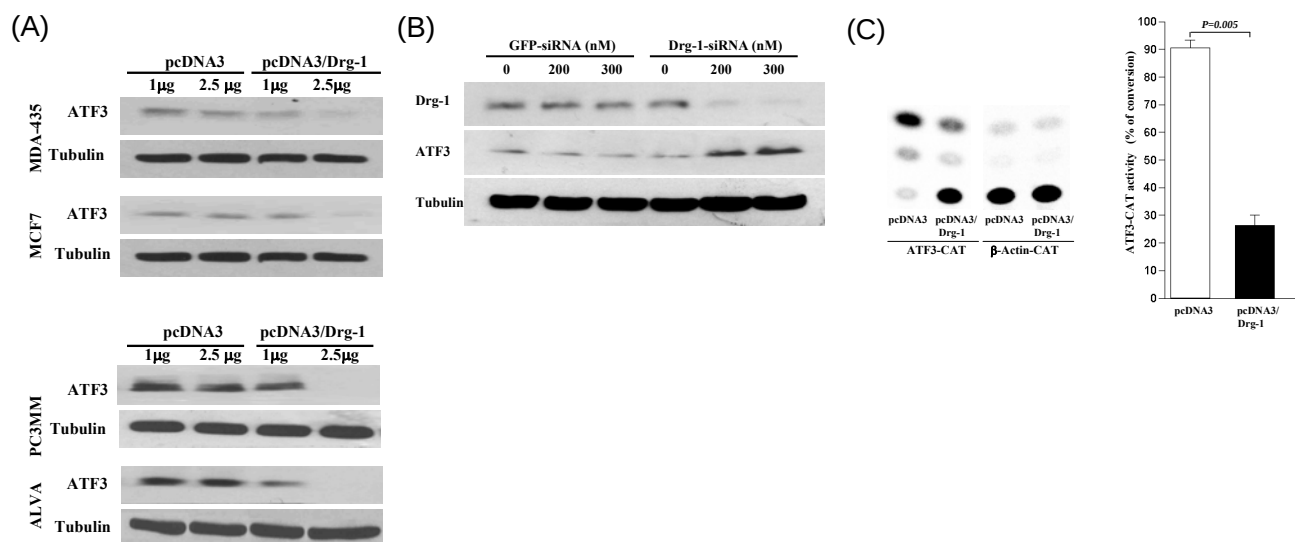


Fig. 3. NDRG1 down-regulates ATF3 expression. (A), Empty vector pcDNA3 or NDRG1 expression vector, pcDNA3/NDRG1, at the indicated amounts, was transfected into the breast cancer cell lines (MDA-435 and MCF7) and prostate cancer cell lines (PC3MM and ALVA). Forty-eight hour post-transfection, cells were lysed and Western blot was performed using antibodies against ATF3 and Tubulin. (B), siRNA for Drg-1 or GFP was synthesized and various amounts of the siRNA, as indicated, were transfected into PC3MM cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for NDRG1, ATF3 and Tubulin. (C), A CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (-1850 to +34) was co-transfected with NDRG1 expression plasmid (pcDNA3/NDRG1) or empty vector (pcDNA3) into the cells. Forty eight hours later, the cells were harvested, lysed and the lysates were then assayed for CAT activity. Acetylated chloramphenicol was resolved on TLC plate (representative run, left panel) and each spot was quantified (right panel). A reporter plasmid containing the β-actin promoter (βactin-CAT) was used as a control.

Table 1. Effect of ATF3 on tumor metastasis

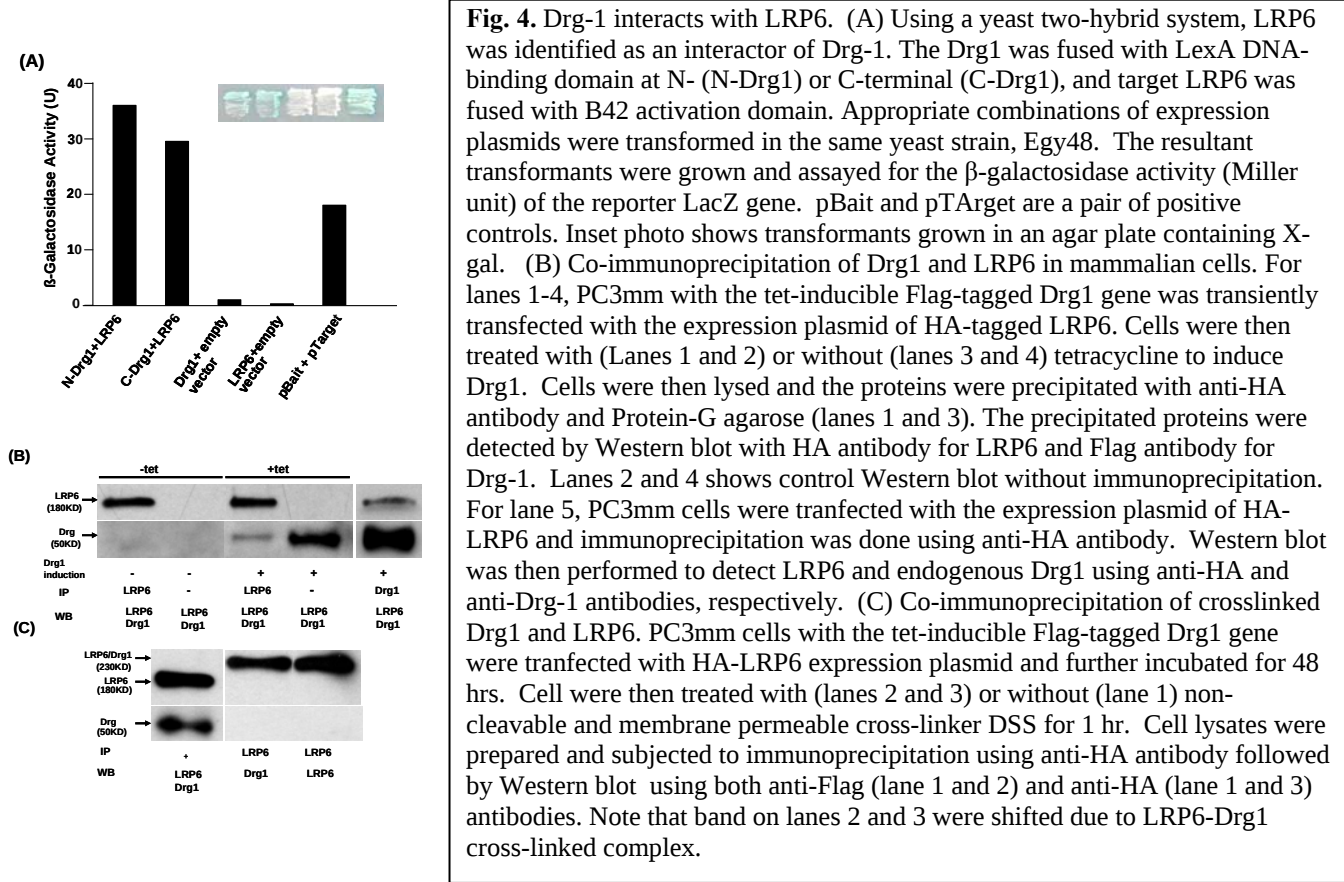
Cell line	ATF3 ^a	Tumor incidence ^b	<i>In vivo</i> doubling time	Lung metastasis (mean+/-S.E.) ^c	P value
AT2.1	-	5/5	4.1+/-0.5	5+/-1.9	
Vector only	-	5/5	3.9+/-0.3	5.2+/-3.3	0.96
ATF#9	-	4/4	3.7+/-0.8	2.5+/-0.9	0.51
ATF#4	+	5/5	4.3+/-0.3	39+/-11.3	0.02*
ATF#111	+	5/5	3.7+/-0.2	32+/-9.0	0.02*
ATF#207	+	5/5	2.5+/-0.2	46+/-3.2	<0.001*

a. ATF3 expression was examined by western blot.

b. Number of tumor-bearing SCID mice / no. of tumor-inoculated SCID mice.

c. Number of metastatic lesions on lungs per SCID mouse.

To further elucidate the mechanism of NDRG1 as a metastasis suppressor, we performed the Yeast two hybrid analysis. After screening approximately 1.2 million c-DNA clones of normal human tissues, we obtained four clones that reproducibly interacted with NDRG1 (Fig.4). Among these



clones, LRP6, which is known to be a co-receptor of the Wnt protein, showed the strongest interaction with NDRG1, and we decided to proceed with further analyses. To show the interaction of NDRG1 and LRP6 in mammalian cells, we first tagged NDRG1 and LRP6 with Flag and HA, respectively, for the purpose of a co-immunoprecipitation experiment. Flag-Drg1 was subcloned into the tetracycline inducible system followed by transfecting and establishing a cell line. The expression plasmid of HA-LRP6 was then transiently transfected into the tet-inducible Flag-NDRG1 cells, followed by induction of NDRG1 by tetracycline. The cells were lysed and proteins were precipitated by using anti-HA antibody. The sample was then subjected to Western blot analysis using anti-Flag antibody. As shown in Fig.4B, the results of our co-immunoprecipitation experiment indicate that Flag-NDRG1 was pulled-down with HA-LRP6, suggesting that these two proteins are indeed interacting in the cell. We also performed a similar co-immunoprecipitation experiment using HA-LRP5 and found that NDRG1 also interacts with LRP5 (data not shown). LRP6 functions as an essential co-receptor together with Frizzled for the Wnt signaling pathway. Apparently, LRP6 constitutes the distal signal-initiating component. The Wnt gene was originally identified as a developmental gene in *Drosophila* but it was later found to play a key role in mammalian embryogenesis as well as in tumorigenesis in various types of human cancers. Recently, the Wnt pathway has also been found to play a critical role in epithelial-mesenchymal transition (EMT). The EMT is a typical characteristic change of tumor cells from primary to metastatic cell, which accompany β -catenin re-localization, loss of E-cadherin and

ability to invade ECM. In fact, over-expression of the Wnt ligand or the receptor has been shown to promote invasiveness of tumor cell and the following metastases. Therefore, it is conceivable that NDRG1 suppresses the metastatic process by blocking the Wnt signal by binding to LRP6.

In order to examine the effect of NDRG1 in a signal pathway, we prepared the lysate of cells which had the tetracycline-inducible NDRG1 gene after treatment with or without tetracycline. This pair of cell lysate was tested for screening “activated” signal molecules using 26 different phospho-specific antibodies (Kinexus) by Western blot analysis. We found that, among these molecules, GSK3 β was significantly phosphorylated at Tyr279/216 while Akt was strongly de-phosphorylated at Thr308. To confirm these results, we have performed Western blot analysis using the lysate prepared from cells with or without induction of NDRG1. As shown in Fig. 5A, Akt was significantly de-phosphorylated by over-expression of NDRG1 while total Akt showed no difference. On the other hand, GSK3 β was significantly phosphorylated by induction of NDRG1. These results suggest that NDRG1 activates GSK3 β but inactivates Akt.

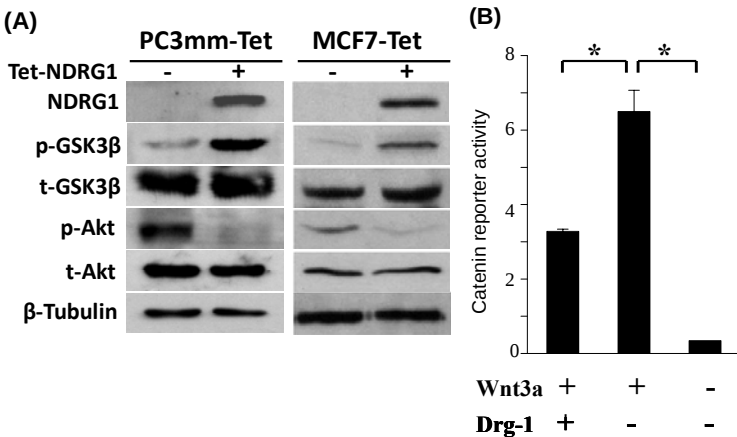


Fig. 5. Drg1 blocks the Wnt signal by augmenting GSK3 Tyr-phosphorylation and inhibition of AKT serine phosphorylation. (A) Cells with the tet-inducible expression plasmid of NDRG1 were treated with or without tetracycline and cell lysates were prepared. The samples were subjected to Western blot analyses using antibodies against phospho-AKT (ser473), total AKT, Drg1, and phospho-GSK3b (Tyr279/216). (B) NDRG1 suppresses β -catenin activity. Cells with the tet-inducible expression plasmid of NDRG1 were transfected with the reporter plasmid containing catenin/TCF binding sites (TOPflash system) and the luciferase gene in the presence or absence of Wnt followed by the treatment with or without tetracycline to induce NDRG1 expression. The cells were then harvested and the luciferase activity of the cell lysates was assayed. Renilla luciferase activity was used as an internal control.

GSK3 β is known to be a key signal mediator of the Wnt pathway. The protein makes a complex with APC and this complex stimulates ubiquitination of β -catenin followed by their degradation. When Wnt binds to the receptor, Frizzled and LRP5/6, GSK3 β is de-phosphorylated at tyr437 residue and “inactivated”. As a consequence, β -catenin accumulates and is transported to the nucleus followed by activation of various “pro-oncogenic” genes. Since our results indicate that NDRG1 binds to LRP6 and also phosphorylates GSK3 β at Tyr279/216 which activates this molecule, it is plausible that the interaction of NDRG1 to LRP6 blocks the process of GSK3 β de-phosphorylation in the Wnt pathway. It should be also noted that Akt phosphorylates GSK3 β at serine residues which results in inactivation of GSK3 β . As shown in Fig.5, NDRG1 appears to de-phosphorylate Akt and “inactivate” this protein kinase, suggesting that Drg1 further “activates” GSK3 β function by down-modulation of the Akt activity. To test the possibility that NDRG1 indeed blocks the Wnt pathway, we examined the β -catenin/TCF activity using the Topflash reporter plasmid which contains 8 tandem repeat sequences of the TCF binding site upstream of the luciferase reporter gene. The reporter plasmid was transfected to the cells containing tetracycline-inducible NDRG1 plasmid in the presence or absence of Wnt, followed by a treatment of the cells with and without tetracycline. The cell lysates were then assayed for luciferase activity. The results of the experiment showed that the luciferase activity was up-regulated more than 30 times in the presence of Wnt, while the induction of Drg1

significantly suppressed the reporter gene activity (Fig. 5B). These results strongly support our notion that NDRG1 blocks the Wnt pathway by GSK3 β activation.

To further validate our in vitro results, we examined the expression of β -catenin and NDRG1 in both breast and prostate cancers by immunohistochemical analysis. As shown in Fig. 6A, membrane β -catenin was strongly expressed in normal gland, while it is significantly reduced in high grade tumors. On the other hand, NDRG1 was highly expressed in normal tissue while it is significantly down-regulated in the high grade tumor. The results of chi-square test (Fig. 6B) for these two markers indicate that there is significant positive correlation between membrane β -catenin and NDRG1 expression in both breast and prostate cancer, which strongly support our hypothesis that NDRG1 blocks the Wnt signaling.

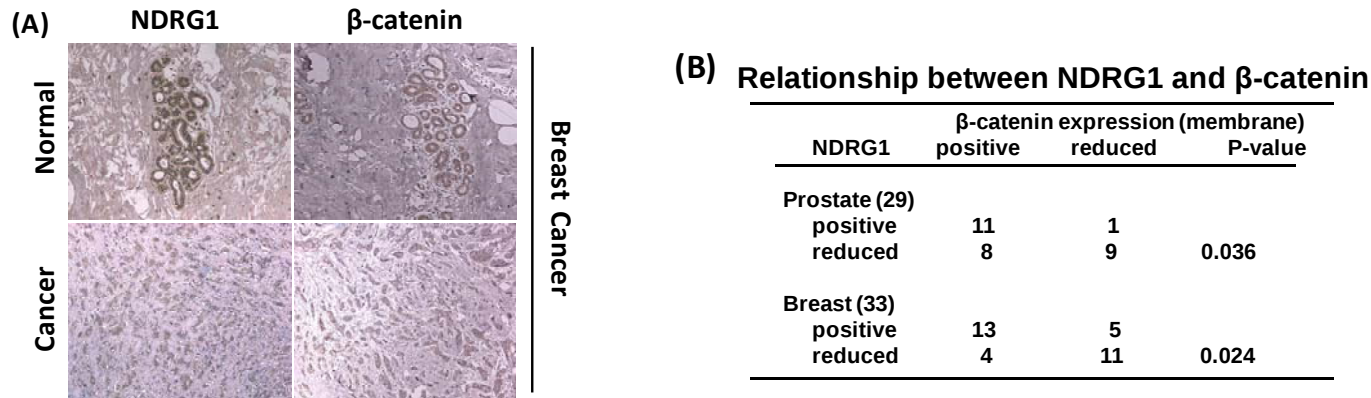
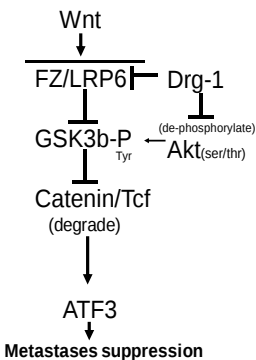


Fig. 6. Immunohistochemical analysis of membrane β -catenin and NDRG1 in breast and prostate cancer. A total of 33 breast and 29 prostate cancer samples were stained with antibodies to β -catenin and NDRG1. The relationship between β -catenin and NDRG1 expression was analyzed by chi-square test.

Because ATF3 was significantly down-regulated by NDRG1 (Fig. 3) and NDRG1 modulates Wnt signaling, we tested a possibility that NDRG1 suppresses ATF3 through the Wnt pathway. We first searched consensus sequence of β -catenin/TCF responsive element on the ATF3 promoter and found

	-34 TATAAAGGGTGA -21	Reporter Activity (Drg-1 : vector)	P-value
Wild type	ATF3 CAT	0.39 $\pm$ 0.10	0.023*
Mutant	TATAAAACCGTGA	0.88 $\pm$ 0.16	0.532

Fig. 7. The β -catenin/TCF responsive element on ATF promoter is responsible for NDRG1 suppression. β -catenin/TCF consensus sequence on the ATF promoter was mutated and the resultant reporter plasmid was transfected to the cells with or without expression of NDRG1. After 48 hrs, cell lysates were prepared and the reporter activities (CAT) were measured.



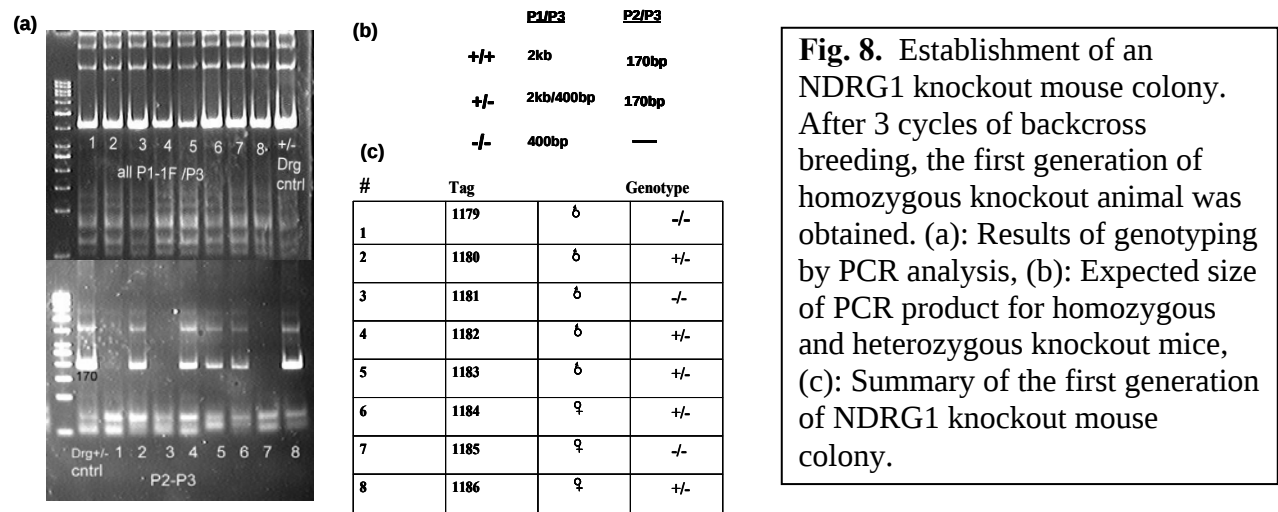
that there is indeed such sequence at -34 region of the promoter. We constructed a ATF3 promoter reporter plasmid and changed the sequence of the β -catenin/TCF responsive element by site-specific mutagenesis and assayed the promoter activity in the cells with or without over-expression of NDRG1. As shown in Fig. 7. The mutation of the TCF binding site on the ATF3 promoter significantly reduced the ability of the promoter to respond to NDRG1, implying that NDRG1 indeed modulates the expression of ATF3 through Wnt pathway. Overall, the work for Task 1 has been fruitful, and we consider that this aim was successfully accomplished. The generated data provided us with a strong foundation for future directions of further

investigation of NDRG1 function.

Task 2. To clarify the control mechanism of the Drg-1 gene by PTEN

Task 2 has lagged behind mainly because Task 1 generated very promising results and we spent more effort and time on Task 1. However, the reporter plasmid of NDRG1 was successfully constructed and we also generated systematic deletion mutants of the promoter region of the NDRG1. The PTEN expression vector is in our hand. We are now conducting a series of experiments to determine the location of PTEN responding region on the promoter of NDRG1 gene. We expect that we will obtain the results of this experiment shortly. Once we identify the region, we will then introduce site-specific mutations to validate the results of deletion analysis.

We have also obtained the NDRG1 knockout mouse from Japan and we are establishing a colony of this knockout mouse at SIU (Fig. 5). These mice will be used for this task in the future. We also established a colony of MMTV-Wnt transgenic mouse at SIU. Because of the critical role of NDRG1 in Wnt pathway, we cross-bred these mice and we are currently analyzing the spontaneous tumor incidence and metastasis status as well as the expression of PTEN in these mice. Although this DOD grant has expired, we will continue working on this task.



Task 3. To evaluate diagnostic/prognostic value of Drg-1 in breast cancer

We performed immunohistochemical analyses for NDRG1 and other clinical parameters on both breast and prostate cancers. As shown in Fig. 9, in both breast and prostate cancers, a significant level of differential expression of NDRG1 was observed between the patients with organ-confined disease and those with metastasis to lymph node or bone. In the case of breast cancer, while 89.7% patients were positive for NDRG1 expression out of 29 localized cases, 60.7% were positive for NDRG1 expression among 56 patients with metastases (Fig. 9A). These results strongly suggest the negative involvement of NDRG1 in the process of metastasis in breast cancer. In the case of prostate cancer, while 28 cases (70%) were positive for NDRG1 out of 40 localized prostate cancer cases, only 5 (25%) were positive for NDRG1 expression out of each of the 20 and 19 cases with lymph node and bone metastasis. Thus, the negative correlation of NDRG1 with metastatic spread to lymph node and

bone is highly significant ($P= 0.003$ and 0.006 respectively), and in fact, is much stronger than the positive correlation with Gleason scores.

(A)

Factor	Total (85)	Drg-1 expression positive	reduced	P value
Age				
< 51	33	25 (75.8%)	8 (24.2%)	
>51	52	35 (67.3%)	17 (32.7%)	0.56
Histological grade				
I/II	30	24(80.0%)	6(20.0%)	
III	55	36(65.5%)	19(34.5%)	0.16
P53				
Wild type	57	40 (70.2%)	17 (29.8%)	
mutant	28	20 (71.4%)	8 (28.6%)	0.99
ER				
Positive	40	27 (67.5%)	13 (32.5%)	
Negative	45	33 (73.3%)	12 (26.7%)	0.73
Tumor status				
T ₁₋₂ N ₀ M ₀	64	46 (71.9%)	18 (28.1%)	
T ₃₋₄ N ₀ M ₀	21	14 (66.7%)	7 (33.3%)	0.86
Metastasis status				
T _x N ₀ M ₀	29	26 (89.7%)	3 (10.3%)	
T _x N ₁₋₂ M ₀₋₁	56	34 (60.7%)	22 (39.3%)	0.01*

(B)

	All (62)	Drg-1 expression positive (34)	reduced (28)	P value
Age (mean +/- S.E. yrs.)	72.0 +/- 1.0	72.6 +/- 1.3	71.2 +/- 1.6	0.9
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015*
P53				
Wild type	59	32	27	
Mutant	3	2	1	0.8
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001*
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044*
Metastasis status				
Organ confined	40	28	12	
Lymph node metastasis	20	5	15	0.003*
Bone metastasis	19	5	14	0.006*

Fig. 9. Association of Drg1 with various clinical parameters. The results of immunohistochemical examination for the expression of NDRG1 in breast (A) and prostate (B) cancer patients were analyzed for the association with various clinical parameters. In each case, chi-squared test was performed to test the significance of association. * indicates statistically significant correlation ($P<0.05$).

Our Kaplan-Meier analysis on 85 patients of breast cancer for a period of 5 years indicate that patients with NDRG1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene ($P=0.002$, log rank test, Fig. 4). Thus, the reduced expression of NDRG1 can be a strong predictor of lymph node and bone metastasis and, in turn, of survival. In multivariate Cox regression analysis involving NDRG1 expression status, primary tumor size and metastasis status, NDRG1 emerged as an independent statistically significant prognostic factor (Table 2). The odds ratio for NDRG1 is 2.435 (95%CI 1.030-5.760, $P=0.043$), implying that the death risk of patients with reduced NDRG1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with NDRG1 positivity.

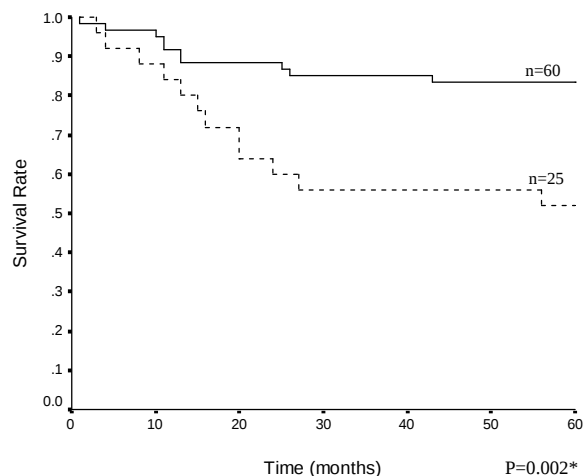


Fig. 10. NDRG1 expression is correlated with survival rate in breast cancer. Disease-free survival rate over a period of 5 years was analyzed in 85 patients in relation to NDRG1 expression. Solid line and dotted line indicate Drg-1 positive patients and patients with reduced expression of NDRG1, respectively. P value was determined by log rank test.

Table 2. Multivariate Cox regression analysis

Table 1. Multivariate Cox regression analysis							
Variables	reference level	b	SE	Wald's x ²	Hazard ratio	95% CI	P
Drg-1	positive	0.890	0.439	4.107	2.435	1.030 – 5.760	0.043*
Tumor status	T ₁₋₂ N _x M _x			2.264			0.132
Metastases	T _x N ₀ M ₀	1.513	0.760	3.963	4.538	1.024 – 20.117	0.046*

Thus, the reduced expression of NDRG1 can be a strong predictor of lymph node and bone metastasis and, in turn, of survival. Therefore, these data underscores the clinical relevance of this gene in advancement of breast cancer.

We have so far performed an immunohistochemical analysis on an archive of more than 80 breast and prostate cancer tissue samples for which we have 5-years survival data. The results showed that NDRG1 was expressed strongly in the epithelial cells of normal ducts and glands in breast tissue sections, while the poorly differentiated tumor cells in the same specimen had significantly reduced level of NDRG1 (Fig. 11). We also found that the expression of PTEN followed a pattern similar to that of NDRG1 (Table 3), which strongly supports our working hypothesis. Therefore, we considered that we successfully accomplished Task 3.

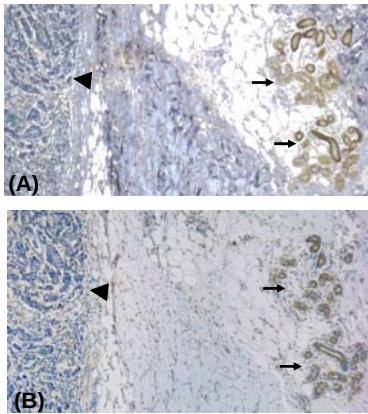


Fig.11. Immunohistochemical analysis of NDRG1 with respect to PTEN and other clinico-pathological parameters in human breast cancer. Immunohistochemistry for Drg-1 and PTEN was performed on paraffin tissue sections. A representative field from a breast cancer specimen immunostained with NDRG1 (A) and PTEN (B) antibodies.

Drg-1 expression				P value
Breast Cancer	All (85)	positive (60)	reduced (25)	
PTEN status				
Positive	62	51 (82.3%)	11 (17.7%)	<0.001*
Negative	23	9 (39.1%)	14 (60.9%)	
Prostate Cancer	All (81)	positive (51)	reduced (30)	
PTEN status				
Positive	63	44 (69.8%)	19 (30.16%)	0.03*
Negative	18	7 (38.89%)	11 (61.11%)	

Table 3. Relationship between NDRG1 and PTEN

KEY RESEARCH ACCOMPLISHMENTS

1. We found that NDRG1 significantly suppress metastatic colonization of breast cancer cells in the lung
2. NDRG1 did not affect the expression of the key genes of metastatic invasion, MMP2 or MMP9.
3. We have identified the ATF3 gene as a down-stream target of NDRG1 by microarray analysis. This result was verified in vitro by over-expression as well as siRNA knockdown of the NDRG1 gene.
4. We found that ATF3 is indeed capable of promoting tumor metastasis without affecting primary tumor growth in an animal model.
5. Using the Yeast two-hybrid system, we identified the receptor of Wnt, LRP6, as the direct target of NDRG1. Binding of NDRG1 indeed blocked the signaling of the Wnt pathway.
6. The expression of membrane beta-catenin is significantly correlated to NDRG1 level.
7. We have examined the expression of NDRG1 in tumor tissues from breast cancer patients and found that the expression of NDRG1 is inversely correlated with 5-year survival of patients and that NDRG1 can be a strong predictor of lymph node and bone metastasis and, in turn, of survival.
8. The expression of both PTEN and NDRG1 has strong correlation with patient survival.
9. Therefore, we consider that Tasks 1 and 3 were successfully accomplished. Task 2 is still ongoing and we plan to continue working on this task.

REPORTABLE OUTCOMES

Peer reviewed publications

(The following works were directly or partly supported by the current grant)

Original articles:

1. Bandyopadhyay, S., Fulk R.S., Pai, SK., Gross, SC., Hirota, S., Hosobe, S., Tsukada, T., Miura, K., Saito, K., Watabe, M., Wang Y., Huggenvik, J. Pauza, ME, Iizumi, M. and Watabe K.(2005) FAS expression inversely correlates with PTEN level in prostate cancer and an Akt inhibitor synergizes with FAS siRNA to induce apoptosis. **Oncogene**, 24, 5389
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3. Bandyopadhyay, S and Watabe, K. The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis in “New developments in Metastasis Suppressors” Ed. By Paul Jackson. Nova Publishers. pp87-102
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 14. Kounosuke Watabe (2008) Roles of CD82 in tumor metastasis. FASEB meeting for Signal Transduction through tetraspanins and other multi-protein cell surface complexes. New Haven CT
 15. Wen Liu, Sucharita Bandyopadhyay, Eiji Furuta and Kounosuke Watabe (2008) Role of tumor metastasis suppressor gene, NDRG1, in breast cancer progression. DOD Breast Cancer Research Program, Era of Hope 2008 Meeting. Baltimore MA

Employment

1. Dr. Sucharita Bandyopadhyay (Postdoc) has been partly supported by the current grant.
2. Dr. Megumi Iizumi (Postdoc) has been supported by the current grant.
3. Dr. Eiji Furuta (Postdoc) has been partly supported by the current grant.
4. Dr. Hiroshi Okuda (Postdoc) has been partly supported by the current grant.

CONCLUSIONS

Overall, the project was extremely fruitful, and we consider that Task 1 and 3 were successfully accomplished. However, since Task 1 has generated very promising results, our effort has been unevenly focused on this task, and work on Task 2 lagged behind. But we have obtained some interesting results and materials for this task and we plan to continue our effort of pursuing this task beyond this grant period. Our finding in Task 1 indicates that NDRG1 directly interacts with the Wnt receptor, LRP6, and this interaction blocks the Wnt signaling. We believe this is a break-through discovery for understanding the suppressor function of NDRG1. Therefore, we plan to further investigate the role of NDRG1 in the Wnt pathway. We also found that ATF3 is the target of NDRG1 and that ATF3 indeed promotes metastases in an animal model, suggesting that ATF3 and NDRG1 serve as prognostic markers and therapeutic targets for metastatic disease. Because ATF3 is a transcription factor, further down-stream target is of paramount interest. We are currently trying to screen potential targets by promoter scanning. We have also shown that PTEN positively regulates the expression of NDRG1 and a combination of these two markers serves as a useful predictor of breast cancer patients.

So what?

Metastatic disease remains the primary cause of death for breast cancer patients. Therefore, it is crucial to identify specific target molecules for better treatment of the patients. Our finding suggests that NDRG1 suppresses tumor metastases by blocking the Wnt pathway followed by inhibiting the function of ATF3. Our results also indicate that PTEN up-regulates the expression of NDRG1. Therefore, a combination of PTEN, NDRG1 and ATF3 can be used for diagnostic/prognostic markers as well as for therapeutic targets. The role of NDRG1 in Wnt signaling is also interesting and our results suggest that this signal pathway is crucial to understand NDRG1 function. Further understanding of the mechanism of NDRG1 function and its relationship to Wnt signal may reveal more rationale targets for the treatment of metastatic disease.

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The Tumor Metastasis Suppressor Gene *Drg-1* Down-regulates the Expression of Activating Transcription Factor 3 in Prostate Cancer

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Abstract

The tumor metastasis suppressor gene *Drg-1* has been shown to suppress metastasis without affecting tumorigenicity in immunodeficient mouse models of prostate and colon cancer. Expression of *Drg-1* has also been found to have a significant inverse correlation with metastasis or invasiveness in various types of human cancer. However, how *Drg-1* exerts its metastasis suppressor function remains unknown. In the present study, to elucidate the mechanism of action of the *Drg-1* gene, we did a microarray analysis and found that induction of *Drg-1* significantly inhibited the expression of activating transcription factor (ATF) 3, a member of the ATF/cyclic AMP-responsive element binding protein family of transcription factors. We also showed that *Drg-1* attenuated the endogenous level of ATF3 mRNA and protein in prostate cancer cells, whereas *Drg-1* small interfering RNA up-regulated the ATF3 expression. Furthermore, *Drg-1* suppressed the promoter activity of the *ATF3* gene, indicating that *Drg-1* regulates ATF3 expression at the transcriptional level. Our immunohistochemical analysis on prostate cancer specimens revealed that nuclear expression of ATF3 was inversely correlated to *Drg-1* expression and positively correlated to metastases. Consistently, we have found that ATF3 overexpression promoted invasiveness of prostate tumor cells *in vitro*, whereas *Drg-1* suppressed the invasive ability of these cells. More importantly, overexpression of ATF3 in prostate cancer cells significantly enhanced spontaneous lung metastasis of these cells without affecting primary tumorigenicity in a severe combined immunodeficient mouse model. Taken together, our results strongly suggest that *Drg-1* suppresses metastasis of prostate tumor cells, at least in part, by inhibiting the invasive ability of the cells via down-regulation of the expression of the *ATF3* gene. (Cancer Res 2006; 66(24): 11983-90)

Introduction

Drg-1 (differentiation-related gene-1), also known as *NdrG1* (N-myc down-regulated gene 1), was originally identified as being strongly up-regulated on induction of differentiation in colon

carcinoma cell lines (1). This gene has been shown recently to play an important role in the context of human cancer progression. We have shown that *Drg-1* suppresses lung metastasis of prostate cancer cells without affecting the growth of primary tumor in a severe combined immunodeficient (SCID) mouse model, strongly indicating the role of the *Drg-1* gene as a metastasis suppressor for prostate cancer (2). *Drg-1* has also been shown to exert a similar metastasis-suppressive effect in colon cancer cells in a mouse model (3). Consistent with our *in vivo* results, we and others have found that expression of the *Drg-1* gene is inversely correlated with Gleason grades in prostate cancer, and importantly, this down-regulation is more significant in patients with metastasis to lymph nodes than those with organ-confined disease (2, 4). Notably, we have observed similar inverse correlation of *Drg-1* expression with metastasis in breast carcinoma patients (5). More recently, *Drg-1* expression has been found to have a significant inverse correlation with depth of invasion in pancreatic adenocarcinoma patients as well (6). These data indicate that *Drg-1* indeed is a critical player in the process of tumor metastasis and it is imperative to understand the mechanism of action of this gene.

The *Drg-1* gene encodes a 43-kDa cytoplasmic protein that has several noticeable features, although the biochemical function of the protein is yet largely unknown. Amino acid sequence of the *Drg-1* protein reveals three serine phosphorylation sites, five calmodulin kinase 2 phosphorylation sites, five myristoylation sites, three protein kinase C phosphorylation sites, one tyrosine phosphorylation site, one thioesterase site, and one phosphopantothine attachment site. It has been shown that protein kinase A and calmodulin kinase 2 are indeed involved in the phosphorylation of this protein *in vitro* (7, 8). At the COOH-terminal end of the *Drg-1* protein, there are three tandem repeats of the amino acids G-T-R-S-R-S-F-T-H-T-S. Murray et al. showed recently that the COOH-terminal stretch of the *Drg-1* protein serves as a substrate for phosphorylation by serum- and glucocorticoid-induced kinase 1, which then primes it for phosphorylation by glycogen synthase kinase 3 (9, 10). However, the physiologic relevance of such phosphorylation remains largely unknown. In addition, based on potentiometric and spectroscopic studies, Zoroddu et al. (11) have proposed that this COOH-terminal stretch may be important for nickel binding. The amino acid sequence of *Drg-1* also indicates the presence of a prominent β -hydrolase fold, although it may not be enzymatically functional (12). Thus, the *Drg-1* protein presents several interesting features; however, the biochemical function of this protein in the context of tumor metastasis suppression remains to be elucidated.

As an initial step toward understanding how *Drg-1* suppresses the process of tumor metastasis, we have done a microarray

Note: S. Bandyopadhyay and Y. Wang contributed equally to this work.

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analysis to find the downstream target of this gene. Here, we present evidence that Drg-1 suppresses expression of the *activating transcription factor (ATF)* 3 gene in prostate and breast tumor cells and that this regulation occurs largely at the transcriptional level. We also show that Drg-1 and ATF3 expression inversely correlate at the clinical level and that ATF3 promotes invasion of prostate tumor cells *in vitro* and spontaneous metastasis *in vivo*.

Materials and Methods

Cell lines. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection (Manassas, VA). Human prostate cancer cell lines, ALVA and PC3MM, were kindly provided by Drs. W. Rosner (Columbia University, New York, NY) and I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX), respectively. Rat prostate cancer cell line AT2.1 was a gift from Dr. C. W. Rinker-Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% FCS, streptomycin (100 µg/mL), penicillin (100 units/mL), and dexamethasone (250 nmol/L) at 37°C in a 5% CO₂ atmosphere.

Expression plasmids and transfection. Drg-1 cDNA was a generous gift from Dr. S.W. Lee (Beth Israel Deaconess Medical Center, Boston, MA). To create the mammalian constitutive expression plasmid pcDNA3/Drg-1, the cDNA was PCR amplified where the forward primer included the Kozak sequence and *EcoRI* linker and the reverse primer included a *XhoI* linker. The PCR product was cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) using standard techniques. The expression of Drg-1 in the transfected cells was confirmed by Western blot. To construct an inducible expression vector of *Drg-1*, the cDNA of this gene was cloned into the pCMV-Tag2 expression vector (Stratagene, La Jolla, CA), and the in-frame fusion between the Flag tag and Drg-1 and the expression of the fused protein were confirmed by sequencing as well as Western blot. The Flag-Drg-1 cDNA was then PCR amplified and cloned into the *EcoRV/XhoI* site of the inducible expression vector pcDNA5/TO (Invitrogen) using standard techniques. To create a cell line with inducible Drg-1 expression, the tetracycline-inducible system T-Rex (Invitrogen) was used. First, the human prostate cancer cell line PC3MM was transfected with the regulatory plasmid pcDNA6/TR encoding the Tet repressor, and a stable cell line (PC3MM/Tet) was generated by blasticidin selection (2 µg/mL). Then, the pcDNA5/TO/Flag-Drg-1 expression plasmid was stably transfected into the PC3MM/Tet cell line and permanent clones were generated by blasticidin and hygromycin selection, and the resultant clones were designated as PC3MM/Tet-Flag-Drg-1. The induction of Drg-1 by tetracycline in this system was confirmed by Western blot. To create a mammalian expression plasmid of ATF3 (pcDNA3/ATF3), the ATF3 cDNA was excised from the pCG-ATF3 expression plasmid (13) and subcloned into the *EcoRI/HindIII* site of the mammalian expression vector pcDNA3 using standard techniques. Construction of the pATF3-CAT reporter plasmid containing the -1850 to +34 region of the ATF3 gene was described before (14). For DNA transfection into ALVA, PC3MM, MDA-435, and MCF7 cells, LipofectAMINE 2000 (Invitrogen) was used, whereas PC3 cells were transfected by TransIT-TKO transfection reagent (Mirus Corp., Madison, WI).

Microarray analysis. The PC3MM/Tet-Flag-Drg-1 cells were treated with 1 µg/mL tetracycline or an equal volume of 70% alcohol when the cells reached 80% confluency. Forty-eight hours after induction, the cells were collected and total RNA was prepared using RNeasy mini kit (Qiagen, Valencia, CA). The RNA was converted to cDNA and biotinylated followed by hybridization to an Affymetrix (Santa Clara, CA) Human Gene Array at the W.M. Keck Foundation Biotechnology Research Laboratory at Yale University.

Real-time reverse transcription-PCR. Forty-eight hours after transfection of appropriate plasmid DNA or forty-eight hours after induction by tetracycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Biosystems, Foster, CA). The cDNA was then amplified with a pair of forward and reverse primers for the *ATF3* gene (5'-AGTCACTGTGACGACAGAC and 5'-TGCTCTCGTTCTTGAG) and for the human β -actin gene. PCRs were

done using DNA Engine Opticon2 System (MJ Research, Waltham, MA) and the Dynamo SYBR Green qPCR kit New England Biolabs (Ipswich, MA). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 minutes followed by 30 cycles of PCR using the following profile: 94°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds.

Western blot. Forty-eight hours after transfection, the cells were collected and subjected to Western blot using antibodies against Drg-1 (1:5,000), ATF3 (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA), or tubulin (1:1,000; Upstate Biotechnology, Lake Placid, NY). The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and visualized by Enhanced Chemiluminescence Plus system (Amersham Life Sciences, Piscataway, NJ).

Small interfering RNA transfection. Four individual small interfering RNAs (siRNA) against the *Drg-1* gene were synthesized by Dharmacon (Chicago, IL) and combined into one pool (SMARTpool). One siRNA duplex targeting the *green fluorescence protein (GFP)* gene was used as a negative control in all the experiments. The siRNA was transfected into the tumor cell lines using the TransIT-TKO transfection reagent according to the manufacturer's protocol.

Chloramphenicol acetyl transferase reporter assay. Forty-eight hours after transfection of plasmid DNAs, the cells were collected and then subjected to chloramphenicol acetyl transferase (CAT) assay as described previously (14). The reaction was done and acetylated [¹⁴C]chloramphenicol was quantified with a PhosphorImager (Packard Instruments, Meriden, CT).

In vitro motility and invasion assay. For motility assay, 10⁵ cells were added to the cell culture inserts (24-well format) with microporous membrane without any extracellular matrix coating (Beckton Dickinson, Bedford, MA). Seven hundred microliter of RPMI 1640 containing 20% fetal bovine serum were added to the bottom chamber. They were then incubated for 24 hours at 37°C, and the upper chamber was removed. The cells that invaded through the membrane were stained with tetrazolium dye and counted under microscope. For *in vitro* invasion assay, the working method was similar as described above, except that the cell culture inserts to which the cells were seeded were coated with Matrigel (Beckton Dickinson). Triplicate tests were done in each case.

Tumor specimens and immunohistochemical staining. Formaldehyde-fixed and paraffin embedded tissue specimens from 64 prostate cancer patients were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four-micron-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L sodium citrate buffer (pH 9.0) at 80°C for 30 minutes (for Drg-1) or by autoclaving the slide in 10 mmol/L sodium citrate buffer (pH 6.0) for 10 minutes (for ATF3). The slides were incubated overnight at 4°C with anti-Drg-1 rabbit polyclonal antibody (1:200) or anti-ATF3 rabbit polyclonal antibody (1:50; Santa Cruz Biotechnology). The sections were incubated with the HRP-conjugated anti-rabbit secondary antibody, and 3,3'-diaminobenzidine substrate chromogen solution (Envision Plus kit, DAKO Corp., Carpinteria, CA) was applied followed by counterstaining with hematoxylin. Results of the immunohistochemistry for Drg-1 and ATF3 were judged based on the intensity of staining combined with percentage of cells with positive staining, and the grading of the Drg-1 and ATF3 expression was done by two independent persons (S.B. and K.W.).

Spontaneous metastasis assay. To examine the growth rate and metastatic ability of the prostate tumor cells expressing ATF3 in animals, 0.5 × 10⁶ cells in 0.2 mL of PBS were injected s.c. in the dorsal flank of 5-week-old SCID mice (Harlan Sprague-Dawley, Indianapolis, IN). Mice were monitored daily, and the tumor volume was measured as an index of the growth rate. Tumor volume was calculated using the equation, volume = (width + length) / 2 × width × length × 0.5236. The doubling time of tumor during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Statistical analysis. For *in vitro* experiments and animal studies, one-way ANOVA was used to calculate the *P* values. The association between Drg-1 and ATF3 expression was calculated by χ^2 analysis. For all of the

statistical tests, the significance was defined as $P < 0.05$. SPSS software was used in all cases.

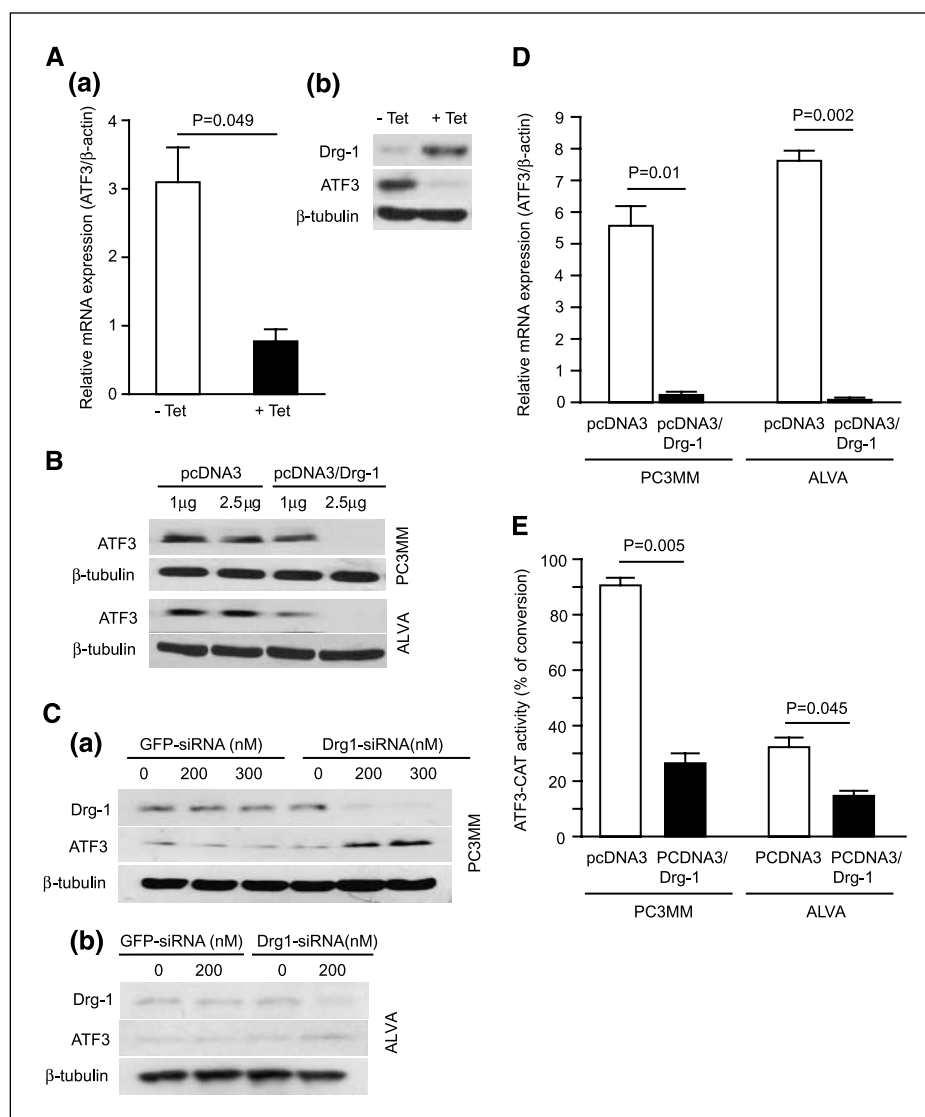
Results

Drg-1 attenuates the expression of the ATF3 gene *in vitro*. To identify the downstream target of the Drg-1 pathway, we did a microarray analysis using the Affymetrix human gene array U133A. For this purpose, we first established tetracycline-inducible expression of Drg-1 in the prostate cancer cell line PC3MM (PC3MM/Tet-Flag-Drg-1), and expression of the *Drg-1* gene was induced by treating the cells with tetracycline or solvent alone for 48 hours. The RNA was then extracted from these cells, converted into cDNA, and hybridized to the microarray. The results of our microarray analyses indicated that the *ATF3* gene, a member of ATF/cyclic AMP-responsive element binding protein (CREB) transcription factor family, was most significantly suppressed by induction of the *Drg-1* gene. Because recent evidence suggests potential involvement of the *ATF3* gene in tumor progression (15–20) and we are particularly interested in the genes up-regulated by suppression of Drg-1 because these may serve as

potential therapeutic targets, we decided to examine further the roles of ATF3 in the metastasis suppressor function of Drg-1. First, to confirm the results of the microarray analysis, we induced Drg-1 expression in the same cell line (PC3MM/Tet-Flag-Drg-1) and examined the level of ATF3 mRNA and protein by real-time reverse transcription-PCR (RT-PCR) analysis and Western blot, respectively. As shown in Fig. 1A, Drg-1 significantly abrogated ATF3 expression at both mRNA and protein levels, suggesting that induction of Drg-1 indeed leads to attenuation of expression of the *ATF3* gene.

To examine the effect of Drg-1 on endogenous ATF3 expression in various prostate tumor cells, the Drg-1 expression plasmid (pcDNA3/Drg-1) or the empty pcDNA3 vector was transiently transfected into the PC3MM and ALVA cells and the level of ATF3 protein was examined by Western blot. As shown in Fig. 1B, Drg-1 attenuated the ATF3 expression in a dose-dependent manner in these cell lines, whereas the empty vector did not have any notable effect. We observed similar effect of Drg-1 on ATF3 expression in breast cancer cell lines MCF-7 and MDA-435 (data not shown). In a complementary approach, we introduced Drg-1 siRNA or GFP siRNA in the prostate cancer cells, PC3MM and ALVA, and as

Figure 1. Drg-1 down-regulates ATF3 expression. **A**, PC3MM cells with tetracycline-inducible Drg-1 expression system were cultured with (+Tet) or without (–Tet) tetracycline. The cells were harvested, and RNA was prepared and subjected to quantitative RT-PCR (a). Another set of cells from identical experiment was lysed and expression of Drg-1, ATF3, and tubulin was examined by Western blot analyses (b). **B**, empty vector pcDNA3 or Drg-1 expression vector, pcDNA3/Drg-1, at the indicated amounts, was transfected into the prostate cancer cell lines PC3MM and ALVA. Forty-eight hours after transfection, cells were lysed and Western blot was done using antibodies against ATF3 and tubulin. **C**, siRNA for Drg-1 or GFP was synthesized as described in Materials and Methods. Various amounts of the siRNA, as indicated, were transfected into PC3MM (a) and ALVA (b) cells. After 72 hours, cells were lysed and the lysates were examined by Western blot with antibodies for Drg-1, ATF3, and tubulin. **D**, the prostate tumor cells, PC3MM and ALVA, were transfected with 2.5 μ g empty vector pcDNA3 or Drg-1 expression vector, pcDNA3/Drg-1. Forty-eight hours after transfection, total RNA was prepared from these cells and the expression of the *ATF3* and β -actin genes was examined by real-time quantitative RT-PCR. **E**, a CAT-reporter plasmid (ATF3-CAT) containing the ATF3 promoter region (–1850 to +34) was cotransfected with Drg-1 expression plasmid (pcDNA3/Drg-1) or empty vector (pcDNA3) into PC3MM and ALVA prostate cancer cells. Forty-eight hours later, the cells were harvested and lysed and the lysates were then assayed for the CAT activity. Acetylated chloramphenicol was resolved on thin-layer chromatography plate and each spot was quantified. A reporter plasmid containing the β -actin promoter (β -actin-CAT) was used as a control.



shown in Fig. 1C, the Drg-1 siRNA specifically abrogated expression of the *Drg-1* gene, which led to concomitant up-regulation of the ATF3 expression in these cells. These data strongly suggest that Drg-1 plays a crucial role in regulation of the *ATF3* gene, and down-regulation of Drg-1 in tumor cells results in augmentation of ATF3 expression. To determine whether the down-regulation of ATF3 by Drg-1 is mediated at the RNA level, pcDNA3/Drg-1 or pcDNA3 empty vector was transiently transfected into the above prostate cancer cell lines, and the level of ATF3 mRNA was measured by a real-time quantitative RT-PCR. Consistent with the results of our microarray analysis, we found that Drg-1 significantly attenuated ATF3 expression in these cells, indicating that Drg-1 down-regulates the *ATF3* gene at the mRNA level (Fig. 1D). We observed similar trends in MCF-7 and MDA-435 breast cancer cells as well (data not shown). To further examine whether down-regulation of ATF3 expression by Drg-1 is mediated at the transcriptional level, prostate cancer cell lines, PC3MM and ALVA, were cotransfected with Drg-1 expression vector (pcDNA3/Drg-1) or an empty vector (pcDNA3) and ATF3-CAT reporter plasmid, and the CAT reporter assay was done. As shown in Fig. 1E, we found that the ATF3-CAT reporter activity was significantly attenuated by Drg-1, thereby strongly suggesting that Drg-1 negatively controls the expression of the *ATF3* gene at the transcriptional level.

ATF3 augments invasiveness of prostate cancer cells *in vitro*. Because we have found previously that stable overexpression of

Drg-1 suppresses the invasiveness of several prostate tumor cells *in vitro* (2), we sought the possibility that ATF3 may be involved in motility and invasive properties of cells. We therefore transiently transfected ATF3 into human prostate cancer cell lines, PC3MM and ALVA, and assayed for the motility and invasiveness of the cells. As shown in Fig. 2A and B, expression of ATF3 significantly augmented invasive ability of these cells when they were tested by an *in vitro* Matrigel assay, whereas the motile ability of the cells remained virtually identical to the cells transfected with empty vector. These data indicate that ATF3 promotes the invasive ability of prostate cancer cells *in vitro* and suggest that attenuation of ATF3 expression by Drg-1 suppresses the invasiveness of tumor cells. To further corroborate this idea, the above prostate cancer cells were transiently transfected with Drg-1 expression vector (pcDNA3/Drg-1), and the invasiveness of these cells was tested. As shown in Fig. 2C, Drg-1 strongly inhibited the invasive ability of these cells compared with the empty vector transfectants. Taken together, these results strongly suggest that Drg-1 suppresses the invasive ability of cells via inhibition of expression of the *ATF3* gene.

Expression of Drg-1 and ATF3 correlates in clinical setting. The result of our *in vitro* experiments prompted us to examine whether there is any correlation between Drg-1 and ATF3 expression levels in the clinical setting. Toward that end, we did an immunohistochemical analysis on an archive of 64 prostate cancer tissue samples. The results of the immunohistochemistry

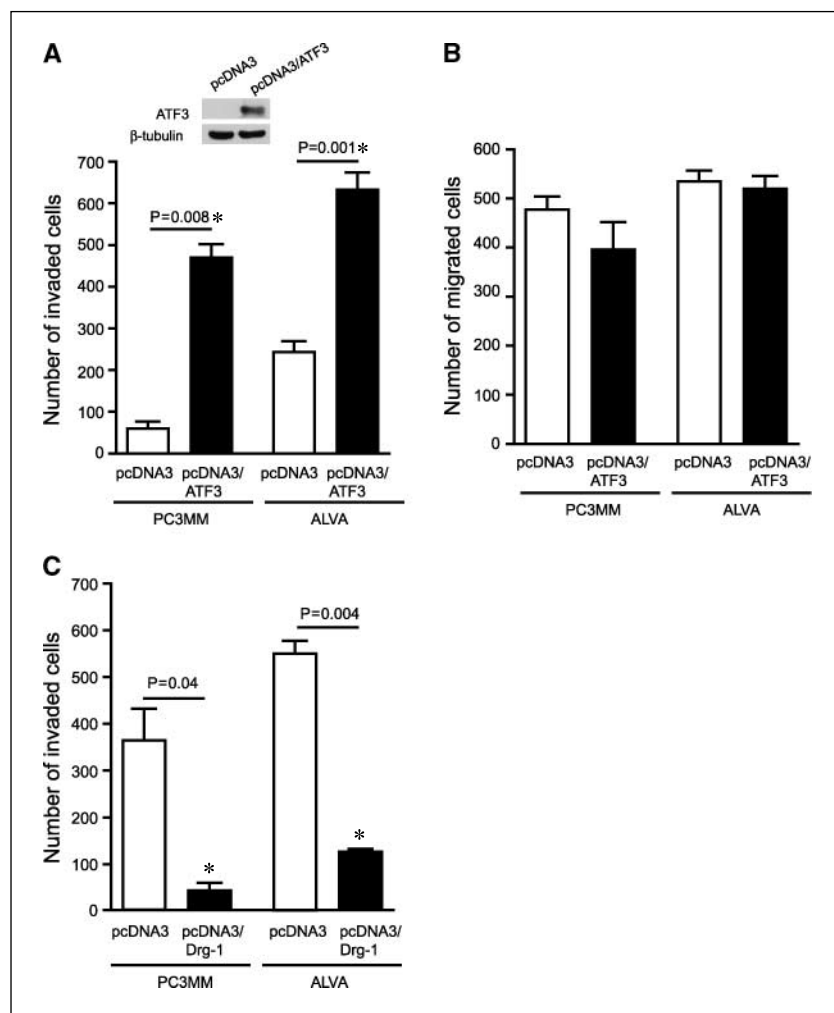
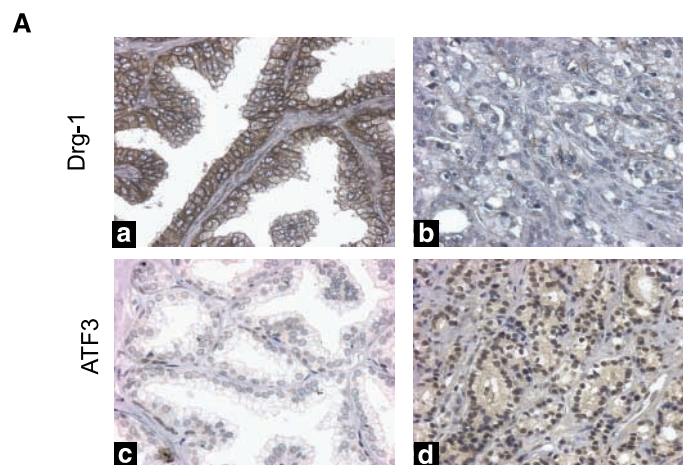


Figure 2. The effect of ATF3 and Drg-1 on the invasiveness and motility of prostate cancer cells *in vitro*. ATF3 expression plasmid (pcDNA3/ATF3) or an empty vector (pcDNA3) was transfected into PC3MM and ALVA prostate cancer cells, and 48 hours after transfection, these cells were examined for invasiveness (A) using Matrigel-coated invasion chamber and for motility (B) using cell culture inserts without any reconstituted extracellular matrix. Expression of ATF3 protein following transient transfection of the expression construct (A, inset). C, PC3MM and ALVA cells were transfected with the empty vector pcDNA3 or the Drg-1 expression plasmid (pcDNA3/Drg-1). Forty-eight hours after transfection, the cells were subjected to invasion chamber assay as described in (A) above. All assays were done in triplicate. *, $P < 0.05$, statistically significant difference.

Figure 3. Immunohistochemical analysis of Drg-1 with respect to ATF3 in human prostate cancer. **A**, immunohistochemistry for Drg-1 and ATF3 was done on paraffin tissue sections from prostate cancer patients. Drg-1 immunostaining in a representative field from a prostate cancer patient sample showing normal prostatic gland (*a*) and poorly differentiated prostate carcinoma cells (*b*). A consecutive section from the same tissue specimen is shown after immunostaining for ATF3 (*c* and *d*). Note the strong nuclear expression of ATF3 in carcinoma (*d*). **B**, nuclear expression of ATF3 inversely correlates with Drg-1 expression and positively associates with metastasis status. Immunohistochemistry was done on prostate tissue specimens as described in Materials and Methods. *, $P < 0.05$, statistically significant correlation, as tested by χ^2 analysis.



B
Relationship between ATF3 and Drg-1 expression

	ATF3 nuclear expression			P value
	All (64)	positive (40)	negative (24)	
Drg-1 expression				
Positive	38	19 (50%)	19 (50%)	0.025*
Reduced	26	21 (80.8%)	5 (19.2%)	
Metastasis status				
Negative	39	19 (48.7%)	20 (51.3%)	0.010*
Positive	25	21 (84%)	4 (16%)	

	ATF3 cytoplasmic expression			P value
	All (64)	positive (27)	negative (37)	
Drg-1 expression				
Positive	38	14 (%)	24 (%)	0.430
Reduced	26	13 (%)	13 (%)	
Metastasis status				
Negative	39	16 (41%)	23 (59%)	1.000
Positive	25	11 (44%)	14 (56%)	

revealed that Drg-1 is expressed strongly in the cytoplasm of the epithelial cells of normal ducts and glands in prostate tissue sections, whereas the poorly differentiated tumor cells in the same specimen had significantly reduced level of Drg-1 (Fig. 3A, *a* and *b*). Notably, Drg-1 expression was undetectable in the nuclei of normal or cancerous tissue or in the stromal cells. On the other hand, in the epithelial cells of normal ducts and glands, the ATF3 protein weakly expressed mostly in the cytoplasm, whereas, in cancerous cells, there was a notable increase and shift of the ATF3 expression in the nuclei (Fig. 3A, *c* and *d*). Statistical analysis indicated that there was no correlation between Drg-1 and cytoplasmic ATF3 expression; however, Drg-1 and nuclear ATF3 had a significant inverse correlation ($P = 0.025$; Fig. 3B). Of 26 patients who had reduced Drg-1 expression, 21 (80.8%) patients also exhibited strong nuclear expression of ATF3, whereas only 5 (19.2%) patients were negative for ATF3 nuclear expression. More importantly, among 25 cases that were positive for bone metastases, 21 (84%) also had positive expression of nuclear ATF3, indicating that ATF3 expression had a significant positive correlation with distant metastasis ($P = 0.010$). The results of this immunohistochemical

analysis are therefore consistent with our notion that Drg-1 down-regulates the expression of ATF3 and suggest a possibility that Drg-1 suppresses metastases of prostate cancer cells by inhibiting the expression of the *ATF3* gene.

ATF3 promotes spontaneous lung metastasis of prostate cancer cells *in vivo*. To investigate the role of ATF3 in primary tumor growth as well as metastasis *in vivo*, the Dunning rat prostate cancer cell line AT2.1, AT2.1 stably overexpressing ATF3, or AT2.1 transfected with the vector alone was individually injected s.c. into the dorsal flanks of SCID mice. As shown in Fig. 4A, Western blot analysis indicated that the clones 4, 111, and 207 expressed ATF3 protein, whereas AT2.1 parental cells, the vector-transfected clone, and the clone 9 did not have any detectable level of ATF3 expression and therefore served as negative controls. The mice were monitored for the formation and the growth rate of tumors for a period of 4 weeks after the inoculation of the cells, and they were sacrificed at the experimental period. Their lungs were then removed and the number of metastatic lesions was grossly counted (Fig. 4B). As shown in Fig. 4C, all the clones and the parental cells formed primary tumors in the animals with similar

growth rates during the 4-week period, indicating that ATF3 did not have an effect on tumorigenesis and growth of prostate cancer cells. AT2.1 has a poor metastatic propensity and consistently, AT2.1, the vector transfectant cell line, or the clone lacking ATF3 expression (ATF3 clone 9) produced a few metastatic nodules in the lungs. The clones that had stable expression of ATF3 (ATF3 clones 4, 111, and 207), however, significantly augmented the degree of lung metastases causing an average of ~40 metastatic foci in the lungs. These results strongly suggest that ATF3 has the ability to promote the metastatic process of prostate cancer cells without affecting primary tumorigenicity *in vivo*.

Discussion

Metastasis is the ultimate cause of death in any type of cancer, and yet this aspect of the cancer biology remains poorly understood because of the complexity of the metastatic process. Metastasis is negatively controlled by the tumor metastasis suppressor genes that by definition suppress the metastatic dissemination of cancer cells without affecting tumorigenicity. Till date, only a few genes have been identified that clearly meet these criteria (i.e., *NM23*, *KAI1*, *Kiss1*, *Brms1*, *MKK4*, *RhoGDI2*, *RKIP*, *CRSP3*, *SSeCK*, *TXNIP/VDUP-1*, *Claudin-4*, and *RRM1*; refs. 21–24). Recent work by our group and others has indicated that Drg-1 serves as one of such metastasis suppressor genes, although mechanistic insight into how Drg-1 suppresses metastasis is still lacking (2, 3, 5). In this report, we have shown that Drg-1 blocks the metastasis process by attenuating the expression of the *ATF3* gene

at mRNA and protein levels and that this regulation occurs for the most part at the transcriptional level.

ATF3 belongs to the mammalian ATF/CREB family of transcription factors (13). Members of this family of proteins bind to a consensus DNA sequence (TGACGTCA) and possess the basic region/leucine zipper (bZip) domain (13). ATF3 acts as a transcriptional repressor as a homodimer, although the same protein functions as a transcriptional activator in heterodimeric form (25–27). ATF3 has been shown to regulate the expression of several genes, including *Thrombospondin*, *Decorin*, *E-selectin*, gluconeogenic enzymes, *Gadd153/Chop10*, and *Osteocalcin* via CREB/activator protein-1 (AP-1) motifs (28–32). *ATF3* is a stress-inducible gene that also affects cell cycle progression and apoptosis in various ways and has been implicated recently in the development of cancer. The *ATF3* gene is localized on human chromosome 1q32 within a region that is found to be frequently amplified in esophageal squamous cell carcinoma (33). ATF3 was also reported recently to be highly expressed in classic Hodgkin's lymphoma but not in the non-Hodgkin's lymphoma, and blockade of ATF3 by siRNA reduced proliferation and viability of the Hodgkin's lymphoma cells (15). A separate study by Iyengar et al. (16) also suggested that ATF3 promotes mammary tumorigenesis by induction of antiapoptotic program. Consistently, antisense ATF3 oligonucleotide was shown to inhibit growth of the colon cancer cell line HT29 *in vivo*, although it had no effect on the growth of these tumor cells *in vitro* (18). These reports strongly suggest a positive role of the *ATF3* gene toward advancement of cancer. It is of interest to note that other members of the *ATF* family have been

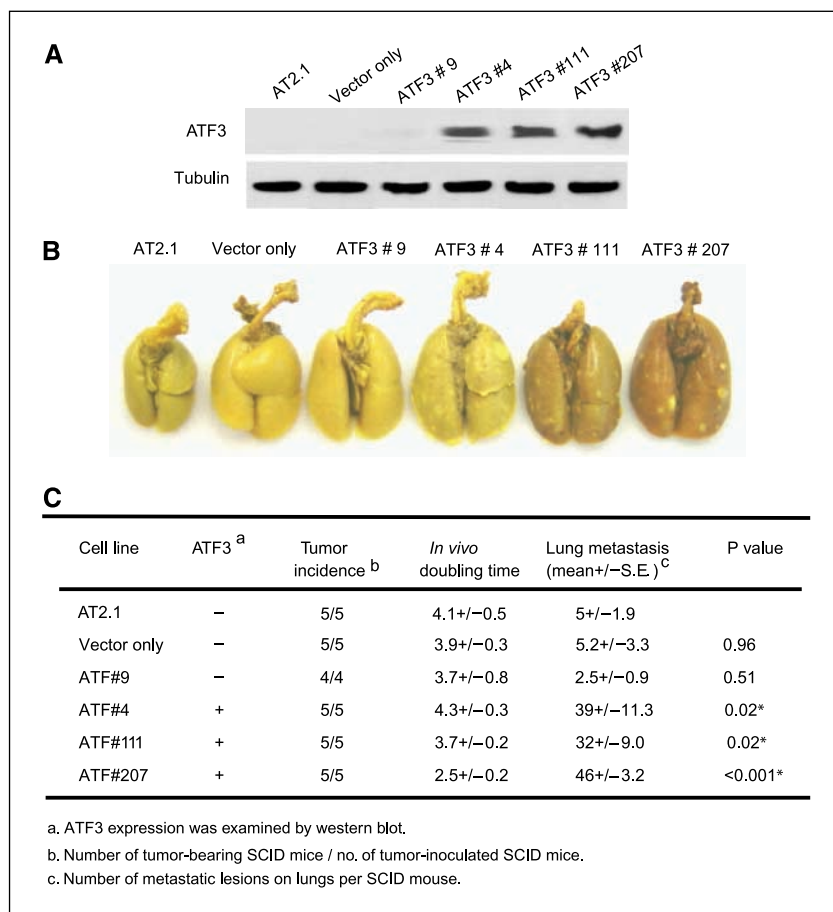


Figure 4. ATF3 augments spontaneous lung metastasis without affecting growth of primary tumor. **A**, the parental cell line (AT2.1), cells transfected with vector (*Vector only*), and ATF3-transfected clones (#9, #4, #111, and #207) were tested for ATF3 and tubulin protein expression by Western blot using anti-ATF3 rabbit polyclonal antibody and anti-tubulin mouse monoclonal antibody, respectively. Each of these cell lines was injected s.c. into SCID mice (five mice per group). After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. **B**, the lungs from two mice from each group are shown as examples. **C**, the number of tumor-bearing mice, primary tumor growth rate, and metastases formation are summarized.

implicated in this process as well. For example, strong nuclear expression of ATF2 is associated with metastasis and poor survival in melanoma patients, and ATF4 has been reported to increase cisplatin resistance of human cancer cell lines (34, 35). However, recent growing body of evidence indicates that much still remains to be learned about the complex roles of the genes of the *ATF* family in the context of tumor progression. In addition to its growth-promoting effect, ATF3 was found to be induced following DNA damage in HCT-116 and RKO colon carcinoma cells and suppressed the growth of HeLa cells (36). In a separate study, ATF3 synergized with curcumin to induce apoptosis in squamous cell carcinoma cell line MDA-1986 (37). Furthermore, Bottone et al. (38) have shown that overexpression of ATF3 in HCT-116 colon carcinoma cells decreased focus formation and invasiveness *in vitro* and also reduced growth of xenograft tumor, although the antisense ATF3 had no effect *in vivo*. Thus, ATF3 plays a complex role in tumor progression, and it is possible that some of the apparent contradictions in terms of the function of the *ATF3* gene arise at least in part due to difference in the cellular context.

In this report, we show that the *ATF3* gene promotes invasion of prostate tumor cells *in vitro*, although migration of these cells was not affected. Previously, Ishiguro et al. showed that antisense ATF3 oligonucleotide inhibited invasion and migration of HT29 colon cancer cells *in vitro*, whereas ATF3 expression correlated with the depth of invasion in clinical samples of colon cancer (18–20). In addition, ATF3 expression was found to be higher in human colon and stomach cancer cell lines that were established from metastatic sites than those derived from primary tumor sites (20). Consistently, the highly metastatic melanoma cells B16F10 has been reported to express ATF3 at a much higher level than its low-metastatic counterpart B16F1 (17). These results are in good agreement with our finding and point toward a proinvasive and prometastatic function of the *ATF3* gene. Furthermore, we and others have shown previously that Drg-1 suppresses invasion and metastasis of colon and prostate cancer cells, and Drg-1 expression has a significant inverse correlation with metastasis in prostate and breast cancer (2, 3, 5). Notably, as shown in this report, we have observed a significant inverse correlation between Drg-1 and ATF3 expression and a positive correlation between ATF3 expression and distant metastases in clinical samples of prostate cancer. These results, together with the results of our *in vitro* experiments, strongly support our notion that the metastasis suppressor gene *Drg-1* attenuates the invasive ability of cells by inhibiting the expression of the *ATF3* gene. How ATF3 promotes invasion remains to be understood at the cellular and molecular levels. Stearns et al. (39) have reported recently that direct binding of ATF3 to the matrix

metalloproteinase-2 (MMP-2) promoter leads to interleukin-10-mediated suppression of MMP-2. However, Yan et al. (40) showed previously that ATF3 represses MMP-2 expression by interfering with p53-dependent transactivation of this gene, independent of the CREB/AP-1 binding motif on the MMP-2 promoter. Consistently, they did not find any effect of ATF3 on the MMP-2 expression in cells where p53 level was low. The PC3MM cells (metastatic derivative of PC3) used in our study are p53 null; therefore, ATF3 is considered to affect the invasive ability of these cells in MMP-2-independent manner (41, 42).

We have shown that ATF3 promotes pulmonary metastases of poorly metastatic Dunning rat prostate tumor cells (AT2.1) in a SCID mouse model without affecting the growth of the primary tumor (Fig. 4). This is the first report indicating that ATF3 promotes spontaneous metastasis and is consistent with the results of an earlier report where ATF3 was found to augment metastasis of murine melanoma cells when the cells were injected i.v. (17). Because we have shown previously that Drg-1 significantly suppressed lung metastases of the highly metastatic Dunning rat prostate cancer cells (AT6.1) and because AT2.1 cells are from the same family as AT6.1 but have low metastatic ability, the results of the animal experiment presented in this report strongly argue for the notion that Drg-1 suppresses the metastatic ability of tumor cells by inhibiting the expression of the *ATF3* gene. Considering the proinvasive activity of the *ATF3* gene noted by us and others, it can be speculated that ATF3 promotes metastasis by augmenting invasion of the cells through the extracellular matrix and/or extravasation of tumor cells at the secondary site, although the cellular and molecular details of this process remain to be understood.

Taken together, we propose a molecular mechanism of action of the metastasis suppressor gene *Drg-1*, where Drg-1 down-regulates the expression of the *ATF3* gene leading to suppression of invasion and metastasis. For metastatic cancer, Drg-1 is significantly down-regulated, which in turn promotes metastatic dissemination of cancer cells, at least in part, by concomitant up-regulation of the *ATF3* gene. Further understanding of the components of this pathway should provide crucial information toward effective therapeutic intervention of metastatic cancer.

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The role of tumor metastasis suppressors in cancers of breast and prostate

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1. ABSTRACT

Despite significant improvement in surgical techniques and chemotherapies, none of the current medical technologies “cure” metastatic disease, and the patients who have acquired metastatic cancer inevitably die from disseminated disease. Thus, there is a need for developing novel therapeutic approaches which can directly target metastatic tumor cells. However, advances in understanding the molecular mechanism of tumor metastases have lagged behind other developments in the cancer field. Tumor metastasis involves complex array of steps with each step requiring a coordination of the actions of many positive and negative factors. A number of tumor metastasis suppressors have been identified which suppress the formation of tumor metastasis without affecting the growth rate of the primary tumor. Such discoveries offer new approaches for curtailing tumor metastasis. This review summarizes our current understanding on these genes and their potential role in the progression of tumor metastases.

2. CLINICAL SIGNIFICANCE OF TUMOR METASTASES

Malignant tumors metastasize to adjacent or distant organs through the blood vascular circuit or lymphatic system. When cancer is detected at an early stage, before it has spread to other distant sites, it can be treated successfully by surgery or local irradiation and the patient will be cured. However, treatments are much less successful when the cancer is detected after it has already metastasized. Unfortunately, most patients present with a metastatic disease at the time of the first visit to the clinic, and in addition, many patients who do not present any evidence of metastasis at the time of their initial diagnosis, metastases will be detected at a later time. Therefore metastatic disease is a serious concern for survival of cancer patients. In spite of this clinical importance of metastasis, much remains to be learned about the biology of the metastatic process.

It is well known, based both on clinical observations and mechanistic studies, that metastasis

formation is an inefficient process (1). Although large numbers of tumor cells are shed into the vascular drainage system from a primary tumor, it has been demonstrated experimentally that, after intravenous injection of highly metastatic tumor cells, approximately only 0.01% of these cells form tumor foci (2, 3). The inefficiency of tumor cells in completing the metastatic cascade results from the fact that successful formation of metastatic foci consists of several highly complex and interdependent steps. Each step is rate-limiting in that, failure to complete any of these events totally disrupts metastasis formation (1). The steps involved in metastasis formation are described below.

3. PROCESS OF TUMOR METASTASES

After the initial neoplastic transformation, the tumor cells undergo progressive proliferation that is accompanied by further genetic changes and development of a heterogeneous tumor cell population with varying degrees of metastatic potential. The oncogenic transformation is a result of the balance between the proto-oncogenes, which gain function by mutation, and the tumor suppressor genes, which contribute to tumorigenesis by loss of function (4, 5). The initial growth of the primary tumor is supported by the surrounding tissue microenvironment, which eventually becomes rate-limiting for further growth. As the tumor grows and the central tumor cells become hypoxic, the tumor initiates recruitment of its own blood supply. This process is referred to as the angiogenic switch and involves a balance between secretion of various angiogenic factors and removal or suppression of angiogenesis inhibitors (6, 7). The numerous positive and negative factors involved in angiogenesis are listed in Table 1. Notably, the process of neovascularization is almost invariably associated with a dramatic increase in the metastatic potential of tumors.

Continued genetic alteration in the tumor cell population results in selection of tumor cell clones with distinct growth advantage and acquisition of an invasive phenotype. Invasive tumor cells down-regulate cell-cell adhesion by modulating the expression of cadherins, alter their attachment to the extracellular matrix by changing integrin expression profiles and proteolytically alter the matrix by secretion of the matrix metalloproteases (1). Collectively, these changes result in enhanced cell motility and the ability of these invasive cells to separate from the primary tumor mass. These cells can detach from the primary tumor and create defects in the extra-cellular matrix that define tissue boundaries such as basement membranes, thus accomplishing stromal invasion. Furthermore, the poorly formed tumor vasculature that is generated in response to the angiogenic switch in the primary tumor mass, as well as thin walled lymphatic channels in the surrounding stroma, are readily penetrated by these invasive tumor cells and offer ready conduits to the systemic circulation (6). Endothelial cells responding to the angiogenic stimulus produced by the primary tumor also express an invasive phenotype and greatly enhance the metastatic process (7).

Once the tumor cells and the tumor cell clumps (emboli) have reached the vascular or lymphatic

compartments, they must survive a variety of hemodynamic and immunologic challenges. Because cancer cells often express tumor specific antigens, they are attacked by non-specific (macrophage and NK cells) as well as specific (T cells) immune systems. However, some tumor cells evade the immune surveillance by a variety of mechanisms such as down-regulation of MHC I (8) and secretion of Fas ligand (9). After survival in the circulation, tumor cells must arrest in distant organs or lymph nodes. This arrest may occur by size trapping on the inflow side of microcirculation, or by adherence of tumor cells through specific interactions with capillary or lymphatic endothelial cells, or by binding to exposed basement membrane. In most cases, arrested tumor cells extravasate before proliferating. After exiting the vascular or lymphatic compartments, metastatic tumor cells may proliferate in response to paracrine growth factors or become dormant. After extravasation, tumor cells migrate to a local environment more favorable for their continued growth. Findings using *in vivo* video-microscopy demonstrate that the poor growth of tumor cells after extravasation from the circulation is a major factor contributing to the inefficiency of the metastatic process (10).

According to a century-old theory, a disseminated cancer cell acts like a seed, growing only if it finds suitable soil at a secondary site. Support for this idea comes from the observation that the target organ of metastasis is typically better than non-target organs in stimulating the growth of cancer cells *in vitro* (11). For example, researchers have noted that the bone marrow, in contrast to various other organs, strongly stimulates prostate cancer cell growth *in vitro* but has little or no effect on cancer cells that metastasize to non-bone organs (12). Similar correlations have been made for cancer cells *in vivo*. In a study of mammary cancer sublines with varying patterns of metastasis, the preferred organ of metastasis in each case was the organ allowing the most rapid growth of cancer cells (13). A traditional alternative to the “seed and soil” argument, known as the anatomical-mechanical hypothesis, challenges the importance of the soil in regulating cancer cell growth. It argues instead that metastasis develops in the organ of any capillary bed in which a disseminated cancer cell becomes mechanically lodged (11). Consistent with this hypothesis, it was noted in the 1940s that specific veins draining the prostate encountered their first capillary bed in the lumbar spine, which is a common site of prostate cancer metastasis (14). More recent findings also suggest that the cancer cell may have an important role in modifying the environment that it encounters. The environment reacts to this modification by inducing changes in the tumor cell and the cycle repeats (15). Hence, according to this model, the regulatory interaction between seed and soil is dynamic and reciprocal.

4. TUMOR METASTASES SUPPRESSOR GENES AND THEIR ROLES IN CANCER PROGRESSION

As described above, the process of tumor metastases involves multiple steps with high complexity and each step requires a coordination of the actions of

Table 1. Factors involved in the process of tumor metastases

Factor	Function	Expression in cancer	Location	Reference
Positive Factor				
Twist	Transcription, Cell adhesion	Breast, Prostate	7p21.2	109, 110
MMP2	Degrades extracellular matrix	Breast, Lung	16q13-q21	111, 112
MMP7	Degrades extracellular matrix	Colorectal, Gastric, Lung	11q21-q22	113-115
Catenin alpha 1	Cell signaling	Pancreatic	5q31	116
Catenin beta 1	Cell signaling	Breast, Prostate	3p21	117, 118
uPA	Serine protease	Breast, Prostate, Colorectal	10q24	119-121
Reptin	ATPase, DNA helicase activity	Prostate	19q13.3	118
VEGF	Angiogenesis	Breast, Prostate, Colorectal	6p12	121-123
PLGF	Angiogenesis	Breast	14q24-q31	124
FGF 1	Cell proliferation, Angiogenesis	Prostate	5q31	125
FGF 4	Cell proliferation, Angiogenesis	Prostate	11q13.3	125
TGF beta	Cell proliferation, differentiation	Breast, Prostate	19q13.1	126, 127
EGF	Cell proliferation, mitogenicity	Breast, Prostate	4q25	128, 129
PDGF	Embryological development	Breast, Prostate	22q13.1	130, 131
GCSF	Cell growth, Survival	Prostate	17q11.2-q12	132
IL-8	Angiogenesis	Breast, Prostate, Colorectal	4q13-q21	121, 133, 134
Angiogenin	Angiogenesis	Breast, Prostate	14q11.1-q11.2	135, 136
CD44	Cell adhesion, migration	Breast, Prostate	11p13	137
HGF	Cell growth, motility	Breast, Prostate, Lung	7q21.1	138-140
AMF	Glycolysis, Neurotropic factor	Breast, Prostate	19q13.1	141, 142
Snail homolog 2	Transcriptional repressor	Breast, Liver	8q11	143, 144
Negative Factor				
E-cadherin	Cell adhesion	Breast, Prostate, Lung	16q22.1	145-147
Fibronectin 1	Cell adhesion molecule	Breast	2q34	148
Vimentin	Cell adhesion molecule	Prostate	10p13	149
Thrombospondin 1	Angiogenesis	Breast	15q15	150
Angiostatin	Angiogenesis	Breast, Prostate	6q26	151, 152
Endostatin	Angiogenesis	Hepatoma	21q22.3	153
Vasostatin	Angiogenesis	Lung	14q32	154

many positive and negative factors. The fact that fusing a non-metastatic cell with highly metastatic cancer cell results in suppression of metastatic ability of the tumor cell raised a hypothesis that tumor metastasis is negatively regulated by tumor metastasis suppressor genes (16). They are defined as genes that suppress the formation of metastases, without affecting the growth rate of the primary tumor. Search for such genes using multiple approaches such as micro-cell mediated chromosome transfer (MMCT), microarray analyses and subtractive hybridization, has been quite effective, and to date, there are fourteen identified genes that clearly meet this criterion (Table 2). The following section summarizes the current information on each of these genes.

4.1. NM23

NM23 was the first gene isolated as a tumor metastasis suppressor. To identify a differentially expressed gene involved in tumor metastasis, Steeg *et al.* utilized a series of related murine melanoma cell lines of varying metastatic potential (17). By subtractive hybridization between the mRNAs from cell lines with low and high metastatic potential, the NM23 gene was isolated (17). They noted that NM23 mRNA levels did not correlate with cells' sensitivity to host immunological responses and therefore must be associated with intrinsic aggressiveness. In addition to the clinical observation of the down-regulation of NM23 gene expression in breast carcinoma (18), transfection of NM23 into highly metastatic breast, melanoma, colon, and oral squamous cell lines reduced *in vivo* metastatic potential of these cells (19-21). In addition, transfection of human NM23 into human breast carcinoma cells reduced *in vitro* motility to numerous attractants and inhibited colonization in soft agar (19). The metastasis suppressive activity of NM23 was

previously correlated with its histidine protein kinase activity although physiological substrates for this unusual kinase activity have not been identified (22). Hartsough *et al.* reported that NM23 co-immunoprecipitated with the KSR (kinase suppressor of Ras) protein and phosphorylated ser-392 and ser-434 on KSR (23). It has been hypothesized that phosphorylation of KSR by NM23 alters its scaffold function, which could lead to reduced ERK activation in response to signaling. In agreement with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced ERK activation levels compared with vector alone control transfectants, while a histidine-kinase-deficient mutant of NM23 showed high levels of activated ERK, compared to those of the controlled transfectants (23). Therefore, altered levels of NM23 in metastatic versus non-metastatic tumor cells might impact ERK activation through a complex interaction with the KSR scaffold protein.

4.2. KAI1

The KAI1 gene was isolated originally by microcell mediated chromosome transfer technique (MMCT) as a prostate-specific tumor metastasis suppressor gene. It is located in the p11.2 region of human chromosome 11 (24, 25). When the KAI1 gene was transferred into a highly metastatic prostatic cancer cell line, KAI1-expressing cancer cells were suppressed in their metastatic ability, whereas their primary tumor growth was not affected (24, 25). Therefore, this gene behaves as a classical tumor metastasis suppressor. DNA sequencing analysis of the KAI1 gene revealed that it is identical to CD82, a surface glycoprotein of leukocytes, which encodes 267 amino acids (27). The protein has four hydrophobic and presumably transmembrane domains and one large extracellular N-glycosylated domain. Consistent with the view that KAI1

Suppressor of tumor metastases

Table 2. Tumor metastases suppressor genes

Gene	Suppressed in cancer	Location	Function	In vitro Motility	In vitro Invasion	Tested in Animal	Immunohistochemistry (% negative in met patients)	Reference
Drg-1	Breast, Prostate, Colon	22q12.2	Inhibit invasion	↓	↓	+	60% (P=0.04) (Breast), 74% (P=0.003) (Prostate)	102, 105, 106, 108
KAI1	Breast, Prostate	11p11.2	Integrin Interaction, EGFR desensitization	↓	↓	+	94.9% (P=0.025) (Breast), 100% (Prostate)	26, 29
BRMS1	Breast, Melanoma	11q13-q13.2	Gap junctional communication	↓	↓	+		49, 50
KiSS-1	Breast, Melanoma	1q32-q41	G-protein-coupled receptor ligand	↓	↓	+	56% (P=0.482) (Melanoma)	43, 155
NM23	Breast, Prostate, Melanoma, Colon	17q21.3	Histidine Kinase	↓	↓	+	66.7% (P=0.013) (Breast), 73% (P=0.289) (Prostate)	17, 156-158
RhoGDI2	Bladder	12p12.3	Regulates Rho & Rac function	↓	↓	+		89
CRSP3	Melanoma	6q22.33-q24.1	Transcriptional coactivator	↓	↓	+		64
MKK4	Prostate, Ovary	17p11.2	MAPKK, JNK kinases	↓	↓	+	67.7% (P<0.0001) (Ovary)	39, 42
VDUP1	Melanoma	1q21.1	Thioredoxin inhibitor					64
E-Cadherin	Breast, Prostate, Gastric, Colorectal, Thyroid, Ovary	16q22.1	Inhibit shedding from primary tumor		↑↓	+	47.7% (P=0.147) (Breast), 27.3% (P=0.004) (Prostate)	55, 159, 160
RKIP	Breast, Prostate, Melanoma	12q24.23	Inhibits Raf-mediated MEK phosphorylation	↓	↓	+	39.2% (p=0.367) (Breast)	66, 161
SSeCKS	Prostate	6q24-25.2	Scaffolding protein for PKC & PKA	↓		+		72
Claudin 7	Breast, Cervical, Gastric	17p13	Tight junction protein					76
RRM1	Lung	11p15.5	Ribonucleotide reductase	↓	↓	+		80, 82

is a metastasis suppressor gene, the immunohistochemical analysis of human tumor samples revealed that the expression of the gene in most cases was downregulated during the tumor progression of not only prostate, but also lung (28), breast (29), bladder (30), and pancreatic cancers (31). The down-regulation of the KAI1 gene expression is also correlated with poor survival in patients with those cancers. Furthermore, in a study of prostate tumors including 120 cases, PCR-single-strand conformational polymorphism and microsatellite analyses revealed that the KAI1 expression was down-regulated consistently during the progression of human prostatic cancer and that this down-regulation did not commonly involve either mutation or allelic loss of the KAI1 gene (26). Therefore, the expression of this gene appears to be down-regulated in advanced tumor cells at or post-transcriptional level, presumably by the loss of an activator or gain of a suppressor.

In order to understand the basic regulatory mechanism of the KAI1 gene expression, the 5' upstream region of the KAI1 gene was cloned by screening a human placental genomic library in our laboratory (32). The KAI1 promoter revealed a p53 consensus binding site and in addition, reverse transcription-PCR analysis revealed that

the expression of endogenous KAI1 mRNA was augmented significantly by p53. The results of the promoter analysis using a reporter plasmid containing the 5' upstream sequence indicated that the KAI1 gene was indeed positively controlled by p53 at the transcriptional level in prostatic tumor cells. By subsequent analysis of the promoter sequence of the KAI1 gene by site specific mutagenesis and gel-shift mobility assay, we found that the region of 272 bp, which was approximately 860 bp upstream of the transcriptional initiation site, was responsible for this p53 activation (32). Results from these experiments clearly indicate that p53 activates the KAI1 gene at the transcriptional level through its binding to the specific site of the 5' upstream region.

In the search for a specific agent which re-activates the expression of the KAI1 gene, it was found in our laboratory that etoposide, a topoisomerase II inhibitor, is able to activate the expression of the KAI1 gene in a dose-dependent manner in human prostate cancer cell lines as well as in human lung carcinoma cells (33). Our results suggest that the augmentation of the KAI1 gene expression by etoposide is independently controlled by both p53 and c-Jun at the transcriptional level in the human prostate tumor cell lines. Furthermore, treatment of these cell lines with

etoposide resulted in a significant reduction of cellular invasion (33). Because etoposide has been shown to be effective on advanced prostate cancer when used in combination with other regimens, our results provide a further rationale to use this drug as an anti-metastatic agent.

How the KAI1 gene suppresses the metastasis process remains the most intriguing question. Recently, Odintsova *et al.* found that KAI1 physically associates with the EGF receptor and rapidly desensitizes the EGF-induced signal that could lead to suppression of cell migration (34). However, it is yet unclear whether this mechanism indeed accounts for the metastasis suppression *in vivo*. The crucial clue to understand the biochemical function of the KAI1 gene came from the results of the recent studies on T-cell activation. KAI1/CD82 is barely detectable on resting peripheral T and B lymphocytes, while its expression is highly up-regulated upon activation of these cells (35). This up-regulation is associated with some morphologic change and expression of activation markers such as CD82 and MHC II antigens. Lebel-binay *et al.* described that the co-engagement of KAI1/CD82 and TCR by anti-CD82 mAb and anti-CD3 mAb, respectively, was able to activate T cell and that, when a T-cell is stimulated *in vitro* by anti-KAI1/CD82 mAb, KAI1/CD82 appears to transmit a signal which results in tyrosine phosphorylation, a rapid increase in intracellular Ca^{2+} level and IL-2 production (36). Interestingly, this activation was associated with a change in cellular morphology and inhibition of cell proliferation (37). Therefore, it is tempting to speculate that tumor cells of epithelial origin may also employ a similar signal pathway upon activation of KAI1/CD82, which results in growth arrest of tumor cells. In fact, it was shown that NGF was capable of up-regulating the expression of KAI1 in prostate cancer cell lines, and this activation was associated with remarkable down-regulation of cell proliferation *in vitro* and *in vivo* (38). Although it remains to be tested whether the KAI1 up-regulation is coupled to the inhibition of cell proliferation, this raises an attractive possibility that activation of KAI1 may lead to growth suppression in tumor cells of epithelial origin similar to that in cells of haematopoietic origin under certain conditions. Thus the existing information points to a very diverse mode of activation of KAI1/CD82 as revealed in the *in vitro* experiments.

4.3. MKK4

The MKK4 gene was originally identified as a metastasis suppressor for prostate cancer by combination of MMCT and differential expression approaches (39). Following identification of metastasis suppressor activity of a 70cM region on human chromosome 17 in an *in vivo* animal model (40), Yoshida *et al.* examined the genes located within this region and having a biological function suggesting a potential role in metastasis suppression (39). Putative candidate genes that were not specifically retained or expressed by microcell mediated chromosome 17-transferred prostate cancer cells and normal prostate tissue were eliminated from further consideration. MKK4/SEK1 was identified as a candidate gene based on its physical location, 17p11.2, within the 70-cM metastasis suppressor region, and the fact that its normal cellular function in the

stress-activated signaling pathway suggests that alteration of this gene may have pleiotrophic effects on the cell (39). The same group of investigators also observed that expression of the MKK4 gene in a metastatic prostate cancer cell line significantly reduced the number of macroscopic lung metastases in SCID mice as compared with the lungs from control animals, without affecting the primary tumor growth (39). Detailed histological examination of sections from the lungs of tumor-bearing animals indicated that lungs from control mice had large metastatic foci while the lungs from mice bearing MKK4-positive tumors contained significantly small foci. In addition, cuffs of cells approximately two to three layers thick were observed around blood vessels in several of the sections from the MKK4-positive samples, suggesting that the tumor cells may co-opt existing host vasculature for growth (39).

In order to understand the clinical significance of the MKK4 gene in cancer progression, Kim *et al.* performed immunohistochemical studies on clinical samples of prostate cancer (41). The study revealed high levels of MKK4 expression in the epithelial but not the stromal compartment of normal prostatic tissues with a significant down-regulation of expression in the neoplastic tissues, and a statistically significant inverse relationship between Gleason pattern and MKK4 was observed (41). These results demonstrate that the MKK4 gene is consistently down-regulated during prostate cancer progression and supports the notion that dysregulation of the MKK4 signaling cascade plays a crucial role in progression of metastatic disease. Similar results have been reported for ovarian cancer as well (42). To test the possibility that down-regulation of MKK4 protein is the result of allelic loss, Kim *et al.* examined the metastatic prostate cancer lesions for loss of heterozygosity (LOH) within the MKK4 locus and found that the downregulation of MKK4 expression in cancer patients does not frequently involve allelic loss or mutation of this gene (41). Although MKK4 is a central molecule in the cell's stress response pathway, how this gene inhibits the metastasis process is yet to be understood.

4.4. KiSS-1

The KiSS-1 gene was originally identified as a metastatic melanoma suppressor gene by combining the aspects of the strategies of both MMCT and differential display. After the introduction of human chromosome 6 into human metastatic melanoma cell lines C8161 or MelJuSo by MMCT resulted in a significant suppression of metastasis without affecting tumorigenicity or local invasiveness, a subtractive hybridization between the highly metastatic parental C8161 and the chromosome 6-C8161 hybrid cells led to the identification of the KiSS-1 transcript (43). The functional role of KiSS-1 in metastasis suppression was evident when the full-length KiSS-1 transfectants suppressed the lung colonization of tumor cells in spontaneous metastasis assay without affecting the growth of the tumor cells *in vivo* (43). Based on the observation that chromosome 1q is frequently deleted in late-stage human breast carcinomas, Lee *et al.* tested whether the KiSS-1 gene that maps to chromosome 1q32-

q41 could suppress metastasis of the human breast carcinoma cell line MDA-MB-435 (44). They found that the expression of KiSS-1 almost completely abrogated the metastatic potential as compared to control cells but did not suppress tumorigenicity. Therefore, KiSS-1 acts as a metastasis suppressor for breast carcinoma as well. The same investigators also noted that metastasis suppression by KiSS-1 correlated with a decreased three-dimensional growth of cells in soft agar but invasion and motility were unaffected. Based on the predicted structure of the KiSS-1 protein, these results imply a mechanism whereby KiSS-1 regulates events downstream of cell-matrix adhesion, perhaps involving cytoskeletal reorganization.

Yan *et al.* have recently found that colon carcinoma cell lines HT-1080 stably transfected with a KiSS-1 expression construct, demonstrated substantially lower MMP-9 enzyme activity and *in vitro* invasiveness (45). The lower MMP-9 enzyme activity reflected reduced steady-state mRNA level that in turn was due to attenuated transcription. Moreover they noted that while activation of ERKs and JNKs by phorbol 12-myristate 13-acetate and tumor necrosis factor alpha, respectively, were able to increase the MMP-9 expression, this MMP-9 activation was not antagonized by KiSS-1 expression, suggesting that MAPK pathways modulating MMP-9 synthesis are not the target of KiSS-1 (45). They further observed that although MMP-9 expression is regulated by AP-1, Sp1 and Ets transcription factors, KiSS-1 did not alter the binding of these factors to the MMP-9 promoter. However, NF- κ B binding to the MMP-9 promoter required for expression of this collagenase was reduced by KiSS-1 expression. Diminished NF- κ B binding reflected less p50/p65 in the nucleus secondary to increased I- κ B levels in the cytosols of the KiSS-1 transfectants (45). Their results suggest that KiSS-1 diminishes MMP-9 expression by effecting reduced NF- κ B binding to the promoter. Another important clue for KiSS-1 function came from the study of Ohtaki *et al.* (46), who isolated a 54 amino acid peptide from human placenta that turned out to be encoded by Kiss-1 C-terminus and served as the endogenous ligand for an orphan G-protein-coupled receptor (hOT7T175). Named as 'Metastin', this peptide inhibits chemotaxis and invasion of hOT7T175-transfected CHO cells *in vitro* and attenuates pulmonary metastasis of hOT7T175-transfected B16-BL6 melanomas *in vivo*. These results suggest possible mechanisms of action for KiSS-1 and a potential new therapeutic approach. Interestingly, since then, similar results have been reported by two other groups independently (47, 48).

4.5. BRMS1

Several regions spanning the q-arm of chromosome 11 have been found to be associated with a majority of breast cancer cases, the most common being amplifications and deletions involving regions near band 11q13 (49). In particular, reports of high-frequency deletions involving 11q13-q14 in late-stage, metastatic breast carcinomas were suggestive of the existence of a metastasis suppressor gene in this region (49). This was further corroborated by the finding that introduction of a normal human chromosome 11 into the metastatic MDA-

MB-435 human breast carcinoma cells by microcell-mediated transfer significantly suppressed metastasis without affecting tumorigenicity. Then, DD-RT-PCR for highly metastatic (MDA-MB-435) parental cells versus the metastasis-suppressed clones led to the identification of three novel cDNA fragments, one of which was identified as BRMS1 (50). Over-expression of BRMS1 in metastatic breast carcinoma cells suppressed metastasis in both spontaneous and experimental breast cancer metastasis models (50). In addition, the same gene was also found to act as a metastasis suppressor for melanoma (51). Stable transfection of BRMS1 in the human melanoma cell lines MelJuSo and C8161.9 did not alter the tumorigenicity of either cell line, but significantly suppressed metastasis compared to vector-only transfectants (51). However, the expression of this gene has not yet been examined in clinical setting.

Toward analyzing mechanisms underlying suppression of metastasis by BRMS1, Samant *et al.* observed that expression of BRMS1 in tumor cells did not make significant difference in adhesion to extracellular matrix components (laminin, fibronectin, type IV collagen, type I collagen) or invasion and only modestly inhibited the motility of the cells and, in some cases, inhibited the ability of the cells to grow in three-dimension in soft agar (52). The results of their study also ruled out the possibility of BRMS1 upregulating expression of other metastasis suppressors, such as NM23, KAI1, KiSS1 or E-cadherin. Some clue regarding function of BRMS1 came from a study by Saunders *et al.*, who reported that transfection and re-expression of BRMS1 restored the ability of human breast carcinoma cells (MDA-435) to form functional homotypic and heterotypic gap junctions (53). Cx43 and Cx26 (connexins) are the predominant gap junction protein in normal breast epithelial tissue but are often reported to be lost in neoplastic breast tissue. Metastatic MDA-MB-435 cells express Cx32 but not Cx43 or Cx26, and restoring BRMS1 expression in this cell line resulted in re-establishment of gap junction but only partly restored Cx43 expression. Based on these observations Saunders *et al.* suggested that re-expression of the BRMS1 gene restores the Cx expression profile from that of a metastatic cell to that more similar to a normal breast epithelial cell and that the composition of gap junctions contributes to metastatic propensity (53).

4.6. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screening a cDNA library of the human liver (54). The E-cadherin is a calcium-dependent adhesion molecule and constitutes a main component of the adherence junction in epithelia cells. Calcium ions bind to the extracellular domain of E-cadherin at the adhesion site of cell-cell junction, while the intracellular domain of this molecule interacts with beta-catenin to mediate actin binding. E-cadherin also sequesters the function of beta-catenin by blocking nuclear translocation which results in inhibition of transcription of c-myc and cyclin D1 (55). The expression of E-cadherin is generally reduced in a variety of human cancers at advanced stages. It is believed that tumor cells with a low level of E-cadherin can be readily detached from

adjacent cells, and these cells invade and metastasize to other distant organs. Several groups have indeed reported that decreased expression of E-cadherin was associated with a poor prognosis of cancer patients (56). Most importantly, over-expression or maintenance of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness (55). Therefore, E-cadherin is considered to function as a metastasis suppressor. Interestingly, E-cadherin has recently been found to be regulated by Snail and Slug (57) that are zinc-finger transcription factors and involved in the process of cell differentiation and apoptosis (58). In breast carcinomas, Snail and Slug have been recently shown to be involved in tumor progression and invasiveness (57), and it is postulated that these proteins repress the expression of E-cadherin (57).

4.7. VDUP1 (TXNIP) and CRSP3

The VDUP1 (Vitamin D3 upregulated protein 1) gene was first identified by the differential display technique as a gene induced by 1,25-dihydroxyvitamin D-3 (59). VDUP1 is able to interact with a reduced form of TRN (60), which results in inactivation of TRN. TRN is an inhibitor for apoptosis signal-regulating kinase 1 (ASK-1) which is known to be a central component of stress-induced apoptosis (61). Therefore, VDUP1 is also considered to participate in this signal pathway through the binding to TRN (62). In fact, the expression of VDUP1 has been shown to arrest cell growth of NIH3T3 cells (63). Consistent with these *in vitro* results, immunohistochemical analyses for tumor specimens revealed that the expression of VDUP and TRN were inversely correlated in many tumors. Over-expression of VDUP1 in a metastatic cell line followed by injection into mice significantly reduced the incidence of lung metastases, suggesting that VDUP1 functions as a metastasis suppressor. The regulatory mechanism of the VDUP1 gene has not been well understood, however, Goldberg and colleagues recently found that VDUP1 is controlled by a transcription factor, CRSP3, and suggested that CRSP3 may also act as a metastasis suppressor and as an up-stream regulator of VDUP and KiSS-1 in human melanoma (64). CRSP3 is known as a co-factor in Sp1 (Specificity protein 1) mediated transcription, and transfection of an expression plasmid of CRSP3 into melanoma cells significantly increased the expression of KiSS1 and VDUP1 genes. Consistent with the notion that CRSP3 is a metastases suppressor gene, over-expression of the CRSP3 gene in metastatic melanoma cells and transplantation of these cells into mice significantly decreased the rate of lung metastasis. Furthermore, the expression of VDUP1 and CRSP3 genes has been shown to be inversely correlated with the progression of melanoma by using quantitative real-time RT-PCR. Therefore, both VDUP1 and CRSP3 apparently act as metastases suppressors via the KiSS1 pathway. However, mechanism of metastases suppression by these genes is not yet clear.

4.8. RKIP

Raf kinase inhibitor protein (RKIP) is a member of the phosphatidylethanolamine binding protein (PEBP) family. RKIP encodes a protein which inhibits the Raf/mitogen-activated protein kinase /extracellular signal-

regulated kinase (ERK) pathway. This signaling plays an important role in determining cell fate and choosing between diverse responses such as proliferation, differentiation and survival. Interestingly, RKIP was recently identified as a gene significantly down-regulated in a metastatic cell line (C4-2B) of prostate cancer by microarray analyses (65). This result was further corroborated by immunohistochemical examination of clinical tissue samples from cancer patients. It was found that RKIP was usually expressed in benign tissues while it was significantly down-regulated in tumors, especially in metastatic cells. These results suggest that RKIP is associated with suppression of metastasis. In consistence with these data, over-expression of RKIP in a metastatic cell line derived from prostate cancer has been shown to have no effect on cell proliferation or colony-formation ability in soft agar but significantly lower the invasive potential of these cells. Furthermore, overexpression of RKIP drastically decreased the lung metastases of these cells when transplanted into animals without affecting primary tumor growth (66).

Since RKIP is an inhibitor of Raf which phosphorylates MEK and ERK, Fu *et al.* examined the status of phosphorylation of these target proteins in various prostate cancer cell lines and found that both MEK and ERK had higher basal levels of the phosphorylated forms in metastatic cells than in non-metastatic cell line, without significant changes in the total protein level (66). Conversely, the degree of phosphorylation of these target proteins was lower in metastatic cell with RKIP over-expression than in mock transfected cells. In this context, it should be noted that treatment of a metastatic cell line with a MEK kinase inhibitor significantly reduced the invasiveness of the cells, suggesting that RKIP suppresses tumor invasion through MEK activity (66). Interestingly, RKIP has also been shown to promote apoptosis of cancer cell, and low level of RKIP expression significantly increases resistance to chemotherapeutic-induced apoptosis. Thus RKIP also appears to contribute to response of cancer cells in chemotherapy (67).

4.9. SSeCKS

SSeCKS (Src-Suppressed C Kinase Substrate) was originally isolated by using PCR-based subtractive hybridization (68, 69). Over-expression of the SSeCKS gene via a retroviral vector caused a significant reduction in cell proliferation compared to a normal control cell or *src*-transfected cell, suggesting that SSeCKS encodes a regulator of mitogenesis. SSeCKS was also known as an orthologue of human Gravin/AKAP12 (A kinase anchor protein 12) which was previously identified as a cytoplasmic antigen recognized in sera from patients with myasthenia gravis (70) and later found to be the cytoplasmic scaffolding protein for protein kinase A and C (71, 72). Recently, Xia *et al.* showed that both RNA and protein levels of SSeCKS/Gravin were significantly decreased in metastatic prostate cancer cell lines of human and rat origin compared to non-metastatic cell lines (72). They also found that the expression of SSeCKS/Gravin inhibited anchorage-independent growth without affecting the cell proliferation. Furthermore, over-expression of

SSeCKS/Gravin in metastatic cell line followed by injecting it into mice significantly decreased the incidence of lung metastasis. Therefore, SSeCKS/Gravin appears to function as a metastasis suppressor.

4.10. Claudin

Claudins, a family of integral membrane proteins, are the basic molecules involved in tight junction structure and function (73). Tight junctions are responsible for controlling the paracellular permeability, cell adhesion and cell polarity. These functions of tight junctions that are often lost in cancer may play a crucial role in tumor growth and metastasis (74). Claudins as prime constituents for tight junctions have been found to be abnormally regulated in human breast and prostate cancers. Claudin-3 and claudin-4 are typically over-expressed in adenocarcinomas including prostate and breast cancers. On the other hand, recent study with pancreatic cancer suggests that claudin-4 functions as an inhibitor of the invasiveness of cells (75). Interestingly, claudin-7 has been found to be significantly down-regulated in invasive ductal carcinomas (IDC) of the breast and there is an inverse correlation between the expression of claudin-7 and cellular discohesion in breast carcinomas (76). These results suggest that claudin-4 and 7 are putative metastasis-suppressors, although the role of claudin-4 in the metastasis process remains to be clarified further.

4.11. RRM1

RRM1 (ribonucleotide reductase M1 polypeptide) encodes the regulatory subunit of ribonucleotide reductase which is known to catalyze the rate limiting step of deoxyribonucleotide formation (77-79). RRM1 is located on chromosome 11p15.5 which is often lost in lung cancer at advanced stages and is also significantly associated with metastatic spread in lung cancer patients (80, 81). A recent study by Bepler and colleagues showed that over-expression of RRM1 induced expression of the known tumor suppressor gene, PTEN, in human and mouse cell lines, and also in animal model (82). These authors found that a lung derived stable cell line over-expressing RRM1 significantly reduced migration and invasive abilities compared with a control cell line. The overexpression of RRM1 also strongly induced the expression of PTEN in these cell lines. Importantly, the expression of RRM1 suppressed spontaneous metastasis to the lung and prolonged survival in animals. Therefore, RRM1 appears to function as a metastasis suppressor through induction of PTEN in lung cancer. In fact, immunohistochemical analyses of clinical samples revealed that the expression of RRM1 was significantly correlated with PTEN and RRM2 (ribonucleotide reductase M2 polypeptide) (83). Furthermore, high expression of RRM1 was found to be predictive of long survival independent of tumor stage, performance status, and weight loss (83, 84).

4.12. RhoGDI2

The Rho proteins belong to a guanine nucleotide family and they exist in two different forms as being active when bound to GTP and inactive when bound to GDP.

RhoGDIs (GDI: GDP-dissociation inhibitor) are the class of proteins that inhibit the dissociation of GDP and stabilizes the inactive form of Rho proteins. RhoGDI2 is a 200 amino acid protein with a molecular weight of 229 kDa and it was first discovered by Leffers *et al.* (85). It was found to be expressed in human and murine hematopoietic tissues, predominantly in B and T lymphocytes (86) as well as in non-hematopoietic neoplastic cells (87). RhoGDI2 is phosphorylated in response to stimulation of T lymphocytes and myelomonocytes cells, and it is involved in inducing hematopoiesis (88). On the other hand, recent study of Gildea *et al.* (89) has shown that inducible expression of exogenous RhoGDI2 in metastatic cells blocked lung metastasis and significantly suppressed invasiveness and motility of cultured cells but did not affect the *in vitro* growth rate, colony formation or *in vivo* tumorigenicity. The intricacy of mechanism by which RhoGDI2 restricts metastasis is yet to be elucidated, but it is speculated that RhoGDI2 suppresses the metastatic process by impeding the tumor cells from invading and colonizing the lung upon reaching the pulmonary vasculature. RhoGDI2 has also been identified as a potent metastatic suppressor in bladder cancer. Therefore, RhoGDI2 is considered as a general metastases suppressor.

4.13. Drg-1

The Drg-1 gene was originally found to be induced *in vitro* by cellular differentiation and hence named as Differentiation-Related-Gene-1 (90). Since then, three more genes, namely, Drg-2, 3 and 4 have been identified that encode proteins highly related to Drg-1 (91, 92). These genes constitute the NDRG gene family although the members vary in the pattern of tissue-specific expression and possibly in function. Drg-1 is identical to the human RTP, cap43 and rit42, and homologous to the mouse genes TDD5 and Ndr1 and rat Bdm1 (93-98). The protein encoded by the Drg-1 gene has a molecular weight of 43 kDa and possesses three unique 10-amino acid tandem repeats at the C terminal end. Analysis of the amino acid sequence predicted that there were seven or more phosphorylation sites, and Drg-1 indeed has been shown to be phosphorylated by Protein Kinase A *in vitro* (99). Drg-1 mRNA is detected in most of the organs, and the level of expression is particularly high in prostate, ovary, intestine and kidney. It was shown that the expression of this gene was repressed by c-myc and N-myc/Max complex *in vitro* (97). On the other hand, p53 was found to be able to induce expression and nuclear translocation of Drg-1 in response to DNA damaging agents (95). The expression of the gene was also augmented by hypoxia and PTEN, and the combination of Drg-1 and PTEN has indeed been shown to be an indicative marker for outcome in patients with both breast and prostate cancers (100-102). In addition, the Drg-1 gene has been shown to be upregulated by hormones such as androgen (96) and by various chemical agents including homocysteine, mercaptoethanol, tunicamycin (98), lysophosphatidylcholine (103), nickel compounds (94) and synthetic retinoids (104). Therefore, the Drg-1 gene is controlled by multiple factors and responsive to various stimuli.

Table 3. Relationship between Drg-1 and other clinical parameters in prostate cancer

Drg-1 expression	All	Positive	Reduced	P value
Gleason grade				
≤ 7	38	26	12	
> 7	24	8	16	0.015 ¹
P53				
Wild type	59	32	27	
Mutant	3	2	1	0.8
Differentiation				
Well	16	14	2	
Moderate	19	14	5	
Poor	27	6	21	<0.001 ¹
Nuclear grade				
I	32	22	10	
II / III	30	12	18	0.044 ¹
Metastasis status				
Organ confined	40	28	12	
Lymph node	20	5	15	0.003 ¹
Bone	19	5	14	0.006 ¹

¹ Statistically significant. Ref 62

Cell line	Expression of Drg-1*	Tumor in animal	Metastases in lung**	
parental AT6.1	 (-)	5/5	153.7 +/- 2	
Drg-1 #7	 (+)	5/5	5.8 +/- 2.5	
Drg-1 #8	 (+)	5/5	11.4 +/- 5.5	
Drg-1 #12	 (-)	5/5	176 +/- 33.1	

Figure 1. Drg-1 suppresses spontaneous lung metastasis without affecting growth of primary tumor. The parental cell line (AT6.1) and Drg-1-transfected clones (#7, #8, and #12) were tested for Drg-1 protein expression by Western blot. Each of these cell lines was injected subcutaneously into SCID mice. After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from mice from each group are shown as examples.

Since the Drg-1 gene is strongly correlated with differentiation and tumor progression is invariably associated with loss of differentiation, we analyzed the Drg-1 expression status in clinical samples of human prostate and breast cancer (105, 106). In both cases, Drg-1 was found to be highly expressed in the epithelial cells of normal glands and ducts where the protein was localized mostly in the cytoplasm. The Drg-1 protein was detected consistently in all cases of normal prostate tissue as well as PIN (Prostatic Intraepithelial Neoplasia) and BPH (Benign Prostatic Hyperplasia), and normal mammary gland cells, while the Drg-1 expression was significantly reduced in the tumor cells of cancer patients (105, 106). In the case of prostate cancer, the reduction in Drg-1 expression correlated significantly with the Gleason grade. A study by Caruso *et al.* also found similar trend of downregulation of Drg-1 expression in prostate cancer, and interestingly, they also observed a significant correlation between Drg-1 expression pattern and ethnic origin of the patients (107).

Most interestingly, in both prostate and breast cancers, we observed a significant level of differential expression of Drg-1 between the patients with organ-confined disease and those with metastasis to lymph node or bone (Table 3,106). In case of prostate cancer, the negative correlation of Drg-1 with metastatic spread to lymph node and bone is highly significant, and in fact, is much stronger than the positive correlation with Gleason scores. In breast cancer, a similar and significant negative correlation of Drg-1 with metastases has been observed (106). These results strongly suggest the negative involvement of Drg-1 in the process of invasion and metastasis in both prostate and breast cancer.

The significant inverse correlation of Drg-1 expression with the extent of metastasis at the clinical level raised the next important question as to whether the down-regulation of Drg-1 is cause or result of metastases. To address this issue, we over-expressed the Drg-1 gene in a highly metastatic prostate cell line and implanted it into SCID mice. The result of this experiment indicated that all the clones formed primary tumors in the animals with similar growth rates (data not shown), suggesting that Drg-1 does not have an effect on tumorigenesis and tumor growth. On the other hand, the clones that were positive for Drg-1 expression exhibited a significantly lower incidence of lung metastases compared with the vector-transfected cell line (Figure 1). Similar metastasis suppressor effect of Drg-1 was also observed in colon carcinoma cells by Guan *et al.* (108). Furthermore we observed that Drg-1 significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay (105, 106). Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that Drg-1 is a metastasis suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How Drg-1 suppresses the tumor metastases is an intriguing question which is under active investigation.

5. CONCLUSION AND FUTURE DIRECTIONS

The development of metastases is a major obstacle to the successful treatment of a patient with any cancer. Much of the lethality of malignant neoplasms is directly attributable to their ability to develop secondary growths in organs at a distance from the primary tumor mass, while few patients die from their primary neoplasm. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the cancer field. This is because of the fact that metastasis involves multiple steps with high complexity. A possible breakthrough in our understanding of cancer progression has emerged with the hypothesis that tumor metastasis is negatively controlled by tumor metastasis suppressor genes. Thus far fourteen genes have been identified that are defined as tumor metastases suppressors. Almost all of them are also significantly down-regulated in advanced stages in a variety of cancers. However the mechanism of metastases suppression for most of the genes is yet to be clarified. A

cross-talk between these proteins remains an intriguing question. The mechanism of down-regulation of these genes in tumor cells also needs to be addressed. Recent studies in this field have begun to shed light on these questions and understanding the molecular mechanism of tumor metastases suppression would eventually lead to the development of therapeutic approaches to intervene in the process of metastatic disease.

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Suppressor of tumor metastases

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Interaction of KAI1 on tumor cells with DARC on vascular endothelium leads to metastasis suppression

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CD82, also known as **KAI1**, was recently identified as a prostate cancer metastasis suppressor gene on human chromosome 11p1.2 (ref. 1). The product of **CD82** is **KAI1**, a 40- to 75-kDa tetraspanin cell-surface protein also known as the leukocyte cell-surface marker **CD82** (refs. 1,2). Downregulation of **KAI1** has been found to be clinically associated with metastatic progression in a variety of cancers, whereas overexpression of **CD82** specifically suppresses tumor metastasis in various animal models³. To define the mechanism of action of **KAI1**, we used a yeast two-hybrid screen and identified an endothelial cell-surface protein, **DARC** (also known as **gp-Fy**), as an interacting partner of **KAI1**. Our results indicate that the cancer cells expressing **KAI1** attach to vascular endothelial cells through direct interaction between **KAI1** and **DARC**, and that this interaction leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of **TBX2** and **p21**. Furthermore, the metastasis-suppression activity of **KAI1** was significantly compromised in **DARC** knockout mice, whereas **KAI1** completely abrogated pulmonary metastasis in wild-type and heterozygous littermates. These results provide direct evidence that **DARC** is essential for the function of **CD82** as a suppressor of metastasis.

We screened the human normal prostate cDNA library using the full-length **CD82** cDNA as bait in a yeast two-hybrid interaction trap⁴ and identified Duffy antigen receptor for chemokines (**DARC**, also known as **gp-Fy** and encoded by **DARC**) as a potential interactor for **KAI1**. A liquid β -galactosidase assay quantitatively showed the strength and specificity of the interaction between **KAI1** and **DARC** (Fig. 1a). **DARC** is an approximately 45-kDa, seven-transmembrane protein expressed on vascular endothelium of various organs, as well as on red blood cells and certain epithelial cells^{5,6}. It binds chemokines of both C-C and C-X-C families, although ligand binding by **DARC** does not induce G-protein-coupled signal transduction or Ca^{2+} flux^{7,8}. The **DARC** gene has two alleles, **Fya** and **Fyb**, which differ only at amino acid residue 44 (ref. 9). Sequence analysis showed that the cloned

DNA identified by our screening represents the spliced isoform of the **Fyb** allele of **DARC** (Fig. 1b). To examine the interaction of **KAI1** and **DARC** in mammalian cells, we carried out a coimmunoprecipitation experiment using a highly metastatic prostate carcinoma cell line, **AT6.1**, which was stably transfected with a Flag epitope-tagged **DARC** gene. The cells were then transiently transfected with a hemagglutinin (**HA**)-tagged **KAI1** plasmid, and the cell lysate was incubated with antibody to Flag. We found that **KAI1** coprecipitated with Flag-tagged **DARC**, suggesting that **KAI1** can interact with **DARC** in mammalian cells (Fig. 1c). To localize the regions of **KAI1** and **DARC** that are essential for this interaction, we tested individual domains as well as serial deletions from the amino terminus of **KAI1** against full-length **DARC** target and vice versa in yeast mating assay. Our results indicate that the first intracellular and transmembrane domains of **KAI1** are dispensable for this interaction (Fig. 1d). On the other hand, deletion of the first extracellular domain of **DARC** at the amino terminus completely abrogated the interaction, suggesting that the amino terminus of **DARC** is essential for binding to **KAI1** (data not shown).

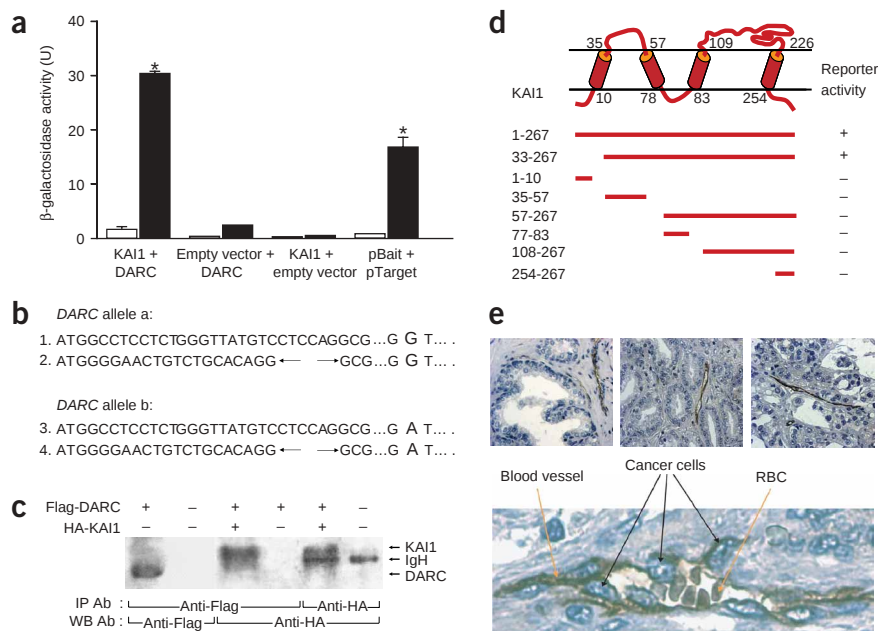
To assess the relevance of the interaction between **KAI1** and **DARC**, we next examined the localization of **DARC** in prostate cancer tissue by immunohistochemistry. We found that **DARC** is highly expressed in the prostate endothelium, particularly in the small veins and venules, as well as in lymphatic vessels, whereas it was undetectable in the epithelial cells and stroma (Fig. 1e). The expression of **DARC** in endothelium was found to be essentially the same in normal, hyperplastic glands and high-grade carcinomas. We observed a similar pattern of expression of **DARC** in breast and lung cancer samples (data not shown). On the other hand, **KAI1** is highly expressed in the normal epithelial cells in these organs, and its expression is substantially reduced in carcinoma, as reported previously³. Because expression of **DARC** in these organs is restricted to the vasculature, it is unlikely that **KAI1** on epithelial cells interacts with **DARC** protein in the same cell. Instead, it suggests that such an interaction takes place when cancer cells expressing **KAI1** intravasate and encounter the endothelial lining of small blood vessels. Consistent with this hypothesis, a previous study using epifluorescence microscopy detected

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Figure 1 KAI1 interacts with DARC *in vitro*.

(a) Quantification of interaction between KAI1 and DARC. Yeast cells transformed with an appropriate combination of expression plasmids were grown in minimal medium in the presence of glucose (white bar) or galactose (black bar) as indicated. The β -galactosidase activity is expressed in Miller units (U). pBait and pTarget are a pair of positive control interactors provided by the manufacturer. (b) Alleles and splice variants of DARC. The junctions of two exons in the biexonic isoforms (#2, #4) are indicated by arrows. (c) Coimmunoprecipitation of DARC and KAI1 in mammalian cells. AT6.1/Flag-DARC permanent clone or the parental cell line was tested for DARC expression by immunoprecipitation with monoclonal antibody to Flag covalently crosslinked to agarose beads followed by western blot with monoclonal antibody to Flag (lanes 1, 2). For coimmunoprecipitation, AT6.1/Flag-DARC cells were transiently transfected with HA-tagged KAI1 expression plasmid, proteins were pulled down by Flag-specific agarose beads and KAI1 was detected by western blot with antibody to hemagglutinin (lane 3). To confirm the HA-KAI1 position, the AT6.1/Flag-DARC cells were transfected with HA-KAI1 as above and immunoprecipitation and western blot were performed with monoclonal antibody to hemagglutinin and protein G agarose followed by western blot with the same monoclonal antibody (lane 5). AT6.1/Flag-DARC cells without KAI1 transfection or parental AT6.1 cells served as negative controls (lanes 4, 6). IgH appeared in lanes 5 and 6, as antibody to hemagglutinin was not crosslinked to the agarose beads during immunoprecipitation. (d) Analysis of interactions of various domains of KAI1 with DARC. Regions of KAI1, as indicated by the amino acid sequence numbers, were tested: '+' indicates positive interaction and '-' indicates lack of interaction. (e) DARC is expressed only in the vascular endothelium of prostate tissue. Immunohistochemistry was performed on clinical samples using the polyclonal antibody to DARC. Representative fields of normal prostate gland and various grades of prostate carcinoma are shown in the upper panel. DARC is detectable only in the vascular endothelium and red blood cells (RBC). The lower panel represents a magnified view of a blood vessel from a high-grade cancer section.



metastatic tumor cells attached to the endothelium of precapillary arterioles and capillaries in intact mouse lungs¹⁰. In agreement with this observation, in our archive of specimens, examination of small blood vessels in a high-grade cancer area indicated that cancer cells are often attached to endothelium of blood vessels (**Fig. 1e**).

We next tested the possibility that KAI1 on tumor cells interacts with DARC on endothelial cells by performing a cell-to-cell binding assay *in vitro* in which green fluorescent protein (GFP)-tagged AT6.1 (KAI1⁻) or AT6.1/Flag-KAI1 (KAI1⁺) cells were overlaid on DARC⁺ endothelial cells, human bone marrow endothelial cells (HBMEs) and human umbilical vein endothelial cells (HUVECs). We observed a significantly higher percentage of attachment of KAI1⁺ cells compared with KAI1⁻ cells to both types of endothelial cells in a time-dependent manner. Moreover, antibody to KAI1 abrogated this binding, indicating the direct involvement of KAI1 in the process (**Fig. 2a**). We next carried out the same binding assay by overlaying the tumor cells on AT6.1 cells with or without expression of DARC. KAI1⁺ tumor cells exhibited a binding affinity specifically to the DARC⁺ AT6.1/Flag-Fy cells (**Fig. 2a**), confirming that the binding of KAI1⁺ cells to these endothelial cells is indeed due to the expression of DARC. To show a direct interaction between these two membrane proteins in a cell-to-cell manner, we mixed the KAI1⁺ tumor cells HT-38 and DARC⁺ HUVECs in the presence of the membrane-impermeable cross-linker 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP), lysed the cells and performed a coimmunoprecipitation experiment. KAI1 coprecipitated with DARC (**Fig. 2b**), whereas another tetraspanin (CD81) did not, indicating a specific interaction between KAI1 and DARC. These results indicate that KAI1-expressing tumor cells can bind to endothelial cells via the interaction between KAI1 and DARC, and suggest the possibility that the metastasis suppressor function of KAI1

is partly due to the trapping of the tumor cells on the endothelial linings of vessels.

It was previously reported that treatment of Jurkat cells with a monoclonal antibody to KAI1 inhibited proliferation of the cells *in vitro*¹¹. Therefore, we sought to determine whether this antibody would elicit a similar response in tumor cells expressing KAI1. We found that this antibody significantly inhibited DNA synthesis in KAI1⁺ prostate tumor cells (**Fig. 2c**). We also obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown). These results suggest that the growth of KAI1-expressing tumor cells is suppressed when KAI1 on the tumor cell surface is engaged by an appropriate ligand. Consistent with this idea, it was previously reported that exposure of prostate tumor cells to nerve growth factor led to upregulation of KAI1, which was also associated with downregulation of cell proliferation *in vitro*¹². To examine whether the signaling pathway leading to growth arrest of tumor cells is also activated when KAI1 binds to DARC, we measured the rate of DNA synthesis in tumor cells when they were allowed to contact cells that either did or did not express DARC. The rate of DNA synthesis was significantly reduced only when the cells expressing KAI1 (AT6.1/Flag-KAI1) contacted the DARC⁺ endothelial cells (HBMEs or HUVECs) or the prostate carcinoma cell line (AT6.1/Flag-DARC; **Fig. 2d**). We obtained similar results for the breast and lung carcinoma cell lines MDA-MB-435 and A549, respectively (data not shown).

To further corroborate the notion of growth arrest of tumor cells upon interaction with DARC on the endothelial cell surface, we mixed GFP-tagged AT6.1 and AT6.1/Flag-KAI1 cells with HBMEs or HUVECs and then selected for GFP⁺ tumor cells. We found that the ability of tumor cells to form colonies significantly decreased when

AT6.1/Flag-KAI1 cells (KAI1⁺), compared with AT6.1 cells (KAI1⁻), interacted with HBMEs or HUVECs (Fig. 2e). We confirmed that this effect is mediated by DARC in the endothelial cells by performing similar experiments in which AT6.1/Flag-KAI1 or AT6.1 cells were mixed with cells with or without DARC expression (AT6.1/Flag-DARC or AT6.1; Fig. 2e). Therefore, our data suggest that the interaction between KAI1 and DARC leads to a growth-suppressive effect on the KAI1-bearing cell; thus, the status of KAI1 expression on tumor cells has a key role in determining their fate once they intravasate into the blood vessels.

To examine whether the interaction between KAI1 and DARC is essential for the metastasis suppressor function of KAI1 *in vivo*, we used *Darc*^{-/-} mice¹³. We chose the syngenic metastatic tumor cell lines B16BL6 and B16F10 to establish tumors in these mice and generated several KAI1⁺ clones or empty-vector transfectants in these cells (Fig. 3a). We then injected the B16BL6 derivatives subcutaneously into *Darc*^{-/-} mice and heterozygous and wild-type littermates. We found that primary tumors developed in all mice. The growth rate and final volume of tumors did not significantly vary with the KAI1 level in the tumor cells or with DARC status of the mice (Table 1). The

KAI1⁺ clones, however, developed significant numbers of pulmonary metastases in *Darc*^{-/-} mice, whereas metastasis was almost completely abrogated when the same clones were injected in the heterozygous and wild-type littermates (Fig. 3b and Table 1). The tumor cells lacking KAI1 (B16BL6/vector), however, metastasized equally in all three groups of mice. Thus, in the absence of DARC, even the tumor cells expressing large amounts of KAI1 recapitulated the metastatic phenotype of downregulation of CD82. To further corroborate the effect of DARC on the metastatic ability of KAI1-bearing cells, we used an experimental metastasis model in which the metastatic cell line B16F10 stably transfected with KAI1 expression plasmid or an empty vector was injected intravenously into *Darc*^{-/-} mice and their control littermates. The KAI1⁺ clones resulted in a significantly higher number of pulmonary metastases in the DARC knockout mice, whereas the empty vector transfectant metastasized regardless of the DARC status of the host (Table 1). These results support our hypothesis that DARC has a crucial role in the metastasis suppressor function of KAI1 *in vivo*.

DARC is known to be a promiscuous chemokine receptor; however, our *in vitro* data indicate that this function of DARC is not likely to

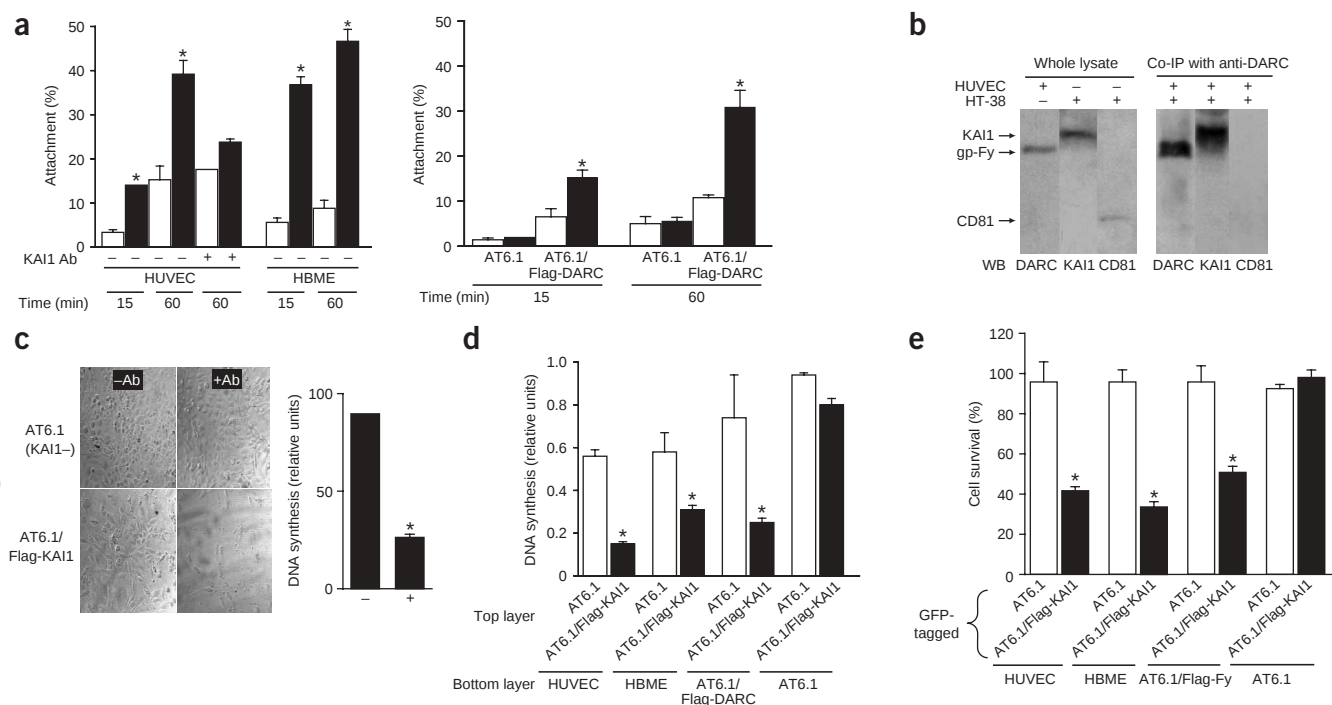


Figure 2 Interaction of KAI1 and DARC leads to growth arrest of cancer cells. **(a)** KAI1 selectively binds to cells expressing DARC in a cell-to-cell binding assay *in vitro*. HBMEs and HUVECs (DARC⁺; left), AT6.1 and AT6.1/Flag-DARC (right) were grown to confluency. Then, approximately 10³ cells of AT6.1 tagged with GFP (KAI1⁻, white bar) or AT6.1/Flag-KAI1 tagged with GFP (KAI1⁺, black bar) were added on the confluent cell layers, in the presence (+) or absence (-) of monoclonal antibody to KAI1 as indicated. After 15 min or 1 h, wells were washed and the percentage of attachment was calculated as described in Methods. **(b)** Endogenous KAI1 and DARC coimmunoprecipitate in mammalian cells. Lanes 1–3: expression level of KAI1 and CD81 in HT-38 cells and level of DARC in HUVECs were tested by western blot using antibodies to KAI1, CD81 and DARC, respectively. Lanes 4–6: HT-38 and HUVECs were mixed in the presence of a cell-impermeable crosslinker DTSSP for 30 min followed by immunoprecipitation with DARC antibody and western blot with antibodies to KAI1, DARC or CD81, as indicated. **(c)** Monoclonal antibody to KAI1 inhibits growth of KAI1⁺ prostate epithelial cells. AT6.1 (KAI1⁻) or AT6.1/Flag-KAI1 (KAI1⁺) were seeded and monoclonal antibody to KAI1 was added to the wells indicated by '+ Ab' and the rate of DNA synthesis was measured. **(d)** Suppression of DNA synthesis by DARC in prostate cancer cells. DARC⁺ endothelial cells (HUVECs, HBMEs) and cells with or without DARC expression (AT6.1, AT6.1/Flag-DARC; bottom layer) were grown to full confluency and incubated with 30 μ M mitomycin C for 18 h. The cells were then washed extensively, and AT6.1 (white bars) or AT6.1/Flag-KAI1 (black bar) cells (top layer) were added on the monolayer, ³H-thymidine was added to the wells and the incorporation of radioisotopes into DNA of the attached cells was assayed. **(e)** Growth arrest in prostate cancer cells caused by interaction between KAI1 and DARC. Prostate cancer cells expressing both CD82 and GFP genes (AT6.1/Flag-KAI1, black bars) or cells expressing only GFP (AT6.1, white bars) were mixed with cells with or without DARC expression for 1 h followed by plating in the presence of hygromycin, which allowed growth of only GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells. After 5 d, the number of colonies was counted under a fluorescent microscope. **P* < 0.05.

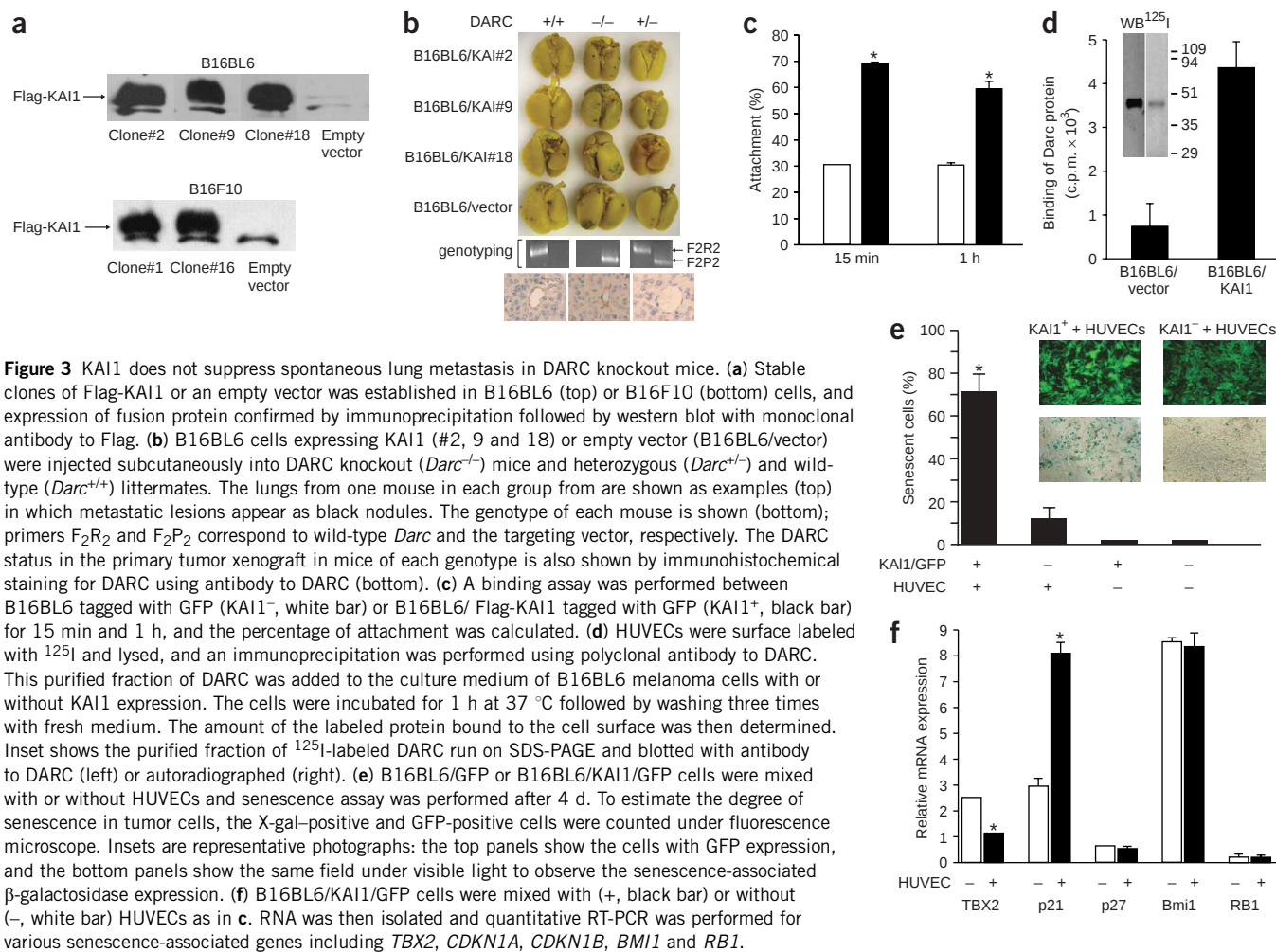


Figure 3 KAI1 does not suppress spontaneous lung metastasis in DARC knockout mice. **(a)** Stable clones of Flag-KAI1 or an empty vector was established in B16BL6 (top) or B16F10 (bottom) cells, and expression of fusion protein confirmed by immunoprecipitation followed by western blot with monoclonal antibody to Flag. **(b)** B16BL6 cells expressing KAI1 (#2, 9 and 18) or empty vector (B16BL6/vector) were injected subcutaneously into DARC knockout (*Darc* $^{-/-}$) mice and heterozygous (*Darc* $^{+/-}$) and wild-type (*Darc* $^{+/+}$) littermates. The lungs from one mouse in each group from are shown as examples (top) in which metastatic lesions appear as black nodules. The genotype of each mouse is shown (bottom); primers F2R2 and F2P2 correspond to wild-type *Darc* and the targeting vector, respectively. The DARC status in the primary tumor xenograft in mice of each genotype is also shown by immunohistochemical staining for DARC using antibody to DARC (bottom). **(c)** A binding assay was performed between B16BL6 tagged with GFP (KAI1 $^{-}$, white bar) or B16BL6/Flag-KAI1 tagged with GFP (KAI1 $^{+}$, black bar) for 15 min and 1 h, and the percentage of attachment was calculated. **(d)** HUVECs were surface labeled with 125 I and lysed, and an immunoprecipitation was performed using polyclonal antibody to DARC. This purified fraction of DARC was added to the culture medium of B16BL6 melanoma cells with or without KAI1 expression. The cells were incubated for 1 h at 37 °C followed by washing three times with fresh medium. The amount of the labeled protein bound to the cell surface was then determined. Inset shows the purified fraction of 125 I-labeled DARC run on SDS-PAGE and blotted with antibody to DARC (left) or autoradiographed (right). **(e)** B16BL6/GFP or B16BL6/KAI1/GFP cells were mixed with or without HUVECs and senescence assay was performed after 4 d. To estimate the degree of senescence in tumor cells, the X-gal-positive and GFP-positive cells were counted under fluorescence microscope. Insets are representative photographs: the top panels show the cells with GFP expression, and the bottom panels show the same field under visible light to observe the senescence-associated β -galactosidase expression. **(f)** B16BL6/KAI1/GFP cells were mixed with (+, black bar) or without (–, white bar) HUVECs as in **c**. RNA was then isolated and quantitative RT-PCR was performed for various senescence-associated genes including *TBX2*, *CDKN1A*, *CDKN1B*, *BMI1* and *RB1*.

have a role in the metastasis-suppression action of KAI1. Rather, DARC seems to directly engage in the interaction with KAI1, which triggers an unknown signal pathway of growth arrest. To obtain mechanistic insight into the interaction between KAI1 and DARC that led to metastasis suppression in our *in vivo* model system, we first carried out a cell-to-cell binding assay using melanoma cells. We found that B16BL6 cells overexpressing KAI1 exhibited a significantly higher binding to the endothelial cells over different time points (Fig. 3c), which is consistent with our observation in the case of prostate tumor cells. We then tested the binding of 125 I-labeled purified fraction of DARC to the cell surface of B16BL6 melanoma cells with or without KAI1 expression. A significantly ($P < 0.05$) higher amount of DARC bound to the melanoma cells expressing KAI1 compared with the empty vector transfectant (Fig. 3d), supporting our notion that KAI1 and DARC interact at the surface of the tumor cells. Such interaction leads to growth arrest of tumor cells (Fig. 2d,e). However, we did not detect apoptosis in the KAI1 $^{+}$ tumor cells by TUNEL assay upon coculturing with DARC $^{+}$ cells (data not shown). We therefore examined whether the interaction with DARC leads to senescence in the KAI1 $^{+}$ tumor cells by mixing HUVECs with GFP-tagged B16BL6 cells with or without KAI1 expression. We found that a significant percentage of KAI1 $^{+}$ tumor cells underwent senescence as a result of interaction with HUVECs (Fig. 3e). Furthermore, we found that expression of the senescence-associated gene *TBX2* was reduced and *CDKN1A* (encoding p21) was

upregulated in these cells upon interaction with HUVECs, whereas *CDKN1B* (encoding p27), *BMI1* or *RB1* did not show any appreciable change in expression level (Fig. 3f). Notably, several previous publications showed a potential link between tumor progression and senescence^{14–18}. Particularly consistent with our results, *TBX2* has been found to inhibit senescence by directly repressing p21 expression in melanoma cells, suggesting that the *TBX2*-p21 pathway has a crucial role in tumor progression¹⁹.

Collectively, our results indicate that when tumor cells dislodge from the primary tumor and intravasate into the blood vessels, tumor cells expressing KAI1 attach to the endothelial cell surface, whereby KAI1 interacts with DARC. This interaction transmits a senescent signal to the tumor cells, whereas those that lost KAI1 expression proliferate in the circulation, potentially giving rise to metastases. Notably, KAI1 as a tetraspanin was previously shown to interact with several other cell-surface proteins including $\alpha 4\beta 1$ integrin²⁰. The presence of these integrins on tumor cells promotes attachment to vascular endothelial cells²¹. Therefore, the association of integrin and KAI1 may have a part in the KAI1-DARC interaction, although this possibility needs to be explored further. Nonetheless, our model of the mechanism of action of KAI1 explains how KAI1 suppresses metastasis without affecting formation of primary tumors. It highlights a previously unappreciated function of *DARC* and identifies *DARC* as a new candidate for potential therapeutic intervention for metastatic cancer.

Table 1 Spontaneous and experimental metastases of B16BL6/KAI1 cells in DARC knockout mice

Spontaneous metastases of B16BL6/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Tumor volume (mean $\pm$ s.e.m.)			Incidence of pulmonary metastasis			
		<i>Darc</i> ^{+/+}	<i>Darc</i> ^{-/-}	<i>Darc</i> ^{+/-}	<i>Darc</i> ^{+/+}	<i>Darc</i> ^{-/-}	<i>Darc</i> ^{+/-}	<i>P</i> value
2	Positive	4.9 $\pm$ 0.03	4.5 $\pm$ 0.02	4.5 $\pm$ 0.01	2/15 (13.3%)	9/15 (60%)	1/15 (6.7%)	0.02 ^a , 0.008 ^b
9	Positive	4.6 $\pm$ 0.05	4.5 $\pm$ 0.03	4.9 $\pm$ 0.04	1/15 (6.7%)	6/13 (46.2%)	1/15 (6.7%)	0.05 ^a , 0.05 ^b
18	Positive	4.5 $\pm$ 0.05	4.2 $\pm$ 0.03	3.9 $\pm$ 0.04	0/13 (0%)	6/12 (50%)	0/13 (0%)	0.04 ^a , 0.04 ^b
Empty vector	Negative	4.9 $\pm$ 0.05	4.8 $\pm$ 0.05	4.9 $\pm$ 0.03	6/15 (40%)	5/14 (35.7%)	5/14 (35.7%)	0.8 ^a , 0.89 ^b

Experimental metastases of B16F10/KAI1 cells in DARC knockout mice

Clone #	KAI1 expression	Number of pulmonary metastases			<i>P</i> value
		<i>Darc</i> ^{+/+}	<i>Darc</i> ^{-/-}	<i>Darc</i> ^{+/-}	
1	Positive	4.7 $\pm$ 2.4 (<i>n</i> = 9)	47.86 $\pm$ 5.9 (<i>n</i> = 7)	2.8 $\pm$ 0.8 (<i>n</i> = 6)	<0.001 ^a , <0.001 ^b
16	Positive	4.4 $\pm$ 2.4 (<i>n</i> = 7)	32.14 $\pm$ 3.6 (<i>n</i> = 7)	9.4 $\pm$ 2.7 (<i>n</i> = 5)	<0.001 ^a , 0.001 ^b
Empty vector	Negative	40.0 $\pm$ 8.4 (<i>n</i> = 5)	56.0 $\pm$ 11.8 (<i>n</i> = 5)	32.5 $\pm$ 4.8 (<i>n</i> = 6)	0.3 ^a , 0.08 ^b

^aComparison between *Darc*^{-/-} and *Darc*^{+/+}. ^bComparison between *Darc*^{-/-} and *Darc*^{+/-}.

METHODS

Yeast two-hybrid screening. We cloned full-length *KAI1* cDNA cloned into the yeast vector pEG202-NLS (Origene Technologies) as bait, and performed yeast two-hybrid screening and mating assay according to the manufacturer's protocol.

Quantitative β -galactosidase assay. We performed the β -galactosidase assay (Miller test) as previously described²².

Cell culture. The rat prostatic carcinoma cell line AT6.1, the human breast carcinoma cell line MDA-MB-435, HBMEs and the mouse melanoma cell lines B16BL6 and B16F10 were provided by C. Rinker-Schaeffer (University of Chicago), B.E. Weissman (University of North Carolina at Chapel Hill), K. Pienta (University of Michigan Medical School) and I.J. Fidler (M.D. Anderson Cancer Center), respectively. We purchased the human lung epithelial carcinoma cell line A549 and colon carcinoma cell line HT-38 from American Type Tissue Culture Collection. We cultured the cells in RPMI-1640 medium (Invitrogen) supplemented with 10% FCS, 250 nM dexamethasone and antibiotics. We obtained HUVECs from Clonetics and cultured them in endothelial growth medium (EGM, Clonetics) as per the manufacturer's instruction.

Immunoprecipitation and western blot. For coimmunoprecipitation experiments using the AT6.1 cells, approximately 48 h after transfection, we harvested cells and lysed them in ice-cold lysis buffer (1% NP40, 10 mM Tris, pH 8.0, 150 mM NaCl, 3 mM MgCl₂, 2 mM PMSF) for 45 min and centrifuged them at maximum speed for 15 min. For immunoprecipitation with monoclonal antibody to Flag, we used Flag-specific M2 affinity gel (Sigma). For immunoprecipitation with antibody to hemagglutinin, we incubated the lysate with monoclonal antibody to hemagglutinin (Boehringer Mannheim) and used protein G-Sepharose beads. After immunoprecipitation, we thoroughly washed the beads, and analyzed bound proteins by western blot using monoclonal antibody to hemagglutinin or monoclonal antibody to Flag (Sigma) at dilutions of 1:400 and 1:500, respectively. For coimmunoprecipitation of endogenous KAI1 and DARC, we mixed the KAI1⁺ tumor cell line HT-38 with DARC⁺ HUVECs in the presence of the cell-impermeable cross-linker DTSSP for 30 min at 24 °C. We lysed the cells in the same lysis buffer as above, centrifuged them and immunoprecipitated the lysate with rabbit polyclonal antibody to DARC in the presence of protein G agarose beads. After immunoprecipitation, we analyzed bound proteins by western blot using antibody to DARC (1:500), mouse monoclonal antibody to KAI1 (1:1,000, a gift from O. Yoshie, Shionogi Institute for Medical Science) or mouse monoclonal antibody to CD81 (1:20, Chemicon).

Immunohistochemistry. We carried out immunohistochemical analysis on paraffin-embedded, surgically resected specimens of prostate, breast and lung, using polyclonal antibody to DARC. Briefly, we deparaffinized sections, rehydrated them and heated them at 80 °C for 20 min in 25 mM sodium citrate buffer (pH 9) for antigen exposure. We treated sections with 3% H₂O₂ to block endogenous peroxidase activity and then incubated them with primary antibody (1:50 dilution) for 1 h at 24 °C. After washing in Tris-buffered saline/0.1% Tween-20, we incubated sections with horseradish peroxidase-conjugated rabbit-specific IgG (Dako Corp.). We washed sections extensively, and applied DAB substrate chromogen solution followed by counterstaining with hematoxylin. The Southern Illinois University Institutional Review Board approved obtaining human specimens for this study.

Cell-to-cell binding assay. We seeded HBMEs, HUVECs, AT6.1 or AT6.1/Flag-DARC (DARC⁺ permanent clone established in AT6.1) cells in 24-well plates and grew them to full confluency. We trypsinized cells used for overlaying (AT6.1/GFP and AT6.1/Flag-KAI1/GFP, or B16BL6/GFP and B16BL6/Flag-KAI1/GFP) and resuspended them in RPMI medium, and added 10³ cells on the confluent bottom cell layers in the presence or absence of antibody to KAI1. After 15 min or 1 h, we washed the wells with RPMI medium three times and incubated the cells for 12 h at 37 °C. The numbers of cells attached on confluent monolayers were then counted by observing GFP signal under a confocal microscope and the percentage of attached cells was calculated. For each data point, experiments were performed in triplicate wells and ten random fields were counted in each well.

Treatment of tumor cells with monoclonal antibody to KAI1. We seeded approximately 10³ cells of AT6.1 and AT6.1/Flag-KAI1 in 96-well plates. We then added ³H-thymidine with or without monoclonal antibody to KAI1 (provided by H. Conjeaud, Cochin Hospital) to the wells, which we then incubated at 37 °C for 48 h. The ³H-thymidine incorporation by the AT6.1/KAI1 cells was normalized with respect to the incorporation by the AT6.1 cells. Each experiment was performed in triplicate.

Measurement of DNA synthesis. We cultured HUVECs, HBMEs, AT6.1 and AT6.1/Flag-DARC cells to confluency and then treated them with mitomycin C for 18 h to block DNA synthesis. After washing the wells extensively with RPMI media, we seeded 10³ AT6.1 cells that did or did not express KAI1 (AT6.1/Flag-KAI1 or AT6.1) on the monolayer of mitomycin C-treated cells and added ³H-thymidine to the wells. We incubated the cells at 37 °C for 48 h, then washed the wells with RPMI media three times and measured the incorporation of ³H-thymidine in the attached cells. The rate of DNA synthesis by the cells

seeded on monolayers was normalized by that of cells seeded directly on the plastic plate. Each experiment was performed in triplicate.

Colony formation assay. We trypsinized HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells, resuspended them in RPMI medium and mixed them with AT6.1 cells, which expressed the gene encoding GFP with or without KAI1 (AT6.1 or AT6.1/Flag-KAI1, both GFP tagged), for 1 h, then plated the mixture in RPMI medium containing hygromycin. The GFP-tagged AT6.1 or AT6.1/Flag-KAI1 cells were also plated without mixing with HUVECs, HBMEs, AT6.1 or AT6.1/DARC cells for the purpose of normalization. We incubated the cells at 37 °C for 5 d and counted the number of colonies expressing GFP under the fluorescence microscope. The number of colonies formed by GFP-tagged AT6.1 or AT6.1/Flag-KAI1 mixed with HUVECs, HBMEs, AT6.1 and AT6.1/DARC cells was normalized with the number of colonies formed by the GFP-tagged cells alone. Each experiment was done in triplicate.

In vivo metastasis assay. For spontaneous metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml of PBS of various B16BL6 clones subcutaneously in the dorsal flank of the DARC knockout mice as well as heterozygous and wild-type littermates. We monitored mice daily for the growth of primary tumor. After 6 weeks, mice were killed, tumor volume was calculated using the equation $\text{Volume} = (\text{Width} + \text{Length})/2 \times \text{width} \times \text{length} \times 0.5236$, and metastatic lesions were counted macroscopically. For experimental metastasis assay, we injected approximately 0.5×10^6 cells/0.2 ml PBS of various B16F10 clones intravenously into the tail vein of the DARC knockout mice as well as control littermates. Mice were killed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically. All protocols were approved by the Southern Illinois University Institutional Review Board.

In vitro binding assay. The DARC⁺ cells were surface labeled with ¹²⁵I using Iodo-beads (Pierce) according to the manufacturer's protocol. We lysed the cells and immunoprecipitated them using antibody to DARC and protein G agarose. We washed the agarose beads extensively to remove unbound proteins and eluted the bound proteins using 0.1 M glycine, pH 3.5, immediately followed by neutralization with 0.5 M Tris, HCl, pH 7.4, 1.5 M NaCl. This eluate was further concentrated by Centricon P10. B16BL6 cells with or without KAI1 expression were seeded in 24-well plates and grown to confluency. We added the purified protein to the cells in culture and 48 h later, washed the wells three times with fresh medium and determined the amount of the bound protein.

Senescence assay. We trypsinized B16BL6/GFP or B16BL6/Flag-KAI1/GFP cells, resuspended them in medium and mixed them with the DARC⁺ HUVECs for 1 h followed by plating the mixture. We also plated the GFP-tagged cells without mixing with HUVECs as control. We incubated the cells at 37 °C for 4 d. We then performed a senescence assay using a senescence-associated β -galactosidase detection kit (Calbiochem) according to the manufacturer's instruction, and counted the X-gal-positive and GFP-positive cells under a fluorescence microscope.

Real-time RT-PCR. We mixed B16BL6/Flag-KAI1/GFP cells with or without the DARC⁺ HUVECs for 1 h, and then plated the mixture and incubated it at 37 °C for 4 d. We isolated total RNA from the cells and reverse-transcribed it. We then amplified the cDNA with a pair of mouse-specific forward and reverse primers for the following genes: *TBX2* (forward, 5'-CACCTCCGCACCTAT GTC-3'; reverse, 5'-CAAACGGAGAGTGGGACGCTT-3'), *CDKN1A* (forward, 5'-CCGTGGACAGTGAGCAGTT-3'; reverse, 5'-CCAATCTGCGCTTG GAGTGA-3'), *BMI1* (forward, 5'-AATCCCCACTTAATGTGTGTC-3'; reverse, 5'-TCACCTCTCCTTAGGCTTCTC-3'), *CDKN1B* (forward, 5'-GTGGAC CAAATGCCTGACT-3'; reverse, 5'-GGCGTCTGCTCCACAGTG-3'), *RBI* (forward, 5'-TGATGAAGAGGCAAACGTGG-3'; reverse, 5'-TGGCCACAGCG TTAGCAAAC-3') and β -actin. We performed PCR using DNA engine opticon2 system (MJ Research) and the Dynamo SYBR Green qPCR Kit (Finnzyme

Corp). The thermal cycling conditions comprised an initial denaturation step at 95 °C for 15 min followed by 30 cycles of PCR using the following profile: 94 °C for 30 s; 57 °C for 30 s; 72 °C for 30 s.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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The Tumor Metastasis Suppressor Gene Drg-1 in Cancer Progression and metastasis

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ABSTRACT

The process of tumor metastasis is negatively regulated by metastasis suppressor genes and understanding the mechanism of action of these genes provides critical insight into the complex process of metastasis. This chapter is devoted to a recently discovered metastasis suppressor gene, Drg-1. Here we summarize the work from the laboratory of ours and others, providing evidence for metastasis suppression by Drg-1, describing the clinical relevance of this gene, and the current understanding of regulation and function of this gene in the context of tumor metastasis.

INTRODUCTION

The most important aspect of cancer, from the medical point of view, is metastasis which almost invariably is the ultimate cause of death from any type of cancer. Metastasis refers to the dissemination and establishment of tumor cells from the site of origin to a distant site that involves a complex multi-step process. Following primary tumor formation, a population of tumor cells acquires invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion, and proteolytic degradation of the matrix. When tumor cells become further aggressive, these cells intravasate into neighboring blood vessels and disseminate through the circulation. Those cells that survive in the circulation are arrested at distant organ sites, extravasate and lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis. Despite its obvious clinical relevance, because of the complexity of the phenomenon, metastasis remains poorly understood at the molecular and biochemical levels. Recently, there has been significant advancement in understanding several crucial aspects of this intricate biological process with the discovery of the ‘metastasis

suppressor genes', which by definition, suppresses the process of metastasis without affecting tumorigenesis. Till date, 13 such genes have been identified and these include nm23, KAI1, Kiss1, BRMS1, MKK4, RhoGD12, RKIP, Drg-1, CRSP3, SSeCK, TXNIP/ VDUP-1, Claudin-4, and RRM1 [1, 2, 3]. This chapter focuses on the regulation and function of the recently discovered tumor metastasis suppressor gene Drg-1.

STRUCTURE AND EXPRESSION PATTERN OF THE Drg-1 GENE

The Drg-1 gene was originally identified as a gene strongly induced by cellular differentiation *in vitro* and hence named as Differentiation-Related-Gene-1 [4]. The Drg-1 gene belongs to the 'NdrG' family of genes, which also includes three other members, Drg-2, 3 and 4 that encode proteins highly related to Drg-1. These members vary in the pattern of tissue-specific expression and possibly in their functions [5, 6]. Drg-1 is almost identical to the human RTP, cap43 and rit42 genes, and is homologous to the mouse genes TDD5, Ndr1 and the rat gene Bdm1 [7, 8, 9]. The cap43 and RTP genes have the same predicted amino acid sequences, although there are a few differences in the 3' untranslated region, and there is a single amino acid difference from Drg-1 [10]. In Drg-1, isoleucine is changed to threonine due to T-to-C transition, but all other Drg-1 homologous genes including the mouse genes have isoleucine at this position. TDD5 has the same amino terminal part of the protein, however, there is a significant difference in the COOH-terminus [10]. In addition to the mammalian homologues mentioned above, genes homologous to Drg-1 exists in a wide variety of organisms, such as zebrafish, fruitfly, nematode, sunflower, and *Arabidopsis* [11]. Thus Drg-1 is highly conserved across species, suggesting its role in important cellular processes.

The Drg-1 gene has been mapped to human chromosome 8q24.2 [12]. Drg-1 mRNA is detected in tissues of most of the organ systems, including the digestive tract, immunological, reproductive and urinary systems [13]. Gene expression study indicated significant variation of expression level of the Drg-1 gene among different organs, and the expression has been found to be particularly high in prostate, ovary, intestine and kidney [13]. The Drg-1 gene encodes a 43kD cytoplasmic protein that has several noticeable features, however, the biochemical function of the protein is yet largely unknown. Amino acid sequence of the Drg-1 protein reveals 3 serine phosphorylation sites, 5 calmodulin kinase 2 phosphorylation sites, 5 myristoylation sites, 3 PKC phosphorylation sites, 1 tyrosine phosphorylation site, 1 thioesterase site and a phosphopantotheine attachment site. It has been shown that Protein kinase A and calmodulin kinase2 are indeed involved in the phosphorylation of this protein *in vitro* [14, 15]. At the C terminal end of the Drg-1 protein, there are 3 tandem repeats of the amino acids G-T-R-S-R-S-F-T H-T-S. Murray *et al.* recently demonstrated that the C-terminal stretch of the Drg-1 protein serves as a substrate for phosphorylation by serum- and glucocorticoid-induced kinase 1 (SGK1) which then primes it for phosphorylation by glycogen synthase kinase 3 (GSK3) [16, 17]. In addition, based on potentiometric and spectroscopic studies, Zoroddu *et al.* have proposed that this stretch may be important for Nickel binding [18]. The Drg-1 protein also contains a prominent beta-hydrolase fold characterized by at least 5 parallel beta strands, a catalytic triad in a specific order (nucleophile-acid-histidine), and a nucleophilic elbow. However, using a Bayesian computational algorithm, Shaw *et al.* have found that all of the residues that could impart hydrolytic functionality have been eliminated in the Drg-1 class of proteins, although the overall structure of the a/b hydrolase fold has been preserved [19]. Studies are underway in the

laboratories of ours and others to understand the exact biochemical function of this protein and its physiological relevance.

CANCER CONNECTIONS: EXPRESSION OF THE Drg-1 GENE IN HUMAN TUMOR TISSUES

Originally aiming at identifying genes involved in differentiation, van Belzen *et al.* utilized a colon carcinoma cell line that could be induced to differentiate *in vitro* and by using a modified differential display approach they identified Drg-1 as a novel gene strongly induced during differentiation [4]. Loss of differentiation is one of the salient features of tumor cells and tumor progression is often characterized by downregulation of differentiation related genes. In line with this idea, the differentiation-related gene Drg-1 has been found to be downregulated in several types of cancers, including prostate, breast, colon and pancreatic carcinoma [20, 21, 22, 23]. As shown in Fig.1(a), in the tissue specimens from both prostate and breast cancer cases, Drg-1 was found to be highly expressed in the epithelial cells of normal glands and ducts, and the basal cell layers also showed high level of Drg-1, where the protein was localized mostly in the cytoplasm. The stroma did not have any detectable level of Drg-1 expression, but the endothelial cells and nerve bundles frequently expressed Drg-1. The Drg-1 protein was detected consistently in all cases of normal prostate and breast tissue, as well as PIN (Prostatic Intraepithelial Neoplasia) and BPH (Benign Prostatic Hyperplasia), while the Drg-1 expression was significantly reduced in the tumor cells of nearly 47% prostate cancer and 30% breast cancer patients [20, 21]. In the case of prostate cancer, when the patients were subdivided into two groups, those with Gleason score lower than or equal to 7 and those with a Gleason score more than 7, the reduction in Drg-1 expression correlated significantly with the Gleason grade

($P=0.015$) (Fig.1b). A study by Caruso *et al.* also found a similar trend of downregulation of Drg-1 expression in prostate cancer, and interestingly, they also observed a significant correlation between Drg-1 expression pattern and ethnic origin of the patients [24]. In our study population, Drg-1 expression had an overall significant inverse correlation with the degree of differentiation ($P < 0.001$) (Fig. 1b). However, one-way ANOVA test with Tukey's W procedure indicated that the down regulation of Drg-1 is not significant between well and moderately differentiated tumors though it is highly significant between moderate and poorly differentiated tumors. These results are in agreement with the idea of this gene being up-regulated by induction of cellular differentiation *in vitro* and also suggest a possibility that Drg-1 suppression may be more important in the late stage of tumor progression. Indeed, in both prostate and breast cancer, we observed a significant level of differential expression of Drg-1 between the patients with organ-confined disease and those with metastasis to lymph node or bone (Fig.1b, ref.21). For instance, in the case of prostate cancer, while 28 cases (70%) were positive for Drg-1 out of 40 localized prostate cancer cases, only 5 (25%) were positive for Drg-1 expression out of each of the 20 and 19 cases with lymph node and bone metastasis. Thus, the negative correlation of Drg-1 with metastatic spread to the lymph node and the bone is highly significant ($P= 0.003$ and 0.006 , respectively), and in fact, is much stronger than the positive correlation with Gleason scores. Similarly, in the case of breast cancer, while 89.7% patients were positive for Drg-1 expression out of 29 cases with localized disease, only 60.7% were positive for Drg-1 expression among 56 patients with metastases [21]. These results strongly suggest the negative involvement of Drg-1 in the process of invasion and metastasis in both prostate and breast cancer, which also is in good agreement with the recent observation by

Maruyama *et al.* that Drg-1 expression has a significant inverse correlation with depth of invasion in pancreatic adenocarcinoma patients [23].

In addition to reduction in expression of the Drg-1 gene in tumor tissue, recent studies have also indicated the prognostic importance of this gene. In the case of prostate cancer, patients with Drg-1 positive expression had significantly more favorable prognosis than those with reduced expression of the gene ($P=0.002$, log rank test) (Fig. 1c). Consistently, in a group of 85 breast cancer cases, patients with Drg-1 positive expression had significantly better prognosis than those with reduced expression of the gene ($P=0.002$) (Fig 1c). Recently, Maruyama *et al.* have also observed that reduced expression of the Drg-1 (cap43) gene is significantly ($P=0.0062$) associated with poor overall survival rate in pancreatic ductal adenocarcinoma [23]. Furthermore, in multivariate Cox regression analysis involving the Drg-1 expression status in breast cancer, primary tumor size and degree of metastasis, we found Drg-1 to be an independent and statistically significant prognostic factor. The odds ratio for Drg-1 was 2.4 (95%CI 1.03-5.76, $P=0.043$), implying that the death risk of breast cancer patients with reduced Drg-1 expression within a specific time was 2.4 times higher than the risk of patients to die within the same time course with Drg-1 positivity [21]. Thus, the reduced expression of Drg-1 can be a strong predictor of lymph node and bone metastasis and, in turn, of survival in breast cancer patients. In Cox regression analysis in univariate mode, the Drg-1 gene expression in prostate cancer also had a significant predictive value ($P=0.0256$), although it was less predictive than lymph node or bone metastasis ($P<0.001$) [20]. Taken together, these data underscore the clinical relevance of the Drg-1 gene in advancement of human cancer.

EVIDENCE OF METASTASIS SUPPRESSION BY Drg-1

The significant inverse correlation of Drg-1 expression with the extent of metastasis at the clinical level suggests a potential role of this gene in the process of tumor metastasis. However, the definitive proof of such action of the gene can only be obtained from experiments in animal model. AT6.1 is a dunning rat prostate cancer cell line and rapidly grows into primary tumor in SCID mice when subcutaneously injected followed by high incidence of lung metastasis. Therefore, it provides a useful model for studying spontaneous metastasis *in vivo*. We transfected the mammalian expression plasmid of Drg-1 into AT6.1 cells, and selected several permanent clones with strong Drg-1 expression as shown in Fig 2a. Each of these clones was individually injected subcutaneously into the dorsal flanks of SCID mice and monitored for tumor formation and growth rate of the tumor. Five weeks after the inoculation of the cells, the mice were sacrificed and the number of metastatic lesions on their lungs was grossly counted. We found that all the clones formed primary tumors in the animals with similar growth rates during the 5-week period, suggesting that Drg-1 does not have an effect on tumorigenesis and primary tumor growth rate. On the other hand, as shown in Fig.2, the clones that were positive for Drg-1 expression showed a significantly lower incidence of lung metastases compared with the vector-transfected cell line and the clone (#12) negative for Drg-1 expression. These results strongly suggest that Drg-1 has the ability to suppress the metastatic dissemination of prostate cancer cells without affecting tumorigenicity *in vivo*.

Similar metastasis suppressor effect of Drg-1 was also observed in colon carcinoma cells by Guan *et al.* [22]. When three Drg1-transfected clones and two empty vector clones were injected into the spleen of athymic nude mice, the tumor burdens of the splenic primary tumors were very similar between the transfected and control groups. However, 75% mice developed liver metastases in the empty vector control groups whereas only 23% had liver metastasis in the

Drg-1-transfected group, strongly suggesting that Drg-1 may function as a suppressor of colon cancer metastasis without altering the ability of the cells to form primary tumor [22].

Furthermore, in consistence with the metastasis suppressor action of the Drg-1 gene, Yoshizumi and colleagues recently demonstrated that treatment of colon cancer cells with a PPAR(Peroxisome proliferator-activated receptor)-gamma ligand and differentiation-inducing agent, thiazolidinedione (TZD), completely inhibited lymph node and lung metastases in a xenograft animal model, and this was associated with a marked increase in Drg-1 expression [25].

On the other hand, Kurdistan *et al.* demonstrated that when EJ bladder cancer cell line overexpressing the Drg-1 gene was injected into nude mice, primary tumor mass was significantly reduced compared to parental cell line [12]. In line with such finding, Stein *et al.* recently observed that Drg-1 is a crucial factor in p53- mediated apoptosis in DLD-1 colon cancer cells [26]. These results result suggests that, depending on the cellular context, Drg-1 is also capable of suppressing primary tumor growth, although the factor that contributes to such dichotomous function of the Drg-1 gene is yet to be understood. It should be noted that Okuda *et al.* recently generated Drg-1 knock-out mouse which does not exhibit any spontaneous tumor phenotype, consistent with the notion that Drg-1 acts as a metastasis suppressor gene without affecting primary tumorigenesis *in vivo* [27]. Therefore, it will be of great interest to cross the Drg-1 knock-out mouse with a spontaneous metastasis mouse model and assess for potential suppression of metastasis.

REGULATION OF THE Drg-1 GENE IN TUMOR CELL

It is evident from both animal studies and clinical studies that Drg-1 acts as a metastasis suppressor gene, and therefore, it is of paramount interest to understand how this gene is down-

regulated in tumor cells so that this information may lead to the design of effective therapeutic strategy to target metastatic cells in cancer patients. Since deletion or loss of human chromosome 8q24.3, where Drg-1 is localized, is not a common event in human cancers, it is plausible that down-regulation of the Drg-1 gene occurs at the transcriptional or translational level. Indeed, RT-PCR analysis on breast cancer samples from patients with metastatic disease revealed that there is a significant reduction of Drg-1 mRNA in the tumor cells in 75% cases compared to the normal counterparts, suggesting that the reduction of the expression of the Drg-1 gene in cancer cells is, for the most part, at the RNA level [21].

Epigenetic regulation of the Drg-1 gene

One notable mechanism of gene regulation at the RNA level that has been observed in different types of human cancers is aberrant methylation of cytosines located 5' to guanines (CpG) in the promoter region of tumor suppressor and metastasis suppressor genes. Scanning of the 5' upstream region of the Drg-1 gene revealed two prominent CpG islands suggesting that DNA methylation may contribute to the regulation of this gene. Indeed, treatment of a panel of human breast carcinoma cell lines with the DNA methylation inhibitor, 5-Azacytidine indicated that demethylation resulted in a significant increase in the expression of Drg-1 at both mRNA and protein levels [21]. Hypermethylation has been shown to down-regulate Drg-1 expression in colon cancer cells as well [22]. These results strongly suggest that Drg-1 expression at the transcriptional level is controlled, at least in part, by hypermethylation of CpG islands and that inhibition of methylation is capable of restoring the expression of the Drg-1 gene. Drg-1 expression is also regulated by histone deacetylation, since Drg-1 mRNA was found to be markedly upregulated by treatment with histone deacetylase inhibitors in colon and nasopharyngeal cancer cell lines [22, 28].

Regulation of Drg-1 by multiple of factors / pathways

In addition to epigenetic mechanisms, Drg-1 is also controlled by multiple factors and is responsive to various stimuli *in vitro* (Fig. 3). The tumor suppressor gene p53 has been shown to regulate the expression of Drg-1 albeit in a cell type specific manner. Kurdistani *et al.* have demonstrated that the tumor suppressor gene p53 is able to induce the expression of Drg-1 in p53-null bladder cancer cell line and fibrosarcoma cells, while Stein *et al.* recently found that p53 induced expression of the Drg-1 gene in non-metastatic colon cancer cell lines DLD-1 and HCT-1 but not in the metastatic lung cancer cell line H1299 [12, 26]. On the other hand, the tumor suppressor gene von Hippel Lindau has been shown to transcriptionally downregulate the expression of the Drg-1 gene in a renal cancer cell line, although such regulation is yet to be clarified *in vivo* [29]. In a separate study, utilizing N-myc deficient mouse embryos, Shimono *et al.* suggested that Drg-1 expression was down-modulated by N-myc, and indeed, N-Myc and Max were found to repress the promoter activity of the Drg-1 gene [8]. They also observed that the Drg-1 promoter was equally repressed by c-Myc and max, suggesting that if N-Myc or c-myc activity is augmented during malignant transformation of the cells, then Drg-1 expression would be repressed [8].

Results of several *in vitro* studies have also indicated that Drg-1 is a stress responsive gene and various chemical agents including homocysteine, mercaptoethanol, tunicamycin, lysophosphatidylcholine, and synthetic retinoids have been shown to induce the expression of this gene in cultured cells [5, 30, 31]. In addition, Richardson *et al.* found that treatment of cells with Fe-chelators specifically upregulated the expression of the Drg-1 gene [32]. Since Fe is a critical factor in cell proliferation, this result suggests that Drg-1 is a novel link between iron metabolism and control of cell proliferation. Furthermore, Drg-1 has been found to be

upregulated in human carcinoma cells following treatment with nickel compounds via elevation of free intracellular Ca^{2+} levels [10]. Consistent with this finding, induction of Drg-1 expression by nickel, calcium ionophore or okadaic acid can be blocked by bis-(O-aminophenoxy)-ethane NNNN tetraacetic acid tetra-(acetoxymethyl)-ester [10]. It has been also demonstrated that acute exposure to nickel results in accumulation of hypoxia-inducible transcription factor (HIF)-1, which strongly activates hypoxia-inducible genes, including Drg-1 [33].

In the case of prostate cancer, it should be noted that the Drg-1 gene was previously shown to be upregulated by androgen in LnCap prostate cancer cell line [34]. On the contrary, Lin *et al.* observed that the rat homologue of Drg-1, TDD5, was repressed by testosterone and dihydrotestosterone [7]. They further suggested that TDD5 is an early responsive androgen target gene, since their animal studies showed that TDD5 mRNA levels were repressed within 8 hours after dihydrotestosterone administration [7]. Thus, regulation of Drg-1 expression by androgen remains controversial. In fact, we did not observe any significant correlation between expression of Drg-1 and androgen receptor in immunohistochemical analysis of clinical samples of prostate cancer, indicating that androgen signaling may not be a critical factor for regulation of Drg-1 expression *in vivo* [35].

Tumor suppressor gene PTEN upregulates the Drg-1 gene

PTEN is one of the most common targets of mutation in human cancers, with a mutation frequency approaching that of p53 [36]. In the case of human prostate cancer, deletion and/or mutations of the PTEN gene are reported in 30% of primary and 63% of metastatic tumors, placing PTEN among the most common genetic alterations in this type of cancer [37, 38]. In a microarray analysis, Unoki *et al.* recently identified Drg-1 as one of the several genes up-regulated by PTEN in two endometrial cancer cell lines [39]. In our study, introduction of

PTEN in PTEN-null prostate and breast cancer cells dramatically upregulated the endogenous level of Drg-1, while knock-down of PTEN gene significantly reduced Drg-1 expression in prostate cancer cells which strongly suggest that Drg-1 is positively regulated by PTEN at least *in vitro* [35]. This regulation of the Drg-1 gene by PTEN occurs at the transcriptional level since we observed that PTEN over-expression significantly augmented the activity of 1.5kb promoter region of the Drg-1 gene [35]. PTEN is a dual specificity phosphatase that inhibits PI3K dependent activation of Akt, and deletion or inactivation of PTEN results in constitutive Akt activation [40]. In line with such cross-talk between PTEN and PI3K, we found that treatment of prostate cancer cells with the PI3K inhibitor Ly-29400 that decreased the phospho-Akt level, also resulted in a concomitant increase in Drg-1 expression [35]. Together, the results of our *in vitro* experiments strongly implicate that PTEN transcriptionally upregulates the expression of the Drg-1 gene via an Akt-mediated pathway.

Expression of the PTEN and Drg-1 genes were also found to have a significant positive correlation in clinical setting of prostate and breast cancer, which is consistent with the notion that PTEN controls the expression of the Drg-1 gene [35]. Furthermore, we found that in univariate survival analysis, patients negative for both PTEN and Drg-1 had significantly worse prognosis than those with positive expression of either one or both markers [35]. Importantly, Cox regression analysis revealed that the combination of PTEN and Drg-1 gene expression was an independent prognostic marker in both prostate and breast cancer, and the death risk of a patient with negative expression of both markers was significantly worse than those positive for both or either PTEN and Drg-1 [35]. These data underscore the prognostic importance of combination of PTEN and Drg-1 and also point toward the clinical relevance of the PTEN-Drg-1 pathway in metastatic advancement of prostate and breast cancer.

The finding that the tumor suppressor gene PTEN gene upregulates the tumor metastasis suppressor gene Drg-1 has several implications especially for the biology of prostate cancer. PTEN has been shown to be frequently mutated in various types of cancers, including glioblastoma, melanoma, endometrial, breast, lung, gastric, colorectal, bladder, and head and neck cancer [36]. In most of these cases, PTEN inactivation was also found to have a significant correlation with invasiveness and metastasis [38, 41, 42]. Interestingly, recent studies using various mouse models have begun to reveal a functional involvement of PTEN in suppressing tumor metastasis. Using a series of hypomorphic PTEN mutant mice with decreasing PTEN activity, Trotman *et al.* have shown that the extent of PTEN inactivation dictates metastatic progression of prostate cancer in a dose-dependent manner [43]. In a separate study, Wang *et al.* demonstrated that mice with prostate specific bi-allelic deletion of the PTEN gene spontaneously develop PIN lesions followed by invasive adenocarcinoma, and more than 50% of the animals develop pulmonary metastasis by 29 weeks of age [44]. More direct link between PTEN and prostate cancer metastasis was demonstrated by Davies *et al.* in an orthotopic mouse model where *ex vivo* treatment of PC3 prostate cancer cells with adenoviral PTEN expression vector completely inhibited lymphnode metastases without inhibiting tumorigenicity [45]. *In vivo* treatment of pre-established PC3 tumors with adenoviral PTEN also markedly diminished lymphnode metastasis formation without causing significant regression of local tumor [45]. These results are in good agreement with the previous observation that reintroduction of the human 10q23-25 region into highly metastatic rat prostate cancer cells significantly suppressed metastasis without affecting their tumorigenic potential [46]. The metastasis suppressor role of PTEN was also suggested in the case of a melanoma mouse model where overexpression of PTEN in B16F10 cells inhibited

experimental pulmonary metastasis [47]. Taken together, the results of these animal experiments implicate a critical role of the PTEN gene in tumor metastasis. Our finding, that PTEN upregulates the expression of the Drg-1 gene, strongly suggests that metastasis suppressor function of PTEN is at least in part mediated by Drg-1.

MECHANISM OF METASTASIS SUPPRESSION BY Drg-1

Results from animal experiments as well as clinical studies provide compelling evidence supporting the notion that the Drg-1 gene is a novel tumor metastasis suppressor, and that the status of the expression of this gene may serve as a diagnostic and prognostic marker. The next most intriguing question that needs to be addressed is how the Drg-1 gene exerts its metastasis suppressor function. Metastasis is a complex process involving a cascade of events and the steps that are affected by Drg-1 are largely unknown, but studies from different laboratories have begun to shed light on the functional role of this gene in various types of cancer. Drg-1 has been found to dramatically suppress the invasive ability of prostate and breast cancer cells in the Matrigel assay *in vitro* [20, 21]. Drg-1 however did not significantly affect the migratory property of the tumor cells in this assay. Notably in separate studies from different laboratories, Drg-1 has also been shown to inhibit invasiveness of colon cancer cells, pancreatic cancer cells and hepatocellular carcinoma cells [22, 23, 48]. These data strongly suggest that Drg-1 suppresses the invasive ability of aggressive cancer cells *in vitro*, which is consistent with the results of immunohistochemical analysis of clinical specimens by us and other groups [20, 21, 23]. In this context, it should be noted that Drg-1 has been found to be up-regulated by the tumor suppressor gene PTEN which is also known to be able to down-regulate metastasis-related genes such as MMP-1, 2 and 13 [49, 50, 51]. Therefore, it is tempting to speculate that Drg-1

may be involved in down regulation of these protease genes by PTEN, which may at least partly account for the metastasis suppressor function of the Drg-1 gene. The effect of Drg-1 on tumor cell proliferation, however, remains elusive. In the case of prostate cancer, it was found that cells stably expressing Drg-1 did not significantly differ from the vector-transfected control cells in terms of growth rate in two-or three-dimensions or any morphological features [20].

Consistently, in separate studies, the rate of proliferation of pancreatic adenocarcinoma and metastatic colon carcinoma (SW620) cells was found to remain unaltered by Drg-1 expression [23, 22]. Interestingly, however, Stein *et al.* recently observed that Drg-1 inhibits proliferation of metastatic lung cancer cells (H1299) but does not affect the growth of non-metastatic colon cancer cells (DLD-1) [26]. In addition, growth-inhibitory property of Drg-1 has been observed in breast (MCF7) and bladder (EJ) cancer cells, and Drg-1 has been also shown to acts as an inhibitor of polyploidy in p53-null tumor cells [12, 52]. Drg-1 may therefore affect cell proliferation albeit in a cell-type-specific and/or context-dependent manner. In terms of metastasis suppression, it is plausible that Drg-1 affects the growth of tumor cells at the secondary site although the factor(s) that trigger the growth-inhibitory property of this gene remains to be understood.

To gain mechanistic insight into the functional role of the Drg-1 gene as a metastasis suppressor, several approaches are underway in our laboratory. Recently, using microarray gene expression analysis technique, we have found that Drg-1 significantly suppressed expression of the ATF3 gene which was previously known as a stress inducible transcription factor [manuscript under preparation]. The ATF3 gene, also known as LRF-1 (Liver regeneration factor-1), belongs to the ATF/CREB family, and as a homodimer acts as a transcriptional repressor on various promoters while it functions as suppressor when it forms a heterodimer [53].

We have also observed that ATF3, when stably transfected into prostate carcinoma cells, significantly promotes invasiveness of the cells. More importantly, ATF3 overexpression significantly enhanced the spontaneous pulmonary metastasis of the rat prostate carcinoma cells AT2.1, which otherwise have a low metastatic potential. At the clinical level also, we have observed a significant negative correlation between Drg-1 and ATF3 expression in the case of prostate cancer. These findings strongly support our notion that Drg-1 suppresses tumor metastasis by inhibiting the function of the ATF3 gene. Consistent with our observation of the metastasis-promoting role of ATF3, Ishiguro *et al.* have previously observed that ATF3 enhanced experimental metastasis of murine melanoma cells and that antisense blocking of the ATF3 mRNA inhibited cell migration and invasion [54, 55].

CAN CANCER METASTASIS BE VIEWED AS A STEM CELL DISEASE?

Cancer stem cells have recently been identified in a number of solid tumors and have been proposed to be the critical cell population for initiation and propagation of cancer [56]. Interestingly, Karhadkar *et al.* have found that blockade of the sonic hedgehog (Shh) pathway by the specific pathway inhibitor cyclopamine led to concomitant upregulation of the Drg-1 gene in metastatic prostate cancer cell lines, PC3 and Du-145, while benign prostate epithelial cells exhibited high basal level of Drg-1 that remained unchanged by cyclopamine treatment [57]. While the Shh pathway plays indispensable role in embryonic pattern formation, it is also essential for maintenance of the pool of adult stem cells in various organs where the inappropriate activation of the pathway leads to tumorigenesis [56]. In the case of human prostate cancer, Shh pathway activity has been found to be dramatically augmented in the cells that have metastasized compared to those that are localized [57]. It is therefore plausible that

Shh promotes metastasis, at least in part, by inhibiting the expression of the metastasis suppressor gene Drg-1 and that Drg-1 plays a crucial role in blocking metastatic dissemination of tumor stem cells.

CONCLUSION

Drg-1 is a recently discovered metastasis suppressor gene which is at the center of wide array of important regulatory factors. Based on our experimental findings as well as current literature, we propose that several factors including the tumor suppressor gene PTEN upregulate the expression of the Drg-1 gene, which in turn suppresses ATF3, thereby inhibiting metastatic colonization at the secondary site. We have just begun to understand the molecular mechanism of action of this gene as a metastasis suppressor, and there are several crucial questions that remain to be answered. We are making an effort to understand what are the interactors of the Drg-1 protein, and how such interaction(s) modulate the activity of Drg-1. It will be also interesting to unravel any cross-talk that may exist between Drg-1 and other tumor metastasis suppressors and perceive the network of action of the metastasis suppressor genes in tumor cells.

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FIGURE LEGENDS

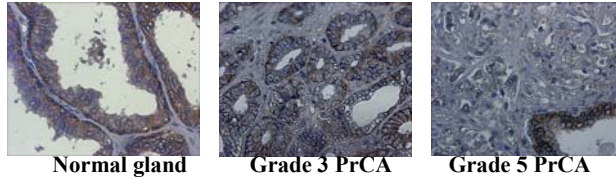
Fig. 1. Immunohistochemical analysis of Drg-1 in human prostate and breast cancer. (a) Using anti-Drg-1 antibody, immunohistochemistry was performed on paraffin tissue sections from prostate and breast cancer patients of various grades. (b) Association of Drg-1 with other clinical parameters in prostate cancer. In each case, chi-squared test was performed to test the significance of association. * indicates statistically significant correlation ($P < 0.05$). (c) Drg-1 expression is correlated with overall survival rate. Overall survival rate over a period of 5 years was measured in patients with prostate and breast cancer, in relation to Drg-1 expression. The solid and dotted line indicate patients with positive and reduced expression of Drg-1, respectively. P value was determined by log rank test.

Fig. 2. Drg-1 suppresses spontaneous lung metastasis without affecting growth of primary tumor. (a) The parental cell line AT6.1, AT6.1 cells transfected with empty vector (vector only), and Drg-1 positive (#4, #7, #8, #10) and negative (# 12) clones were injected subcutaneously into SCID mice (5 mice per group). After 4 weeks, the mice were sacrificed and the lungs were removed. The tumor nodules on the lungs were counted macroscopically. The lungs from two mice from each group are shown as examples. (b) The table summarises the data from the animal experiment described above.

Fig. 3 Proposed regulation and mechanism of action of Drg-1.

(a)

Prostate tissue



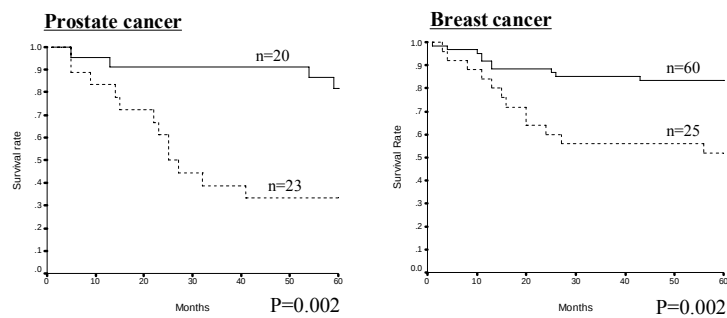
Breast tissue

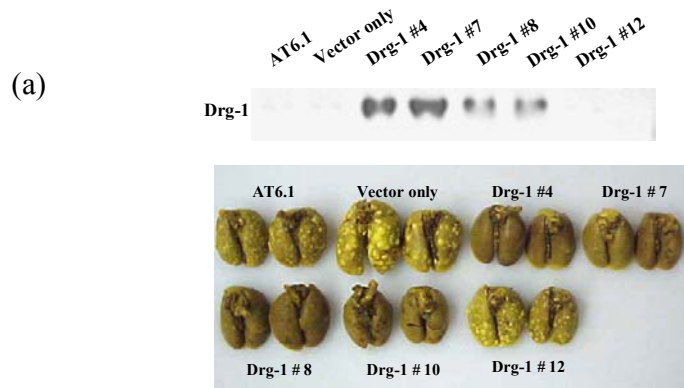


(b)

	All (62)	Drg-1 expression positive (34)	reduced (28)	P value
Gleason grade				
≤ 7	38	26	12	0.015*
> 7	24	8	16	
Differentiation				
Well	16	14	2	<0.001*
Moderate	19	14	5	
Poor	27	6	21	
Metastasis status				
Organ confined	40	28	12	0.003*
Lymph node metastasis	20	5	15	
Bone metastasis	19	5	14	0.006*

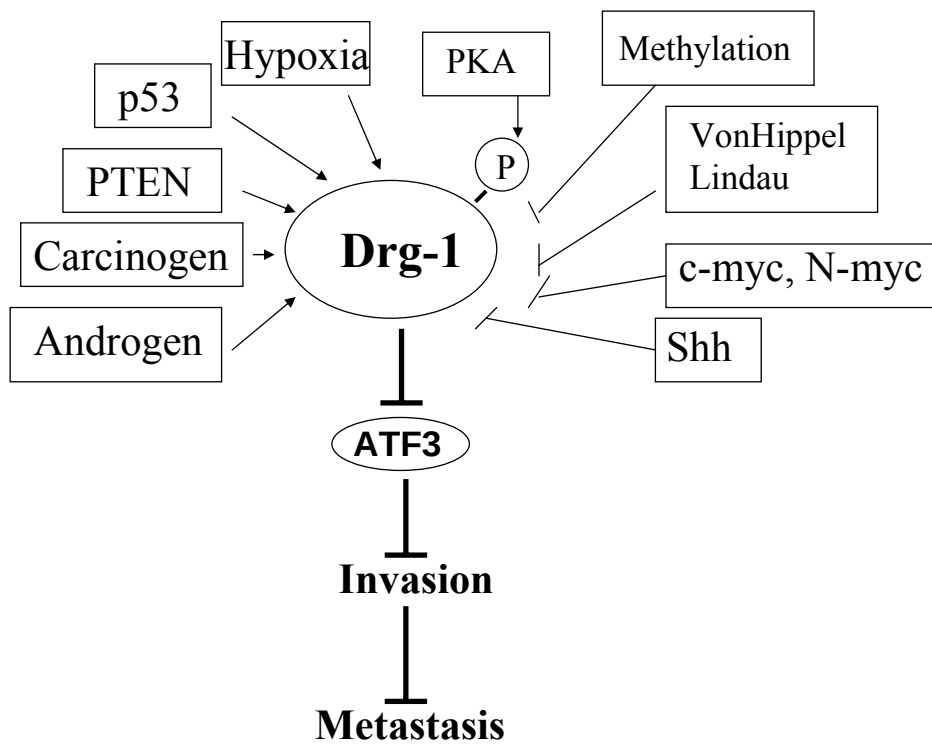
(c)





(b)

Cell line	Drg-1 ^a	Tumor incidence ^b	In vivo doubling time	Lung metastases			P value
				Mean $\pm$ SE ^d	Median ^d	(Range) ^c	
AT6.1	-	5/5	3.4 $\pm$ 0.3	153.7 $\pm$ 22.0	150	(116-195)	
Vector only	-	5/5	3.3 $\pm$ 0.4	134.5 $\pm$ 22.8	110	(78-240)	0.7
Drg-1 #4	+	5/5	3.4 $\pm$ 0.3	13.7 $\pm$ 6.6	10	(3-32)	<0.001*
Drg-1 #7	+	5/5	3.0 $\pm$ 0.2	5.8 $\pm$ 2.5	2	(2-14)	<0.001*
Drg-1 #8	+	5/5	2.9 $\pm$ 0.4	11.4 $\pm$ 5.5	9	(0-11)	<0.001*
Drg-1 #10	+	5/5	3.2 $\pm$ 0.5	1.0 $\pm$ 0.5	1	(0-3)	<0.001*
Drg-1 #12	-	5/5	3.4 $\pm$ 0.4	176 $\pm$ 33.1	180	(80-280)	0.7





Review

Drug development against metastasis-related genes and their pathways: A rationale for cancer therapy

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ABSTRACT

It is well recognized that the majority of cancer related deaths is caused by metastatic diseases. Therefore, there is an urgent need for the development of therapeutic intervention specifically targeted to the metastatic process. In the last decade, significant progress has been made in this research field, and many new concepts have emerged that shed light on the molecular mechanism of metastasis cascade which is often portrayed as a succession of six distinct steps; localized invasion, intravasation, translocation, extravasation, micrometastasis and colonization. Successful metastasis is dependent on the balance and complex interplay of both the metastasis promoters and suppressors in each step. Therefore, the basic strategy of our interventions is aimed at either blocking the promoters or potentiating the suppressors in this disease process. Toward this goal, various kinds of antibodies and small molecules have been designed. These include agents that block the ligand-receptor interaction of metastasis promoters (HGF/c-Met), antagonize the metastasis-promoting enzymes (AMF, uPA and MMP) and inhibit the transcriptional activity of metastasis promoter (β -Catenin). On the other hand, the intriguing roles of metastasis suppressors and their signal pathways have been extensively studied and various attempts have been made to potentiate these factors. Small molecules have been developed to restore the expression or mimic the function of metastasis-suppressor genes such as NM23, E-cadherin, Kiss-1, MKK4 and NDRG1, and some of them are under clinical trials. This review summarizes our current understanding of the molecular pathway of tumor metastasis and discusses strategies and recent development of anti-metastatic drugs.

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1. Introduction

Cancer is the second leading cause of death in the USA, and more than half a million people succumb to the disease every year [1]. Despite significant improvements in screening methods and treatment options, the majority of cancer patients are still diagnosed at an advanced stage, and more than 90% of patients ultimately die from sequel of metastatic disease. Therefore, metastasis is a hallmark of malignancy, and no effective therapeutic option is currently available for those patients. Although the clinical importance of tumor metastasis is well recognized, advances in understanding the molecular mechanism involved in metastasis formation have lagged behind other developments in the field of cancer research. This is attributed to the fact that cancer cells are extremely heterologous in nature and that metastasis involves multiple steps with a high degree of complexity, and each step requires coordinated action of many promoters and suppressors. However, extensive efforts in the past decade have led to the discoveries of many previously unknown factors involved in metastasis and also unveiled several novel concepts in this research field [2,3]. These findings have shed new light on molecular pathways of metastasis, which also provided valuable information about potential targets for the treatment of metastatic disease. This review discusses our current understanding of molecular mechanism of metastatic process and summarizes recent information of drug development specifically targeted to the metastatic pathways.

2. Tumor metastasis involves multi-step process with high complexity

A primary tumor generally consists of heterogeneous cell types including a small number of cancer stem cells that are able to perpetually proliferate without responding to tumor suppressor function. The current theory predicts that these cancer stem cells originate from a normal stem cell or a cancer cell, which acquired a stem cell-like ability [4]. When a tumor grows more than 1 mm³ in size at the primary site, it acquires active supply of oxygen and nutrients by promoting angiogenesis. Tumor cells accomplish this task by generating hypoxic environment followed by secretion of angiogenic growth factors (Fig. 1). Tumor cells that gain growth advantage further proliferate and acquire metastatic phenotypes due to additional mutations. The first step in metastasis is the detachment of

these tumor cells from the primary tumor mass by acquiring an invasive phenotype that results in the loss of cell-cell adhesion and cell-extracellular matrix adhesion followed by proteolytic degradation of the matrix (Fig. 1) [5]. It is believed that autocrine motility factor (AMF) and hepatocyte growth factor (HGF) are critical components of motility and that degradative enzymes including serine-, thiol-proteinases, heparanases and metalloproteinases such as MMP2 and 9 play critical roles in the invasion [6–8]. When tumor cells intravasate surrounding tumor vasculature and neighboring lymphatic vessels, they must survive in this hostile environment that includes mechanical damage, lack of growth factor from the original environment and the host immune system (Fig. 1) [9]. Tumor cells in the circulation often aggregate with platelets and fibrin, and they embolize in the capillaries or directly adhere to the endothelial cells by a mechanism similar to leukocyte adhesion at the inflammatory site [10–12]. In some cases, arrested tumor cells extravasate before proliferating themselves using the same hydrolytic enzymes that are used in the initial step of invasion (Fig. 1) [13]. However, in many cases, cancer cells actually proliferate within the lumen of vessels to create a considerable tumor mass that can eventually obliterate the adjacent vessel wall by pushing aside the barrier composed of endothelial cells, pericytes and smooth muscle cells that previously separated the vessel lumen from the surrounding tissue [14,15]. After extravasation, cancer cells lodge at the secondary sites, where the cells must also proliferate and colonize for successful metastasis (Fig. 1). These processes are controlled by various metastasis promoters and suppressors, and they must be well coordinated to establish successful distant metastasis (Table 1) [2]. Recent advancement of research in this field has revealed the complex interplay of metastatic factors and many novel concepts of signal pathways leading to metastasis (Fig. 2 a,b). Based on this information, the current research is gradually moving toward translational stage by aiming at development of targeted anti-metastatic drugs (Table 1). The following sections summarize up-to-date information of the promoters and suppressors of metastasis that are currently under active investigation for drug development.

3. Metastasis promoters

3.1. Amf

Autocrine motility factor (AMF) was originally isolated as a C-X-X-C cytokine that stimulates random or directed motility of AMF-

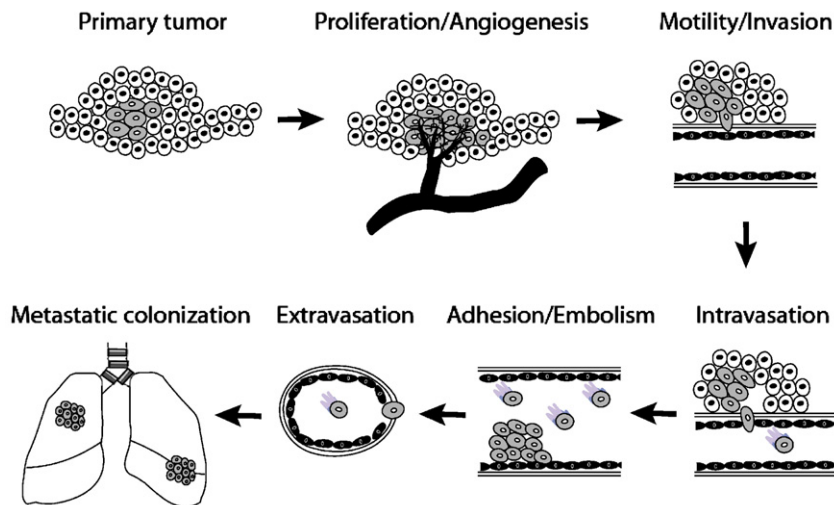


Fig. 1. Process of tumor metastasis. As primary tumor grows, tumor cells induce angiogenic factors to promote vessel formation which facilitates tumor growth and cell invasion into the circulatory system. Some tumor cells gain an invasive ability by expressing motility factors and proteases followed by breaching the basement membrane. Tumor cells then enter the blood vessel where they often aggregate with the platelets and cause embolize. When cells migrate to a distant organ, they adhere to endothelial cells and extravasate by inducing proteases. Cells then colonize and establish metastasis at the distant organ site where appropriate growth factors are provided.

producing tumor cells in an autocrine manner [16]. Elevated serum AMF was found in patients with malignant tumors such as colorectal, lung, kidney, breast and gastrointestinal carcinomas and is well correlated with the development of metastasis [16–19]. AMF is a multifunctional molecule, also known as phosphoglucose isomerase, neuroleukin, and maturation factor [20]. AMF causes tumor cell detachment from the primary site by promoting cell motility in an autocrine fashion. However, recent research revealed that AMF also contributes to malignant progression by stimulating the migration and proliferation of endothelial cells via its receptor AMFR, a unique seven transmembrane receptor (gp78), followed by activation of small Rho-like GTPase [16,21]. Therefore, tumor cells appear to induce aggressive angiogenesis by promoting cross-talk of signals between VEGF-VEGFR and AMF-AMFR which also promotes cell survival via activation of Akt and MAPK-dependent anti-apoptotic pathways (Fig. 2) [22]. A recent report by Raz et al. demonstrated a more direct role of AMF in tumor progression and metastasis. They have shown that overexpression of AMF in normal fibroblasts lead to a gain of tumorigenicity, whereas down-regulation of AMF by siRNA in mesenchymal tumor cells resulted in mesenchymal-to-epithelial transition (MET), the reverse process of epithelial-to-mesenchymal transition, as reflected by a loss of cell polarity, reduced proliferation and invasion *in vitro* and loss of tumorigenic properties *in vivo* [23]. Interestingly, they later also showed that silencing AMF expression in human fibrosarcoma cells resulted in an increased sensitivity to oxidative stress-induced and p21-mediated cellular senescence, which brought a novel insight into the function of AMF in tumor progression [24]. Collectively, neutralizing AMF, disruption of AMFR and blocking their signal pathways are considered to be rational approaches for anti-metastatic drug development.

It has been shown that specific carbohydrate phosphate inhibitors including E4P, D-mannose-6-phosphate and 5-phospho-D-arabinonate (5PAA) are able to block both AMF enzymatic activity and AMF-induced cell motility [25,26]. Treatment of tumor cells with these inhibitors has been shown to decrease the growth, DNA synthesis, migration and invasiveness of several types of cancer cells [22,23,27]. Since these carbohydrate phosphate inhibitors are among the smallest compounds that have AMF inhibitory activity, information of the known crystal structure may help in designing a lead compound to develop more effective AMF inhibitors.

Because AMF is a secretory factor, antibody against AMF may also be a rational approach. In fact, Talukder et al. showed that neutralizing antibodies against AMF were able to partially block HRG-induced invasiveness of human breast cancer MCF-7 cells [28]. Raz et al. also demonstrated that a monoclonal anti-AMF antibody induced apoptosis in human fibrosarcoma cell lines *in vitro* and effectively promoted drug-induced apoptosis *in vivo* [22]. Therefore, humanized anti-AMF holds promise for future therapeutic application. Interestingly, antibody against EGFR2 (Herceptin) was also shown by Talukder et al. to block AMF expression and its promoter activity [27]. Because Herceptin has been used as an effective drug for breast cancer, it is interesting to know whether this antibody also blocks the invasiveness of the tumor.

Ectopic expression of AMF makes some tumor cells become resistant to apoptosis induced by serum deprivation, and this resistance appears to be mediated via PI3K and PKC/MAPK pathways (Fig. 2A). Yanagawa et al. recently indeed showed that PI3K inhibitors (Ly294002 and Wortmanin), PKC inhibitor (GF109203) and MAPK inhibitor (PD98059) were able to recover the expression of Apaf-1 (Apoptotic protease activating factor 1) in the AMF-transfected HT1080 cells followed by induction of apoptosis [22]. In addition, GF109203X and Wortmanin were shown to inhibit AMF-induced expression of fms-like tyrosine kinase (Flt-1) and hence impair the proliferative signals of VEGF in endothelial cells. Therefore, AMF may be a good target for anti-angiogenic therapy, although potential side effects of such drugs are unknown. Finally, it is recently found that the stability of AMF protein is

regulated through ubiquitin-lysosome system, which is mediated by poly (ADP-ribose) polymerase-14 (PARP-14). This new discovery may offer a novel target to block the AMF/AMFR signaling and deserves further investigation [29].

3.2. Hgf/sf

Hepatocyte growth factor (HGF), also known as scatter factor (SF), was identified as the natural ligand for the c-Met receptor tyrosine kinase [30]. HGF/SF interacts with c-Met receptor and transduces multiple biological signalings that control proliferation, disruption of intercellular junctions of EMC, migration and protection from apoptosis [31,32]. HGF/SF signaling has also been demonstrated to play an important role in a wide variety of human cancers of both epithelial and mesenchymal origins [31]. The results of several clinical studies indicate the prognostic value of HGF/SF and c-Met in various types of cancer and that the expression of HGF and/or c-Met is frequently associated with the aggressive nature of the tumors and the poor clinical outcome [31,33]. The exact mechanism of up-regulation of these genes in cancer is not well understood. However, a recent study suggested that the up-regulation of c-Met and HGF may be due to the stress of tumor microenvironment such as hypoxia [34]. Therefore, HGF/SF is considered to be widely involved in the tumor metastatic process. HGF is a potential promoter of cell invasion by directly stimulating the motility and migration of cancer cells as well as affecting the microenvironment [32]. HGF can disrupt cell-cell adhesion and promote cancer cell growth, partly by inducing phosphorylation of β -Catenin and relocation of E-cadherin, which may result in down-regulation of cell cycle regulatory factors such as p27 (Fig. 2A) [35–37]. On the other hand, HGF can increase the adhesion between cancer cells and matrix by activating the FAK and paxillin pathways, which cooperatively regulate the expression of integrins in cancer cells and eventually lead to adhesion as well as migration of cancer cells to matrix [38]. HGF is also able to increase the expression and secretion of proteolytic enzymes from cancer cells including MMP2, MMP7, MMP9 and uPA that are involved in matrix and basement membrane degradation (Fig. 2) [36,39,40]. In addition, HGF is considered as an angiogenesis-promoting factor through its direct morphogenic and adhesive effects and indirect regulation of other angiogenic factors such as IL-8, VEGF and TSP-1 [41,42]. Furthermore, Boccaccio et al. have recently demonstrated that the c-Met oncogene was responsible for the induction of thrombohemorrhagic syndrome, suggesting that c-Met may give survival advantage to tumor cells in the circulation by promoting the aggregation of tumor cells with platelets [43,44]. Therefore, the HGF/c-Met signaling plays a critical role in the metastatic process and this gene as well as the downstream signal can be potential targets for cancer therapy.

Recently, rapid progress has been made toward drug development against HGF/SF for the purpose of cancer therapy. These include HGF antagonists, anti-HGF and anti-cMet antibodies, small molecules targeting c-Met and its signaling pathways as well as compounds interfering with HGF-elicited biological activities [45]. Antagonizing ligand binding that block the activation of downstream signaling is a conventional therapeutic strategy for most carcinomas. NK4 is one of the antagonists that compete with HGF for the c-Met receptor, and it has been known to block HGF-induced cellular adhesion, invasion and metastasis in various types of cancer cells including breast, bladder, colorectal, lung, prostate, glioma, pancreatic and gastric cancers *in vitro* [46]. Moreover, NK4 also acts as angiogenesis inhibitor, and this activity is independent of its action as HGF-antagonist [47,48]. As expected, treatment of mice via intraperitoneal or intratumoral administration of NK4 protein or recombinant adenoviruses expression vector effectively blocked tumorigenesis, angiogenesis and metastasis in various mouse xenograft models including pancreatic and gastric cancers [46,49]. Another antagonist is an uncleavable HGF, which was engineered with a single amino-acid substitution at the

Table 1

Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
AMF	carbohydrate phosphate compounds (E4P,M6P,5PA)	AMF	Inhibit AMF cytokine enzymatic activity		Pre-clinical studies	[25,26]
	Herceptin	EGFR2	Down-regulates AMF protein and promoter activity	Increase the tumor progression time in mice model of xenograft tumor of Her2 over-expression	In clinical use	[27,270]
HGF/c-Met	NK4	HGF	competitive antagonist for HGF binding to the c-Met receptor	Inhibited tumorigenesis, angiogenesis and metastases in mouse tumor xenograft models	Pre-clinical	[46,49]
	uncleavable HGF	HGF	Prevent maturation of pro-HGF and compete with HGF to bind to c-Met receptor	Inhibited tumor growth, angiogenesis and metastases in tumor xenograft models	Pre-clinical	[50]
	AMG102	HGF	Neutralizing anti-HGF antibody	pharmacokinetic and safety profile are passed through in cynomolgus monkeys test	Phase II	[53]
	DN30	c-Met	Binds to extracellular domain of c-Met and prevent its activation	inhibited growth and metastatic spread to the lung of tumor xenograft mouse model	Pre-clinical	[61]
	PHA-665752 SU11274 K252a	Kinase inhibitors	inhibit c-Met phosphorylation	Inhibition of tumor growth in c-Met-dependent lung and gastric carcinoma xenograft animal model	Pre-clinical	[55-60]
TGF- β	SD-208	TGF β 1 receptor	TGF- β typel receptor kinase inhibitor	Inhibited primary tumor growth, angiogenesis and metastasis of xenograft animal model	Pre-clinical studies	[68,73,87-92]
	SD-093 SB-431542 A-83-01 LY2109761 2G7	TGF β	Neutralizing antibody of TGF β	Inhibited abdominal and lung metastasis of xenograft animal model	Pre-clinical	[69]
	β - glycan (sRIII)	TGF β	Soluble extracellular domain of TGF- β type III receptor	Inhibited lung metastasis in human breast tumor xenograft model	Pre-clinical	[96]
	Fc:TpRII	TGF β	Dominant negative TGF- β typell receptor	Inhibited lung metastasis in human melanoma xenograft model and MMTV-Neu model	Pre-clinical	[94,95]
	AP12009	TGF β	Oligonucleotide against human TGF β 2		PhaseI/II (high grade glioma)	[97]
			Pharmacologically developed MMPs inhibitor		PhaseII,III,IV (Pancreatic cancer) phaseIII Non-small-cell lung cancer)	[122,271]
MMP	Marimastat (BB-2516)	MMPs			Phase III, IV (NSCLC)	[122,272]
	Prinomastat (AG3340)	MMPs	inhibitor with selectivity for MMPs 2, 3, 9, 13, and 14	enhance tumoricidal activity after Photodynamic therapy in a mouse mammary tumor model	phaseII (advanced esophageal cancer)	[122,272]
	Tanomastat(BAY12-9566)	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII (Small-cell lung and pancreatic cancer)	[122,271]
	BMS-275291Neovastat	MMPs	Pharmacologically developed MMPs inhibitor		PhaseIII, IV (Non-small-cell lung and Renal cell carcinoma)	[122,271]
	Bisphosphonates (BP)	for use in disorders of bone metabolism	Inhibit proteolytic activity of MMPs	Increase bone mineral density in animal model	In use (osteolytic metastases)	[129]
uPA	WX-UK1	uPA	Protease inhibitor		Phase I,II	[148]
	WX-671	uPA				
	231 Bi-PAI2	uPA	Recombinant PAI-2 (uPA inhibitor-2)	Inhibited micrometastasis in human breast cancer xenograft models	Pre-clinical studies	[160-162]
	1-Isoquinolinyguanidines (UK-356,202) and its derivatives	uPA	Reversibly competitive inhibitors of uPA enzymatic activity	Inhibit exogenous uPA in human chronic wound fluid and in the porcine excisional wound model	Pre-clinical studies	[273]
	Bikunin	Trypsin and plasmin	Down-regulate uPA gene and protein expression	once-daily oral administration of bikunin against ovarian carcinoma in nude mice	Phase I	[153-156]
β -catenin	DX-1000 PEGylated DX-100	plasmin	Down-regulate uPA expression	Inhibited tumor proliferation and vascularization in human tumor xenograft model	Pre-clinical	[157,158]
	Celecoxib	COX-2	Induce degradation of β -catenin via a COX-2-independent mechanism	Diet treatment significantly reduce tumor development without signs of metastasis in TRAMP mice	phase II (advanced colorectal cancer)	[182,183,274]
	R-Etodolac and its analog (SDX-308)	enantiomer of Etodolac	Down-regulates protein and promoter activity, increase β -catenin and E-cadherin complex at the membrane	inhibited tumor development and metastasis in the transgenic mouse adenocarcinoma of the prostate (TRAMP) model	phase II (chronic lymphocytic leukemia)	[182,183]
	Thiazolidinedione (TZD)	PPARs	cause localization shift to cytoplasm, reduced tyrosine phosphorylation of β -catenin	Inhibited lymph node and lung metastases in the xenograft animal model	Pre-clinical studies	[185]

Table 1 (continued)

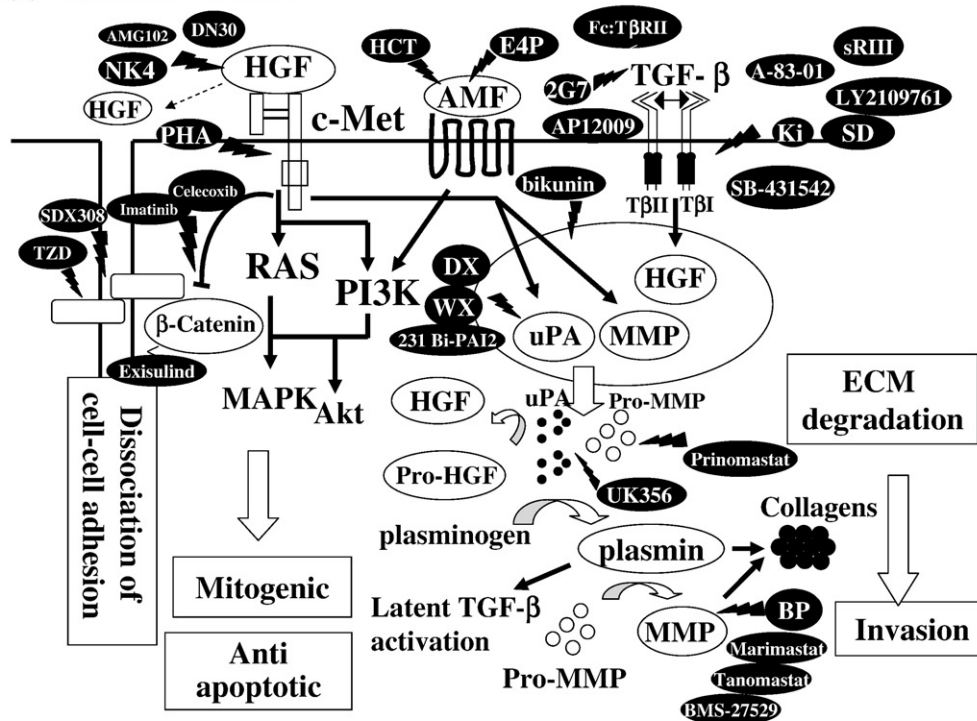
Metastasis promoter	Drug	Original target	Action	Animal	Clinic trial	Reference
β -catenin	Exisulind(Aptosyn)	SAANDs	Down-regulate β -catenin and cyclin D1 via PKG-mediated signalling	Inhibited tumor growth and metastasis of human lung cancer xenograft in athymic nude rats.	Phasae I,II,III	[164,172,173]
	CP461 CP248 Imatinib (Gleevec)	PDGF receptor	Inhibits tyrosine phosphorylation of β -catenin and resultant cell migration		In use (chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) etc)	[176]
Metastasis Suppressor NM23	Medroxyprogesterone acetate (MPA)	Progesterone receptor	MPA elevated NM23 expression and inhibited soft agar colonization	Inhibited lung cancer metastasis in the experimentally metastasis mice model	Phase III(metastatic breast cancer)	[206,275,276]
	Estradiol	Estrogen receptor	Up-regulates NM23-H1 in ERa+ breast cancer cell lines. Inhibits invasion <i>in vitro</i> .	Suppression of lung metastasis <i>in vivo</i> model of chemically induced hepatocellular carcinoma.	Phase II (metastatic breast and prostate cancer)	[212,277]
	Aspirin	Cox1/2 inhibitor	Up-regulates NM23. Decreased metastatic phenotype <i>in vitro</i> .		Phase III (esophageal cancer)	[217,277]
	Indomethacin	Cox1/2 inhibitor	Up-regulates NM23 expression in breast cancer cell lines	Inhibited lung tumor metastasis in the experimental metastasis mice model	Phase II (head and neck cancer)	[219,220,277]
	All-trans retinoic acid (ATRA)	Retinoid receptors	Up-regulates NM23 in hepatocarcinoma cells. Increased adhesion to ECM <i>in vitro</i>	Inhibits the growth of xenograft tumors and gastric cancer cell metastasis to liver.	Currently in clinical use, (acute promyelocytic leukemia)	[226-228,278]
KISS-1	Metastin	orphan G-protein coupled receptor	Regulate the NF κ B signaling pathway		Pre-clinical studies	[279]
MKK4	Anti-death receptor antibody (2E12, TRA-8)	death receptor	Induce apoptosis <i>in vitro</i> . Activate MKK4/JNK/p38 pathways		Pre-clinical studies	[241]
	Bisindolylmaleimide VIII	PKC inhibitor	Enhances affects of anti-death receptor antibodies		Pre-clinical studies	[245]
E-cadherin	pyrazolo [3,4-d] pyrimidines (PP)1, PP2	Src family inhibitor	Reactivate the E-cadherin expression. Reduced migration ability of breast cancer cells	Decrease in pancreatic tumor growth and metastasis in nude mice	Pre-clinical studies	[255,256,280]
NDRG1	Fe chelator (DFO, 311)	Fe	NDRG1 was specifically up-regulated by Fe chelation.	Delay or regression of tumor cell growth in athymic nude mice.	Phase II (Neuroblastoma)	[263,266,281,282]

proteolytic site of HGF [50]. The uncleavable HGF competes with endogenous pro-HGF for the catalytic domain and thus inhibits endogenous pro-HGF maturation. The peptide also binds to the c-Met receptor with high affinity and displaces the mature ligand. More strikingly, both local and systemic administration of uncleavable HGF in a xenograft mouse model significantly suppressed tumor growth and tumor angiogenesis, and notably inhibited the formation of spontaneous metastases without affecting vital physiological functions [50]. In a separate study, neutralizing anti-HGF antibodies were first developed by Cao et al. who demonstrated that a minimum of three antibodies, each of which act on different HGF epitopes, were required to block c-Met tyrosine kinase activation and the biological outcomes [51]. Moreover, Burgess et al. have shown that fully humanized monoclonal anti-HGF antibodies effectively suppressed HGF-dependent tumor growth in tumor xenograft mouse model [52]. Another fully human HGF antibody, AMG102, was recently tested for its pharmacokinetics and safety in monkeys and further clinical investigation was warranted [53].

It is recently suggested that MET functions in certain human cancers as "oncogene addiction", the concept formulated in the late 1990s, indicating a constant requirement of MET in these tumors [54]. Therefore, targeting the activated c-Met holds a great promise as an anti-cancer therapy at least for certain tumor types. Regarding c-Met tyrosine kinase receptor inhibitors, a set of low molecular weight compounds including PHA-665752, SU11274, and K252a, which are able to compete for the ATP binding and prevent receptor transactiva-

tion and recruitment of the downstream effectors, have recently been tested and shown to effectively inhibit the kinase activity and block the subsequent signaling pathways [55–58]. Particularly, PHA-665742 is capable of inhibiting the autophosphorylation of c-Met with a relatively high specificity compared to other tyrosine and serine-threonine kinases [55,59]. In addition, PHA-665752 was shown to induce massive apoptosis in human gastric cancer cell lines that had amplified MET genes, while it did not affect other cell lines without c-Met receptor amplification [59]. Furthermore, Salgia et al. has recently shown that PHA-665752 treatment inhibited tumorigenicity and angiogenesis in a mouse model of lung cancer xenografts [60]. These results strongly support a potential utility of these compounds for a therapeutic application in the future. Designing a drug that binds the extracellular domain of the c-Met receptor and thus impairing receptor dimerization has been considered as another c-Met blocking strategy. Recently, Petrelli et al. showed that a monoclonal antibody, DN30, prevented c-Met activation and abrogated its biological activity [61]. In addition, soluble recombinant Sema proteins or anti-Sema antibodies against the extracellular Sema domain that is involved in ligand binding and receptor dimerization of c-Met have been generated [62]. As expected, they suppressed the downstream signaling triggered by the c-Met receptor even in the presence of HGF. Another alternative strategy for specifically blocking the receptor is a gene silencing technology. Using adenovirus vectors carrying small-interfering RNA targeting c-MET, Shinomiya et al. demonstrated that the siRNA drastically reduced the c-MET gene expression

(a) Metastasis Promoters



(b) Metastasis suppressors

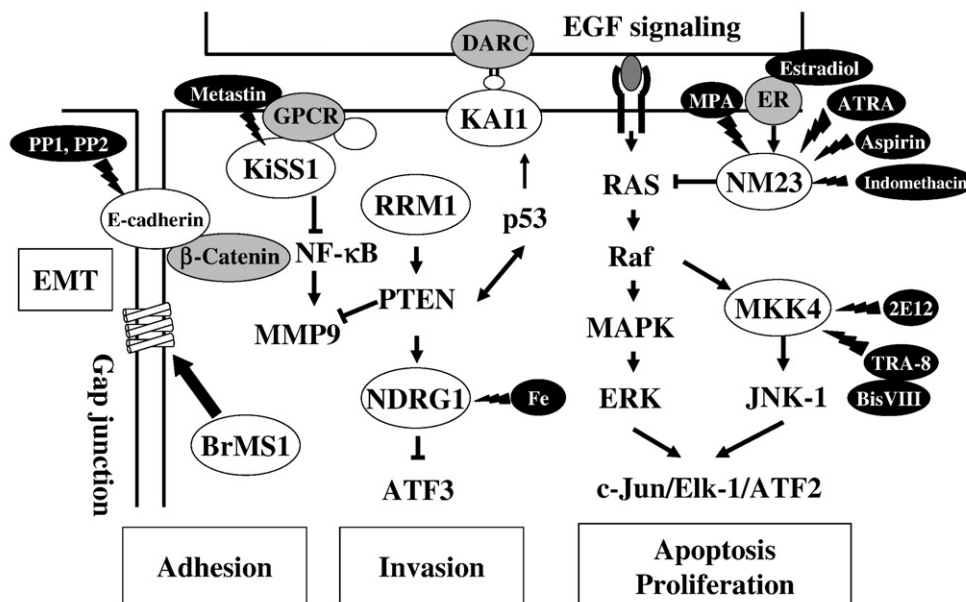


Fig. 2. Signal pathway of tumor metastasis. Tumor metastasis is a result of complex interplay of both positive (a) and negative (b) factors. These pathways and their factors are potential targets for anti-metastatic therapy. The drugs currently under development are shown as black oval shapes.

followed by significant inhibition of proliferation and invasion of various tumor cells lines both *in vitro* and *in vivo* [63]. Collectively, recent information about the mechanistic insight of HGF/c-Met signaling in tumor progression has greatly facilitated the development of a variety of strategies for anti-HGF/cMet therapies, and some of these compounds hold great promises for future clinical application.

3.3. Tgfb

Transforming growth factor- β (TGF β) is a secreted polypeptide cytokine that plays multiple roles in cell proliferation, differentiation,

extracellular matrix production, migration and apoptosis [64–66]. Notably, in normal epithelial cells and at an early stage of tumorigenesis, TGF β inhibits the proliferation of cells by inducing cell cycle arrest, promoting apoptosis, and enhancing genomic stability [65,66]. However, as the tumor develops, cancer cells become resistant to TGF β -mediated growth inhibition because of the loss of TGF β signaling, mutations of cell cycle regulators, or alteration of cross-talk signaling pathways such as activation of Ras [67].

TGF β 1 has been shown to be over-expressed in 74% and 60% of patients with breast and colon cancers, respectively. Interestingly, more intense staining patterns for TGF β 1 are observed in various

types of metastatic cancer including breast, colon, liver, lung, prostate and stomach compared to primary tumors, emphasizing the importance of TGF β signaling for pro-metastatic activity [68]. Transplanting cell lines stably over-expressing TGF β 1 into athymic mice has been shown to cause increased tumor growth and metastases *in vivo* [69,70]. In another study, transgenic mice that co-express MMTV-Neu and MMTV-TGF β 1 developed mammary tumors with the same latency as the control MMTV-Neu transgenic mice; however the co-transgenics showed significantly more local invasion and elevated numbers of circulating tumor cells and lung metastases [71]. Thus, over-expression of TGF β can enhance and stimulate tumor growth and malignant progression at least in particular subtypes of tumors. Therefore, TGF β has been recognized as a tumor promoter at an advanced stage of some tumors, probably by stimulating tumor cell invasion, angiogenesis and immunological surveillance [65,66].

It has been shown that mouse and human carcinomas often over-express TGF β , which promotes Epithelial-mesenchyma Transition (EMT) via the Smad pathway [66]. Furthermore, Shen et al. have shown that TGF β was capable of inducing the expression of guanine exchange factor NET1 via Smad3 followed by activation of the Rho GTPase pathway, which results in local disassembly of the actin cytoskeleton and tight junction breakdown [72]. On the other hand, TGF β can also activate various non-Smad signaling effectors including Ras, Rho GTPase, Erk1/2, PI3K and NF- κ B that all play critical roles in EMT, which eventually promotes tumor metastasis [67,73,74]. It has been shown that the motility of metastatic breast carcinoma cells responding to autocrine TGF β 1 did not require Smad activation but rather the activity of the PI3K pathway [74]. In addition, Vogelmann et al. have shown that in polarized epithelial cells, TGF β blocked cell-cell adhesion by inducing tyrosine phosphorylation of α - and β -Catenin which disrupts the E-cadherin/catenin complexes with actin, and by inducing the expression of transcriptional repressors of the E-cadherin gene such as Snail, Slug and LEF1 [75,76]. Wikstrom et al. showed that the ectopic expression of TGF β in human prostate cancer correlated with increased angiogenesis around the tumor and eventually lead to a high rate of metastasis of prostate carcinoma cells [77]. The ability of TGF β to promote angiogenesis is considered to be the action of either inducing expression of VEGF, which directly stimulates the proliferation and migration of endothelial cells, or its chemoattractant activity for monocytes that release angiogenic cytokines [78]. It should be also noted that, in breast cancer, TGF β stimulates the expression of pTHrP (parathyroid hormone related protein) which promotes osteolytic metastasis and also suppresses late stages of osteoblast differentiation, which leads to net bone loss [79]. Furthermore, TGF β plays a role in helping tumor cells to escape from the immunological surveillance through its ability to inhibit B and T lymphocyte proliferation and differentiation [80]. TGF β is also able to deactivate macrophages and thus protect the tumor cells from the immune surveillance [81]. Collectively, because TGF β often promotes tumor progression in particular subtypes, the components of the TGF β signaling pathway are being considered as prognostic biomarkers for such tumors as well as potential therapeutic targets [68].

On the contrary, to the tumor-promoting activity of TGF β , this molecule also has tumor suppressive function at an early stage in some types of cancer. Therefore, TGF β is considered as a target for chemoprevention for the population with high-risk cancer incidence. To this end, several compounds have been examined and these include FTI-277, Dietary ω -3 fatty acids, Captopril, Suberoylanilide hydroxamic acid (SAHA) and triterpenoids. They are capable of enhancing the expression of TGF receptor (T β RII and T β RI) at mRNA and protein levels, thus increasing the responsiveness of tumor cells to TGF β with respect to growth arrest and cytostatic effect [82–86]. However, considering the pro-tumorigenic actions of TGF β , such drugs may have dreadful effects by promoting tumor invasiveness and

metastasis. Therefore, current effort is more focused on drugs that block the tumor progression at a later stage. These strategies include developing small molecule inhibitors, affinity- or antibody-based drugs and antisense RNA.

Intense high-throughput screenings have led to the development of selective small molecule inhibitors against the enzymatic activity of the T β RII and T β RI kinases. These inhibitors including SD-208, SD-093, SB-431542, A-83-01 and LY2109761 act as ATP-binding analogues and thus competitively block the catalytic pocket of the receptor kinase [68]. SD-208, an orally active specific T β RI kinase inhibitor, was previously tested in a glioma model, which depends primarily on the pro-tumorigenic action of TGF β . In this study, SD-208 was found to effectively inhibit the TGF β -induced glioma cell migration and invasiveness and also to enhance the immunological surveillance [87]. Recently, Reiss et al. also showed that SD-208 treatment resulted in decreased angiogenesis in a mouse model of mammary carcinoma [88]. In addition, Wong et al. showed that SD-208 reduced primary tumor growth and decreased the incidence of metastasis in an orthotopic xenograft mouse model of pancreatic adenocarcinoma [89]. Thus, this inhibitor holds a great promise for future clinical application. Another small molecule for T β RI kinase inhibitor, SD-093, has been shown to strongly decrease the *in vitro* motility and invasiveness of pancreatic carcinoma cells without affecting their growth [90]. Another set of T β RI inhibitors, SB-431542, A-83-01 and LY2109761, all potentially affect TGF β -dependent transcriptional activation and inhibit TGF β -induced EMT [73]. Interestingly, SB-431542 was demonstrated to reduce colony formation of human lung adenocarcinoma cells, which are growth-dependent on TGF β ; however, it also induced anchorage independent growth of human colon adenocarcinoma cells whose proliferation is promoted by TGF β [91]. Furthermore, SB-431542 showed no effect on a cell line that failed to respond to TGF β , which further strengthens the rationale in using this compound as a therapeutic agent of human cancer responsive to tumor-promoting effects of TGF β . A-83-01 is structurally similar to SB-431542 while it has shown even more potent effect of suppressing T β RI [73]. LY2109761 is a specific pharmacologic inhibitor of T β RI and T β RII kinases. It was demonstrated that this drug was capable of inducing the expression of the Coxsackie and adenovirus receptor (CAR), a tight junction component whose expression is required to be down-regulated for EMT [92]. Currently, some of the above-mentioned specific inhibitors of T β RI have already entered the phase I clinical trials for various human cancers (Table 1).

Neutralizing anti-TGF β antibodies and the soluble extracellular domain of T β RII with receptor-binding activity have also been pursued as anti-TGF β approaches. Interestingly, the results of pre-clinical studies have shown that these drugs had a weak and transiently negative effect on primary tumor growth but strongly suppressed metastasis [73]. Pietenpol et al. have demonstrated that the neutralizing antibody 2G7 which has high affinity to three mammalian isoforms of TGF β showed moderate inhibitory effect on the growth of the primary tumor in an animal model of MDA-MB-231 xenograft, while it almost completely blocked the abdominal and lung metastasis [69]. In addition, enforced expression of the extracellular domain of T β RII has been demonstrated to enhance tumor immune surveillance and strongly inhibit metastasis in animal models of human pancreatic carcinoma [93]. These observations led to a development of a fusion protein of immunoglobulin Fc fragment with the soluble extracellular domain of T β RII (Fc: T β RII) as a therapeutic approach [94]. When tested *in vitro*, this fusion protein indeed effectively induced apoptosis and inhibited migration of breast cancer cells. Furthermore, Wakefield et al. found that when Fc:T β RII was expressed in the mammary gland of MMTV-based transgenic mouse model followed by a challenge of melanoma cells or by crossing it to the MMTV-Neu mouse, it completely blocked lung metastasis without any adverse side effect [95]. The clinical potential of this experiment is significant especially because the chronic presence of

Fc:TRII did not show obvious adverse effects. Similarly, Sun et al. have shown that over-expression of soluble extracellular domain of β -glycan (sRIII) antagonized TGF β in the breast carcinoma cells, which resulted in significant inhibition of metastasis of the tumor cells to the lung, while it moderately blocked the tumorigenic ability [96].

Finally, the antisense DNA or RNAi technology have recently brought a promising development in anti-TGF β therapy. The oligonucleotide AP12009, which is directed against human TGF β 2, has been tested by administering into brain tumors with continuous infusion and showed better survival time after recurrence than other current chemotherapy against gliomas [97]. Also, RNAi for both TGF β 1 and TGF β 2 in human glioblastoma has been reported to be effective in restoring the proper immune response, which significantly decreased the glioma cell motility and invasiveness [98]. Further investigations in this research field are expected to provide valuable information to improve the efficacy of these compounds and to develop a better delivery system for eventual clinical use of anti-TGF β therapy.

3.4. *Mmp*

Matrix metalloproteinases (MMPs), a group of zinc-dependent endopeptidases, was originally identified to have roles in ECM disruption and thus associated with invasion and metastasis in late stages of cancer progression (Fig. 2A). Years of intense investigations of MMPs have highlighted the significance of these molecules in cancer. MMPs contribute to the formation of a complex microenvironment that promotes malignant transformation in early stages of cancer, suppresses tumor cell apoptosis, and enhances angiogenesis as well as impairs the host immunological surveillance [99]. Several studies have indicated that cleavage of particular substrates such as insulin-like growth factor binding proteins (IGFBPs) and TGF β by MMPs can have direct effects on tumor growth [100,101]. In transgenic animals, over-expression of certain MMPs such as MMP1 and MMP3 was sufficient to generate fully malignant tumors in the absence of specific carcinogens [102,103]. In the normal cells or at an early stage of tumor, MMPs can target substrates that influence the apoptotic process of the cells, which is also linked to the chemotherapeutic resistance. Particularly, MMP7 is able to release a soluble form of the death protein Fas Ligand (FasL), which has lower death-promoting potency than the membrane anchored form but has more flexibility to interact with its cognate receptor Fas [104,105]. Thus, the weak but constant apoptotic signal acts as a selective pressure for tumor cells that have elevated anti-apoptotic signals and those that have propensity to acquire additional mutations, which further promote tumor progression. This mechanism is also considered to be the basis of induction of chemoresistance to certain types of tumors [106].

MMPs also play critical roles in angiogenesis. Angiogenic factors such as basic fibroblast growth factor (bFGF) and VEGF are usually localized in the matrix and cannot interact with their receptors until freed by MMPs, particularly by MMP9 through ECM proteolysis [107,108]. In addition, MMP9, when recruited to the tumor cell surface and interact with the docking receptor CD44, can proteolytically cleave latent TGF β and thus promote tumor invasion and angiogenesis [100]. Furthermore, an elegant work of Hanahan and Coussens has shown that MMP9 is predominantly expressed in the tumor-associated stromal cells as well as in macrophages, neutrophils, mast cells and endothelial cells rather than in tumor cells themselves in many cases, which regulates the vascular formation and architecture [109–111]. Intriguingly, Hiratsuka and colleagues have recently shown that MMP9 plays a role in priming premetastatic sites for primary tumor. They demonstrated that tumor-associated macrophages (TAM) induced MMP9 in endothelial cells and in TAMs, which facilitated tumor cell invasion and also prepared the lung as premetastatic niche for the growth of tumor cells in a manner dependent on VEGFR-1 [112].

Escaping from host immune response is a significant problem associated with many cancers. Some MMPs alter the behavior of chemokines and cytokines by specific proteolytic cleavage. For example, MMP9 can suppress the development and propagation of T lymphocytes by disrupting IL-2R α signaling, resulting in attenuation of a T cell-mediated anti-tumor response [113]. Likewise, CXCL12, also known as SDF1 has been identified as a substrate of MMP2. MMP2-mediated cleavage renders CXCL12 unable to bind its receptor CXCR4, which consequently influence the metastatic dissemination of tumor cells [114].

The strong correlations between altered expression of MMPs at mRNA and protein levels in different human cancers with poor disease prognosis have been well established [99,115]. The over-expression of many MMPs, including MMP-1,-2,-7,-9,-13,-14, is positively associated with tumor progression and metastasis [115]. On the other hand, human breast tumor cells with reduced expression of MMP-8 were found to acquire the metastatic ability compared to their non-metastatic counterpart [116]. Interestingly, Balb/c mice have revealed that MMP8-null mice exhibit an increased tumor susceptibility compared to the wild type because of the attenuation of adaptive immune responses due to the loss of MMP8 [117]. Similarly, MMP-3 knockout mice exhibited increased rate of initial skin tumor growth [118]. However, altered expression pattern or levels of individual MMPs in tumor or stromal cells do not always correlate in the primary tumors and secondary metastatic sites [115]. Interestingly, over-expression of MMPs is frequently accompanied with a corresponding increased expression of natural inhibitors (TIMPs) of MMPs, which result in reduced tumorigenesis in some model systems but does not necessarily inhibit metastasis [119,120]. These discrepancies point out the complexity of MMP functions *in vivo*.

The link between MMPs activity and malignant progression has stimulated serious effort in developing pharmacological inhibitors of MMPs (known as MMPis) as a potential therapeutic modality since the 1980s [121]. A variety of MMP inhibitors including Marimastat (BB-2516), Prinomastat (AG3340), Tanomastat (BAY12-9566) and BMS-275291 Neovastat were found to be orally active and achieved effective blood levels and displayed high specificity to MMPs while sparing most other types of proteases [122]. These MMPis have been shown to be effective in controlling cancer progression in animals. However, most clinical trials have come to a crashing halt with the repeated failure in multiple large-scale phase III stage [122]. Even worse, some compounds caused severe side effects such as inflammation, musculoskeletal pain and joint stricture [122]. Considering the ability of MMPs to cleave not only ECM but also a variety of other factors, cytokine precursors and chemokines, it may not be surprising to see unwanted chaotic immune responses. Therefore, this area of research requires newer strategies.

A recent work of Taketo and colleagues has provided valuable insights regarding a possibility of targeting the MMP-producing cell instead of inhibiting MMPs themselves [123]. They found that immature myeloid cells expressing CC chemokine receptor (CCR1), MMP2 and MMP9 infiltrated the tumor invasion front and migrated toward the CCR1 ligand CCL9, whereas blocking CCR1 expression resulted in the accumulation of MMP-expressing cells at the invasion front and suppressed tumor invasion in an animal model. Although an application of this “cellular target” concept is still premature and is waiting to be confirmed by multiple studies, it is expected to cause fewer side effects than the systemic “molecular target” therapy using MMP inhibitors. One important lesson we learned from the past clinical trials of MMPs inhibitors is the need for attention to the stage and type of cancer and the critical selectivity of MMPs inhibitors since the expression pattern of MMPs varies in various cancer types and stages [122]. For example, small cell lung cancer is known to over-express MMP11 and MMP14 rather than MMP2, thus the MMP2 specific inhibitors like Tanomastat and Prinomastat would lead to a poor outcome [124]. One possible strategy is to take advantage of both

the frequent over-expression of MMPs in malignant tumors and the catalytic functions of these enzymes, and this strategy led to the development of protease-activatable retroviral vectors, which contain engineered MMP-cleavable linkers [125,126]. Another approach is to employ macromolecular carriers that are linked to anti-cancer drugs or immune response-stimulating drugs that can be released from its carrier when encountered with MMPs in the tumor environment [127,128]. Alternatively, designing an inhibitor which targets substrate-specific binding sites of MMPs resulting in reduced binding and cleavage of specific substrates of the corresponding MMP opened a possibility of blocking the unwanted catalytic activity of MMPs during tumor progression [99]. Finally, re-screening for MMPs inhibitors from the current anti-cancer drug pool may be worth a consideration. Notably, Bisphosphonates (BP), a class of pyrophosphate analogues widely used in the treatment of breast cancer patients with osteolytic tumors for the past 20 years, was found to significantly inhibit proteolytic activity of MMPs without reducing the expression of MMPs [129]. Although past efforts in developing anti-MMP drugs have been less fruitful than expected, there are still strong rationales and hopes to continue this line of research using more innovative approaches.

3.5. *Upa*

The urinary-type plasminogen activator (uPA) is a serine protease and able to proteolytically degrade various ECM components and the basement membrane around the primary tumors. It also activates multiple growth factors and MMPs that further contribute to the degradation of the ECM, and thus facilitates tumor cell invasion and intravasation (Fig. 2) [130,131]. Interestingly, a newly identified metastasis suppressor, p75 neurotrophin receptor (p75^{NTR}), has recently been demonstrated to suppress metastasis in part by down-regulating specific proteases such as uPA [132]. uPA is produced and secreted as a zymogen (pro-uPA) which binds to the cell surface uPA receptor, uPAR. The pro-uPA is then cleaved by plasmin to become an active form of uPA, which has plasminogen-activating property to convert plasminogen to the active matrix-degrading serine protease plasmin [131]. The proteolytic activity of uPA is regulated by the serine protease inhibitors, plasminogen activator inhibitor-1 (PAI-1) and PAI-2. PAI-1 is able to react with uPA/uPAR-complex and induces internalization of the complex, which results in the intracellular degradation of uPA and PAI-1. On the other hand, PAI-2 forms a complex with uPA and uPAR without internalization, and it is degraded once bound to uPA/uPAR [133]. Because the activity of uPA is dependent on its binding to uPAR, this receptor is also considered to play a crucial role in metastasis [130]. Besides the role in proteolysis, uPAR can interact with and regulate other cell surface proteins such as integrins, growth factor receptors and G-protein coupled receptors to exert its biological functions including chemotaxis, cell migration and invasion, adhesion, proliferation and angiogenesis [134].

Several recent studies have shown that uPAR is also involved in activation of the signaling of other metastasis-promoting factors such as basic fibroblast growth factor (bFGF), VEGF, TGF β and HGF (Fig. 2) [130,135,136]. Most normal tissues have little or no detectable uPAR, while uPAR is over-expressed across a variety of carcinomas including colon, breast, ovary, lung, kidney, liver, stomach, bladder, endometrium and bone [131,137,138]. uPAR expression has also been shown to be strongly correlated with advanced metastatic cancer, and it is typically found to be abundant at the invasive boundary between tumor cells and normal tissue [139,140]. This localization of uPAR expression in the invasion front may be due to the fact that uPAR is a hypoxia-inducible gene [141,142]. Importantly, the uPAR expression has been found to correlate with a poor prognosis and mortality of patients with various types of solid tumors [141–143]. Currently, the PAI-1 is considered as one of the most informative prognostic markers in several cancer types and a high PAI-1 level is

significantly associated with a poor prognosis in these cancers [144–147]. The precise role of PAI-1 in tumor growth and metastasis is yet to be elucidated, but PAI-1 shows diverse functions depending on the cell context and the expression level [148]. Interestingly, several reports indicated that unlike PAI-1, PAI-2 functions as a tumor suppressor and blocks metastasis, and therefore, is associated with a favorable outcome in patients [143,149]. In addition, uPA and PAI-1 have also been reported to be associated with resistance to hormone therapy in advanced breast cancer [150]. Therefore, uPA/PAI-1 can also be used to predict resistance to specific therapies for breast cancer patients. These studies of uPA/uPAR and PAI-1 so far indicate the critical roles of these molecules in tumor progression, suggesting that these proteins serve as excellent therapeutic targets for cancer patients.

In the past, various approaches have been developed to inhibit uPA and its signals. WX-UK1 and WX-671, synthetic serine protease inhibitors developed by WILEX, are the first inhibitors of uPA in world wide clinical trials. Both of them have shown to effectively block metastasis formation and to reduce primary tumor growth in pre-clinical studies, and they have already entered the phase I/II clinical trials as a single agent and/or in combination with other chemotherapeutics for the treatment of patients with metastatic tumors [148]. Bikunin, a Kunitz-type protease inhibitor, is discovered as a potent and selective inhibitor for trypsin and plasmin, while it is moderately effective in inhibiting the catalytic activity of uPA [151]. Kobayashi et al. have also shown that Bikunin was able to down-regulate the expression of uPA and uPAR [152]. Furthermore, Bikunin has been shown to inhibit MAPK and PI3K/Akt signaling, and to effectively inhibit growth and invasiveness of several types of tumor cells [153–155]. Recently, the possibility of using Bikunin as oral therapy was examined in an ovarian cancer model in animal. Results of these experiments have shown that once-daily oral administration of Bikunin had no significant side effects and strongly suppressed the expression of uPA and uPAR, suggesting a utility of Bikunin for an anti-metastatic therapy in humans [156].

DX-1000, another Kunitz domain-based inhibitor of plasmin with specificity, has been previously shown to block tumor growth and metastases *in vivo* with few side effects [157]. However, DX-1000 has a quick clearance and short half-life in circulation that challenges the practical utility of this compound in patients. To circumvent these problems, Henderikx et al. conjugated the DX-1000 with polyethylene-glycol (PEG) to prolong *in vivo* half-life. The PEG-conjugated DX-1000 was indeed shown to be effective *in vitro* and significantly blocked tumor proliferation, vascularization and metastasis *in vivo* [158]. More recently, Fische et al. have shown that 1-Isoquinolinyguanidines (UK-356,202) and its derivatives were able to reversibly inhibit uPA enzymatic activity with selectivity over tPA and plasmin, and it has been selected as a candidate for clinical evaluation [159]. There are also several other strategies currently under active investigation and these include receptor ligand analogues to interfere with the cellular uPA/uPAR interaction, antibodies for PAI-1 and recombinant PAI-2 (231Bi-PAI2) [160–162].

3.6. β -catenin

β -Catenin is an essential component of the cadherin-catenin complex and plays a critical role in the Wnt signaling pathway [163]. The product of the tumor suppressor gene APC (adenomatous polyposis coli) forms a complex with axin/axil, protein phosphatase 2A (PP2A) and glycogen synthase kinase3 β (GSK3 β) which leads to phosphorylation of β -Catenin thereby inducing degradation of this protein by ubiquitination-mediated proteasomes [164]. The abnormally activated Wnt signaling due to the mutations of APC results in accumulation of β -Catenin followed by promotion of tumorigenesis. Phosphorylation of β -Catenin also releases E-cadherin, which initiates tumor cell migration and tumor metastasis [165,166]. On the other

hand, β -Catenin together with other proteins such as TCF/LEF complex, Reptin and p50, acts as a transcription factor to regulate metastasis-related gene including MMP-9 and KAI1 [167]. More recently, it has been reported that accumulated β -Catenin binds specifically to androgen receptor (AR) and augments the ligand-independent activity of AR in hormone-refractory prostate cancer [168]. Indeed, aberrant expression of β -Catenin has been reported in many types of cancer including colon, bladder, breast, prostate, lung cancer and adrenocortical adenomas [169]. Furthermore, the Wnt/ β -Catenin signaling pathway has been shown to be involved in the self-renewal of embryonic stem cells and perhaps in progression of tumor stem cells [170]. Several agents targeting the Wnt/ β -Catenin pathway including Exisulind and Imatinib have been shown to inhibit self-renewal of cancer stem cells with varying levels of success [171]. Therefore, targeting β -Catenin and blocking APC/ β -Catenin/TCF signals is considered to be a rational approach for developing new anti-cancer drugs.

Exisulind (Aptosyn) and two analogs CP461, CP248 belong to a new class of compounds of SAANDs (Selective Apoptotic Antineoplastic Drugs), which are oxidative metabolites of the nonsteroidal anti-inflammatory drug (NSAID) sulindac. These drugs reduce β -Catenin activity and block Cyclin D1 followed by an induction of apoptosis and inhibition of tumor cell growth [164,172,173]. Currently, Exisulind is in Phase III clinical trials in combination with several chemotherapeutic agents [174,175]. Imatinib (Gleevec), originally identified as an inhibitor of platelet-derived growth factor (PDGF) receptor, has been used in treating chronic myelogenous leukemia (CML), gastrointestinal stromal tumors (GISTs) and a number of other malignancies. Interestingly, Imatinib has been shown to inhibit tyrosine phosphorylation of β -Catenin, which otherwise releases E-cadherin and promotes cell migration and tumor metastasis [176]. Other strategies including RNAi, antisenseDNA and small molecule inhibitors for blocking β -Catenin have been developed [171,177]. The antisense approach has been used in colon and esophageal cancers as well as leukemia and lymphoma *in vitro*, which lead to reduction of β -Catenin expression and subsequent decrease in the expression of its downstream targets such as Cyclin D1 [177–179].

NSAIDs are also found to be effective in inhibiting the Wnt/ β -Catenin signaling pathway. Among them, aspirin and indomethacin were shown to block the transcriptional activity of β -Catenin/TCF [180]. Celecoxib (a COX-2-inhibitor) blocked β -Catenin activity by inducing its degradation via GSK3 β and APC, leading to diminished tumor cell proliferation and survival [181]. R-Etodolac (an enantiomer of Etodolac) and its analog (SDX-308) have been shown to be able to decrease total and activated forms of β -Catenin via GSK3 β activation [182]. These drugs also increased β -Catenin and E-cadherin complex at the membrane site and inhibited β -Catenin-dependent TCF activity followed by decreasing the level of downstream target gene products, Cyclin-D1 and glutamine synthetase [183,184]. In addition to these efforts of directly blocking the β -Catenin activity, selective disruption of β -Catenin-TCF complex and reversing the localization of β -Catenin from cytoplasmic membrane to the nucleus are also considered to be effective approaches for anti-cancer therapy. Thiazolidinedione (TZD), a peroxisome proliferator-activated receptor- γ ligand, has been demonstrated to completely inhibit lymph node and lung metastases in a xenograft animal model by promoting localization shift of β -Catenin from the nucleus to plasma membrane [185]. TZD also reduced tyrosine phosphorylation of β -Catenin and promoted enhanced expression of E-cadherin [185]. Recently, a crystal structure of β -Catenin-TCF complex has been clarified which shed new light on the molecular mechanism by which this stable and potent transcription factor complex forms [186–188]. Therefore, developing a drug which can disrupt the β -Catenin-TCF complex holds great promise, although how to effectively and selectively disrupt the complex without affecting β -Catenin-E-cadherin or APC complex is still a challenge.

4. Metastasis suppressors

4.1. Nm23

NM23 is the first identified metastasis-suppressor gene in this group. It is located on chromosome 17q21 and codes for an 18.5-kDa protein containing 166 amino acids which functions as nucleoside diphosphate kinase and protein-histidine kinase [189,190]. Clinically, NM23 has been shown to be down-regulated in a variety of tumors including breast and prostate cancers [191,192]. Ectopic expression of NM23 has also been shown to significantly reduce the *in vitro* and *in vivo* metastatic potential of highly metastatic carcinoma cell lines including breast, melanoma, colon, and oral squamous cells [190,193–195]. Recently, Hartsough et al. reported that NM23 formed a complex with Kinase suppressor of Ras1 (KSR1) and phosphorylated this protein at Ser-392 and Ser-434, which resulted in blockade of Ras/MAPK pathway (Fig. 2b) [196]. More recently, Salerno et al. have shown that the NM23 expression level influenced the binding properties, stability and function of the KSR1 in breast carcinoma cells [197]. Hence, NM23 was hypothesized to inhibit MAPK/ERK activation via altering the scaffold function of KSR1 (Fig. 2b). Consistent with this hypothesis, MDA-MB-435 breast cancer cells that over-express NM23 showed reduced MAP kinase activity and cell motility *in vitro* as well as diminished incidence of metastasis *in vivo* [196,198,199]. Therefore, NM23 acts as a metastasis suppressor by inhibiting the MAP kinase pathway through the interaction with the KSR1 scaffold protein.

In an attempt to restore the expression of NM23 in tumor cells, several drugs have been found in the past. Among them, medroxyprogesterone acetate (MPA) and estradiol were reported to suppress metastasis through up-regulation of the NM23 gene (Table 1). Medroxyprogesterone is a progestin and commonly used as a component of hormonal contraceptives. Progesterone binds to the progesterone receptor which is then transferred to the nucleus and acts as a transcription factor by binding to the progesterone response elements (PRE) in the promoter region of target genes. Progesterone receptor is known to directly regulate the expression of Cyclin D1, beta-casein and p21^{WAF1} as well as MAPK [200–205]. MPA has a long history of clinical use at a low dose as the contraceptive Depo-Provera and has also been used for hormone replacement therapy in combination with estrogen [206]. At a high concentration, it has been used for the treatment of advanced breast and endometrial cancers [207]. MPA can competitively bind to several steroid hormones including progesterone (PR), androgen (AR) and glucocorticoids (GR), and thus it is able to up-regulate NM23 by antagonizing the effect of glucocorticoid response element (GRE) on the NM23 promoter [208]. Ouatas et al. previously found that MPA inhibited the soft agar colonization of breast carcinoma cells by up-regulating the NM23 expression [209]. In *in vivo*, Palmieri et al. treated mice xenografted with breast carcinoma cells with MPA and found 27–36% reduction of metastasis incidence in the treated animals.

Estradiol works as an estrogen to modulate gene expression via binding to its intracellular receptor ERs [210]. Interestingly, Estradiol was found to be able to decrease the number of experimental lung metastases in nude mice when they were injected with breast cancer cell line MDA-MB231 with forced expression of ER (Table 1) [211]. Lin et al. reported that the level of NM23 mRNA and protein was induced by Estradiol in breast cancer cell lines with the extent that these effects correlated with the level of ER α expression [212]. In addition, Estradiol was shown to be able to decrease the invasive ability of ER α positive carcinoma cell lines MCF7 and BT-474, while it did not have any effect on BCM-1 cell which had virtually no ER α expression [212]. Therefore, it is suggested that Estradiol was able to suppress tumor metastasis by activating the expression of the NM23 gene in an ER α -dependent manner (Fig. 2b) [212].

Many of the therapeutic effects of nonsteroidal anti-inflammatory agent (NSAIDs) are clearly due to the inhibition of prostaglandin synthesis by inactivation of cyclooxygenase 1 and 2 (COX-1 and COX-2) [213]. The anti-tumor effect of NSAID has been recognized when Aspirin was found to reduce the risk of colorectal adenoma and carcinoma in animal models [214–217]. Interestingly, Yu et al. reported that Aspirin decreased the invasive potential of COX2 negative colon cancer cells via up-regulation of NM23 expression (Table 1) [217].

Another NSAID, Indomethacin, was also found to up-regulate the expression of NM23 in breast cancer cells and to alter the malignant choline phospholipid phenotype toward a less malignant tumor [218]. Reich et al. reported that indomethacin reduced the invasive ability of human fibrosarcoma and murine melanoma cell lines and that murine melanoma cells exposed to indomethacin prior to i.v. injection produced significantly fewer lung metastases (Table 1) [219]. Kundu et al. also reported the anti-metastasis effect of indomethacin by oral administration in a murine model [220]. They transplanted a murine mammary adenocarcinoma cell line 410.4 and found that the metastatic ability of this cell line was reduced by almost 50% with the treatment of indomethacin (Table 1) [220]. Therefore, indomethacin has potential utility as an anti-metastatic drug and it is currently under clinical trial.

All-*trans* Retinoic Acid (ATRA) is known as the first successful targeted drug for cancer therapy. ATRA causes the differentiation of leukemic myeloid cells from mature myeloid cells by attaching to one of several retinoid receptors in the cell nucleus and then directly modulating gene expression [221–223]. The down-regulation of several oncogenes including Ras and c-fms by ATRA has been reported [224,225]. Interestingly, the expression of NM23 was also shown to be up-regulated by ATRA in human hepatocarcinoma cell line and gastric cancer cell lines [226,227]. Liu et al. demonstrated that treatment with either ATRA or transfected NM23 cDNA reduced metastasis-associated phenotypes including chemotactic cell migration and invasion of human hepatocarcinoma cell line [226]. Furthermore, Wu et al. examined the effect of ATRA treatment in xenografted nude mice and found that ATRA treatment significantly decreased the metastasis in liver and increased NM23 protein levels in experimental groups compared with a control group [227]. Since ATRA was also able to reduce cell growth *in vitro* and *in vivo* [227], the specificity of ATRA treatment on tumor metastasis is still unclear. However, a combination treatment of ATRA and IFN- α in a clinical trial was well tolerated, and patients who have metastatic osteosarcoma were found to be in stable complete remission 14 months after the end of therapy [228]. Therefore, further investigation of ATRA as an anti-metastatic drug is warranted.

4.2. KiSS-1

KiSS-1 was originally identified as a metastasis-suppressor gene using a combined strategy of MMCT and differential display [229]. The introduction of an intact copy of whole human chromosome 6 into the C8161 human melanoma cell resulted in significant reduction of metastasis ability of this cell line without affecting tumorigenicity or local invasiveness in animals [229]. Later Lee et al. reported that the KiSS-1 gene was actually mapped on chromosome 1q region which is frequently deleted in late-stage human breast carcinomas [230]. They then transfected the KiSS-1 gene into human breast ductal carcinoma cell line MDA-MB-435 and found that KiSS-1 almost completely suppressed metastatic activity of MDA-MB-435 [230]. Therefore, although the KiSS-1 gene is located on chromosome 1, it is believed that chromosome 6 is responsible at least in part for its metastasis suppressive effects by harboring a gene that positively regulates KiSS-1 expression [231]. Clinically, the expression of mRNA of the KiSS-1 gene was found to be significantly down-regulated in metastatic tumors, which is in accordance with the idea that KiSS-1 is a metastasis suppressor [232].

Ectopic expression of the KiSS-1 gene was shown to significantly reduce the rate of three-dimensional growth in soft agar, but it did not affect invasion or motility [230]. These results suggest that KiSS-1 affects downstream of cell-matrix adhesion and perhaps involves cytoskeletal reorganization. On the other hand, Yan et al. reported that KiSS-1 transfected HT1080 cells showed substantially reduced enzyme activity of MMP9 with specific down-regulation of mRNA level of MMP9 and invasiveness of tumor cells *in vitro* [233]. They have further shown that this effect was partly attributable to the ability of KiSS-1 to reduce NF- κ B binding to the promoter of MMP9 by enhancing I- κ B activity (Fig. 2b) [233].

Metastin is a 54 amino acid peptide whose sequence is identical to a part of the KiSS-1 gene, and this peptide was found to act as a ligand for orphan G-protein coupled receptor (hOT7T175, AXOR12, GPR54) (Table 1) [234,235]. Interestingly, Ohtaki et al. have shown that Metastin significantly attenuated pulmonary metastasis in a mouse xenograft model using the B16-BL6MR melanoma cell, while Metastin had no direct effect on the primary tumor growth [234]. Importantly, Metastin was found to be able to suppress the degree of pulmonary metastasis even when the peptide was administered to the mice that already had metastasis in the lung [234]. Therefore, Metastin is considered to be a promising agent for the treatment of metastatic cancer patients. In this regard, it is encouraging that the expression of the Metastin receptor genes was found to be normal even when KiSS-1 was significantly down-regulated in various types of cancers [236]. These results suggest that Metastin may be effective even in advanced cancer that has lost KiSS-1 expression.

4.3. Mkk4

Chekmareva et al. has previously demonstrated a prostate cancer metastasis-suppressor activity encoded by a discontinuous ~70 cM region of human chromosome 17, which suppresses the spontaneous metastatic ability of highly metastatic Dunning AT6.1 rat prostate cancer cells [237]. Later, Yoshida et al. identified the MKK4/SEK1 (Mitogen-activated protein kinase kinase 4) gene in this chromosomal region as a candidate metastasis suppressor [238]. Ectopic expression of MKK4 in highly metastatic prostate cancer cell line indeed significantly suppressed macroscopic lung metastasis without affecting the primary tumor growth in animals [238]. Furthermore, Kim et al. examined the status of MKK4 expression in clinical samples of prostate cancer by immunohistochemical analysis and found that the expression of MKK4 was inversely correlated with Gleason score and tumor progression [239]. How MKK4 suppresses metastasis is a crucial question and has been under active investigation. MKK4 belongs to MAP kinase family which plays central roles in cell proliferation, differentiation and apoptosis. It is known that MKK4 is activated in response to a variety of extracellular stimuli including stress followed by activation of JNK(c-Jun N-terminal kinase) and/or p38 MAPK pathways (Fig. 2b) [240]. It is plausible that, when a tumor cell reaches a distant organ site, the expression of MKK gene in cancer cell is suppressed in the stressful environment, and therefore, fails to establish colonization.

A strategy of using monoclonal antibodies has been considered to be an attractive approach for cancer therapy due to their high target specificity. Anti-death receptor antibody such as anti-TRAIL antibodies, 2E12 and TRA-8, have been found to activate the MKK4/JNK/p38 pathway, suggesting a potential utility of the antibodies for anti-metastatic therapy [241]. Furthermore, Ohtsuka et al. reported that the combination of the anti-death receptor antibodies and chemotherapy agents led to a synergistical activation of the JNK/p38 MAP kinase which was mediated by MKK4 (Table 1) [241]. In their studies, agonistic anti-TRAIL antibodies 2E12 and TRA-8, when combined with chemotherapeutic agents such as Adriamycin, were able to increase the release of cytochrome c and Smac/DIABLO from mitochondria in parallel with the profound loss of mitochondrial membrane potential, which resulted in apoptosis in breast, prostate and colon cancer cells

[241]. It is interesting to test whether these regimens are able to suppress metastatic potential of MKK-positive cancer cells *in vivo*. Bisindolylmaleimide VIII was originally developed as a synthetic inhibitor of protein kinase C (PKC) [242,243], and it was later found to promote Fas-mediated apoptosis in a PKC-independent manner [244]. Ohtsuka et al. examined a possible effect of Bisindolylmaleimide VIII on TRA-8 induced apoptosis and found that a combination of Bisindolylmaleimide VIII and TRA-8 induced 50–80% of apoptosis in human astrocytoma cell line (1321N1), while the treatment of the cells with TRA-8 alone induced apoptosis only in up to 20% of the cells [245]. In *in vivo*, either Bisindolylmaleimide VIII or TRA-8 alone partially regressed the xenografted tumor in NOD/SCID mice, while the combination of these two drugs almost completely blocked the tumor growth. However, whether Bisindolylmaleimide VIII enhances TRA-8- induced apoptosis via a role in regulating MKK4/JNK/p38 apoptosis kinase signaling and whether the combination of these drugs indeed suppresses metastasis remains to be examined.

4.4. E-cadherin

The transmembrane protein E-cadherin (also known as CDH 1) was originally isolated as human uvomorulin by screening a cDNA library of the human liver [246]. The E-cadherin is a calcium-dependent adhesion molecule which constitutes the adherence junction in epithelial cells [247,248]. Reduced level of E-cadherin is shown in a variety of human cancers at advanced stages. It is believed that a low level of E-cadherin can give advantage to tumor cells on breaking the adhesion junction and detaching from adjacent cells, so that these cells invade and metastasize to other distant organs. Clinically, several groups have reported that decreased expression of E-cadherin was associated with a poor prognosis in cancer patients [249]. On the other hand, over-expression of E-cadherin in invasive cancer cells has been shown to decrease motility and invasiveness [250]. In addition, using a transgenic mouse model of pancreatic β -cell carcinogenesis (Rip1Tag), Perl et al. showed that tumor incidence or tumor volume was not significantly changed between double-transgenic Rip1Tag2xRip1dnE-cad mice and single-transgenic Rip1Tag2 littermates [251]. However, the double-transgenic mouse developed metastases to the pancreatic lymph nodes, an invasive phenotype that was never observed in single-transgenic Rip1Tag2 mice [251]. Therefore, E-cadherin is considered to function as a metastasis suppressor. Generally, E-cadherin plays an important role in epithelial-mesenchymal transition (EMT) during which epithelial cells lose their cell-cell junctions and acquire mesenchymal characteristics to endow the migratory ability to tumor cells [249]. E-cadherin interacts with β -Catenin to mediate actin binding (Fig. 2b) [252]. Therefore, loss of E-cadherin, in addition to reducing cell-cell adhesion, provides an oncogenic stimulus by freeing β -Catenin from the membrane, so that β -Catenin can travel to the nucleus to activate TCF-regulated genes such as c-Myc and Cyclin D1 [253]. Furthermore, E-cadherin has been recently found to be down-regulated by transcription factors Snail and Slug that are involved in the process of EMT, cell differentiation and apoptosis [254]. Therefore, restoring the function of E-cadherin is considered to be a potential therapeutic option for metastatic disease. PP (pyrazolo [3,4-d] pyrimidines)1 and PP2 were originally identified as selective inhibitors for Src, and they were shown to be able to block tumor growth and to reduce metastasis in a mouse pancreatic model. However, these compounds have also been found to reactivate the E-cadherin expression in pancreatic and colon cancer cells (Table 1) [255,256]. Therefore, PP1 and PP2 may serve as effective anti-metastatic drugs although they need to be tested more extensively in a clinical trial.

4.5. NdrG1

N-myc downstream regulated gene 1 (NDRG1) was originally identified by differential displays as being significantly up-regulated

by induction of *in vitro* differentiation of colon carcinoma cells [257]. The protein encoded by the NDRG1 gene has a molecular weight of 43 kDa and possesses three unique 10-amino acids tandem repeats at the C-terminal, among which seven or more phosphorylation sites were predicted and later they were shown to be targets of protein kinase A *in vitro* [258]. The NDRG1 gene is controlled by multiple factors and responsive to various stimuli. The expression of NDRG1 was repressed by C-myc and N-myc/Max complex *in vitro*, while it was induced by p53, hypoxia and PTEN (Fig. 2b) [259]. NDRG1 has been shown to act as a tumor suppressor as well as a tumor metastasis suppressor depending on cell context [259]. In a clinical setting, NDRG1 was found to be consistently expressed in normal prostate tissue as well as PIN (prostatic intraepithelial neoplasia) and BPH (benign prostatic hyperplasia), whereas the expression was significantly reduced in high-grade tumors [260,261]. In addition, the level of the NDRG1 expression was inversely co-related with the status of metastasis in these patients, supporting the notion that NDRG1 is a tumor metastasis suppressor [260]. In breast cancer, a similar and significant negative correlation of NDRG1 with metastasis has been observed, while the expression of NDRG1 does not show any significant correlation with the size or the histological grade of the primary tumor [261]. These results strongly suggest the negative involvement of NDRG1 in the process of invasion and metastasis in both prostate and breast cancer. Furthermore, ectopic expression of the NDRG1 gene in a highly metastatic prostate cancer cell line significantly reduced the incidence of lung metastases, suggesting that NDRG1 was able to block the metastatic process without affecting the primary tumor growth [260,261]. Similar metastasis suppressor effect of NDRG1 was also observed in colon carcinoma cells by Guan et al. [262]. In addition, NDRG1 also significantly suppressed the invasive potential of prostate and breast cancer cells as tested by *in vitro* invasion chamber assay [260,261]. Therefore, evidence from both clinical data and the results of *in vitro* as well as animal experiments overwhelmingly support the notion that NDRG1 is a metastasis-suppressor gene and that the down-regulation of the gene results in acceleration of tumor metastasis. How NDRG1 suppresses the tumor metastasis is an intriguing question which is under active investigation.

Recently, Fe chelators, desferrioxamine (DFO) and 311 were shown to be able to up-regulate the NDRG1 expression in human breast cancer cell line MCF7 [263]. In the past years, dietary Fe restriction has been shown to markedly decrease tumor growth in rodents [264–266], and Fe chelators such as Triapine and desferrioxamine (DFO) were reported to be potentially useful for cancer therapy (Table 1) [266–268]. More recently, Whitnall et al. examined the effect of another Fe chelator, di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone (Dp44mT), on tumorigenesis in xenografted mice models of lung carcinoma, neuroepithelioma and melanoma and found that Dp44mT strongly inhibited the growth of all tested human xenografts in nude mice [269]. Notably, Dp44mT significantly augmented the expression of the NDRG1 gene in the tumor compared to that of control group, suggesting a promising utility of this compound as an anti-cancer as well as anti-metastatic drug [269].

5. Conclusion and future direction

Despite significant improvement in surgical techniques and chemotherapy for cancer treatment in general, none of the current medical technologies “cure” the metastatic disease, and the patients who have already acquired metastatic cancer are left virtually with no options. Therefore, there is an urgent need for developing a novel approach of target-specific therapy to metastatic tumor cells, which requires more comprehensive understanding of the molecular mechanism of metastases. The goals of anti-metastatic therapy are three folds. Firstly, we need to develop a specific drug that blocks secondary metastasis to treat patients who have already acquired metastatic disease but are still at an early stage. Secondly, a drug

should also be developed to treat patients who underwent surgical resection of their primary tumors in order to prevent a possible recurrent disease. However, the ultimate goal is to develop a non-toxic agent which can be taken as diet for prevention of metastasis. In the past decade, the major effort of anti-cancer research has been focused on the development of drugs that can block the proliferation of tumor cells. They take advantage of the fact that tumor cells are more actively proliferating than other normal cells, and therefore, “selectively” kill the cancer cells. However, this “selectivity” has narrow margins and these agents inevitably cause severe side effects even when they are used in combination to lower the toxicity. From these experiences, we have learned an important lesson that the most critical issue for anti-cancer drugs is their specificities. Therefore, to develop an anti-metastatic drug, it is crucial to define a target molecule which is specifically expressed in metastatic cells. Ideally, an agent which can attack the molecule is inactive (pro-drug) when given to patients, and is activated only in the tumor cells. In theory, monoclonal antibodies and siRNA are highly specific to target genes, and active investigations are underway to utilize these technologies for the development of anti-metastatic drugs. If a target is well defined and specific, these agents are considered to be very effective, although there are still many unknown technical questions such as stability and delivery method of these agents. However, recent advancement of bio-technology such as nano-particles has provided us with a hope that we can eventually overcome these problems.

We have learned a great deal of the metastasis cascade, and many new genes and signal pathways involved in this process have been identified. Some genes hold great promises as potential druggable targets. The genes that control EMT and cell motility as well as their signal pathways are rational candidates for the drug development. Although a clinical trial of the drugs that block MMP resulted in a rather disappointing outcome, these molecules are still considered to be excellent targets. The fact that metastatic cells are the only epithelial cells in circulation may provide us with a window of opportunity to attack such cells. In addition, tumor cells are often attracted by various types of chemokines to the distant organ sites, and these chemokines may also serve as molecular targets for anti-metastatic therapy. Reactivation of metastasis-suppressor genes and their signal pathways such as MKK/JNK, PTEN/Akt and NDRG/ATF are also a rational strategy. Recent finding that KAI1 blocks metastasis by inducing senescence upon interaction with endothelial cells also suggests an interesting possibility to develop an effective drug to activate the KAI1 pathway. Perhaps, genome wide shRNA library screening and comprehensive proteomics approach may reveal more suitable targets for metastatic therapy in the near future. The use of computer-driven strategies such as automated determinations of the structures of target molecules and computer-aided design of drug molecules followed by a high-throughput screening has already begun to set this trend into motion.

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RhoC Promotes Metastasis via Activation of the Pyk2 Pathway in Prostate Cancer

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Abstract

RhoC is a member of the Ras-homologous family of genes which have been implicated in tumorigenesis and tumor progression. However, the exact role of *RhoC* is controversial and is yet to be clarified. We have examined the effect of *RhoC* on prostate tumor cells and found that *RhoC* had no effect on cell proliferation *in vitro* or on tumor growth in mice. However, *RhoC* significantly enhanced the metastatic ability of the tumor cells in these animals, suggesting that *RhoC* affects only the metastasis but not the growth of prostate tumor cells. The results of our immunohistochemical analyses on tumor specimens from 63 patients with prostate cancer indicate that *RhoC* expression had no significant correlation with Gleason grade. However, the expression of *RhoC* showed significant positive correlation with both lymph node and distant metastasis, and it was inversely correlated with patient survival. We also found that *RhoC* significantly augmented the invasion and motility of prostate tumor cells by activating matrix metalloproteinases 2 and 9 (MMP2 and MMP9) *in vitro*. The results of our antibody array analysis for signal molecules revealed that *RhoC* significantly activated kinases including mitogen-activated protein kinase (MAPK), focal adhesion kinase (FAK), Akt, and Pyk2. Inhibition of Pyk2 kinase blocked the *RhoC*-dependent activation of FAK, MAPK, and Akt, followed by the suppression of MMP2 and MMP9. Inhibitors of both MAPK and Akt also significantly blocked the activities of these MMPs. Therefore, our results indicate that *RhoC* promotes tumor metastasis in prostate cancer by sequential activation of Pyk2, FAK, MAPK, and Akt followed by the up-regulation of MMP2 and MMP9, which results in the stimulation of invasiveness of tumor cells. [Cancer Res 2008;68(18):7613–20]

Introduction

The family of Ras homologous (*Rho*) genes, which plays a central role in cell proliferation and motility, has been implicated in tumorigenesis as well as metastatic progression (1). The *Rho* subfamily includes *RhoA*, *RhoB*, and *RhoC* and they share 85%

amino acid sequence identity (2). Despite this similarity, each protein has different affinities with various downstream effectors and shows different subcellular localizations, suggesting that they have distinct roles in normal cellular function as well as in tumor pathogenesis (3). *RhoA* seems to be involved in the regulation of actomyosin contractility, and the overexpression of *RhoA* has been shown to promote the invasiveness of tumor cells (2, 4–6). On the other hand, *RhoB* plays a role in controlling cytokine trafficking as well as in apoptosis induced by DNA-damaging agents and has been suggested to act as a suppressor of tumor progression (7, 8).

Recently, *RhoC* has been shown to be up-regulated in various types of cancer including inflammatory breast cancer (9), hepatocellular carcinoma (10), and non-small cell lung cancer (11). However, the exact role of *RhoC* in tumorigenesis and tumor progression has remained controversial and needs further clarification. Pillé and colleagues previously found that blocking *RhoC* expression by short interfering RNA significantly inhibited cell proliferation of breast tumor cells *in vitro* as well as tumor growth in an animal model (12). More recently, Faried and colleagues also reported that ectopic expression of *RhoC* in esophageal carcinoma cells significantly enhanced the growth of tumors in nude mice. These results suggest that *RhoC* plays a critical role in cell proliferation and tumor growth both *in vitro* and *in vivo* (13). On the contrary, Ikoma and colleagues reported that ectopic expression of *RhoC* using retroviral vectors in Lewis lung carcinoma cells showed no significant difference in primary tumor growth in mice. However, the rate of lymph node metastasis was significantly enhanced in these animals (14). In agreement with these results, Hakem and colleagues recently constructed a *RhoC* knockout mouse and found that loss of *RhoC* does not affect tumorigenesis but significantly decreased metastasis in this mouse, suggesting that *RhoC* is involved only in metastasis but not in tumor cell proliferation (15). These apparent contradictory results by different groups may be due to the difference in the systems used or it may be due to the dependency of *RhoC* on cellular context. Therefore, it is critical to take a more systematic approach of testing the gene both *in vitro* and *in vivo* and to validate the outcome results in a clinical setting for each organ or tissue type in order to further clarify the role of *RhoC* in tumor progression. In this study, we found that *RhoC* promotes tumor metastasis but not tumor growth by sequential activation of Pyk2, focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and Akt followed by up-regulation of matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in prostate tumor cells, and that the expression of *RhoC* serves as a marker to predict metastatic status and survival of patients with prostate cancer.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Materials and Methods

Cell culture and reagents. Human prostate cancer cell line PC3 was obtained from American Type Culture Collection, and human prostate cancer cell line PC3MM was kindly provided by Dr. I.J. Fidler (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The PC3MM/tet cell line was previously established as a derivative of PC3MM and contains the tetracycline-inducible suppressor. Rat prostate cancer cell line AT2.1 was a gift from Dr. C.W. Rinker-Schaeffer (University of Chicago, Chicago, IL). All cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin (100 µg/mL), penicillin (100 units/mL), and 250 nmol/L of dexamethasone at 37°C in a 5% CO₂ atmosphere. The phosphoinositide-3-kinase (PI3K)/Akt inhibitor (Ly294002) and the MAPK inhibitor (PD98059) were purchased from Sigma Co. and Calbiochem, respectively. FAK inhibitor (TAE226) was previously described and kindly provided by Dr. Honda (Novartis Pharma AG, Basel, Switzerland; ref. 16).

Construction of expression vectors. To generate a RhoC expression vector, cDNA of the RhoC gene was isolated by PCR amplification from a human cDNA library using a forward primer containing a Flag-tagged Kozak sequence and *EcoRI* linker and a reverse primer including a *XhoI* linker. The PCR product was then cloned into the mammalian expression vector pcDNA3 (Invitrogen). To construct a tetracycline-inducible RhoC expression plasmid, the fragment of the *RhoC* gene in pcDNA3 was subcloned into pcDNA5/TO (Invitrogen) at the *BamHI/XhoI* site. The RhoC expression plasmids or the vector alone were transfected into the AT2.1, PC3MM, and PC3MM/tet cells using LipofectAMINE (Invitrogen). To establish stable clones, transfected cells were treated with G418 or hygromycin, and drug-resistant colonies were selected followed by testing RhoC expression by Western blot.

Short hairpin RNA. Five individual short hairpin RNAs (shRNA) against the *Pyk2* gene were purchased from Open Biosystems. shRNA with a scrambled sequence was purchased from Addgene and used as a negative control. The shRNAs were transfected into the prostate cancer cells using LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and the culture was further incubated for 48 h before harvesting the cells for assays.

Western blot analysis. Cells were collected and dissolved in loading dye solution (125 mmol/L Tris-HCl, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.04% bromophenol blue), boiled for 5 min and subjected to 8% to 12% SDS-PAGE. Proteins were transferred to nitrocellulose membranes that were then treated with antibodies against anti-Flag (Sigma-Aldrich), anti-β-tubulin (Upstate Biotechnology), anti-phospho-Pyk2 (Tyr^{579/580}; Sigma-Aldrich), anti-Pyk2 (Cell Signaling Technology), anti-phospho-Akt (Ser⁴⁷³; Cell Signaling Technology), anti-Akt (Cell Signaling Technology), anti-phospho-FAK (Tyr³⁹⁷; Sigma-Aldrich), anti-FAK (Cell Signaling Technology), or anti-phospho-MAPK (Thr¹⁸³; Sigma-Aldrich) or anti-MAPK (Cell Signaling Technology). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by the enhanced chemiluminescence plus system (Amersham Life Sciences).

Cell growth assay. Cell lines expressing or not expressing the *RhoC* gene were cultured in the RPMI 1650 medium. At each time point, cells were trypsinized, serially diluted, and re-plated in Petri dishes. The resultant colonies were stained with crystal violet and the number of colonies was visually counted. For thymidine uptake assays, cells were treated with or without tetracycline for 24 h and ³H-thymidine was added to the culture. After 3 and 12 h, cells were collected and acid-insoluble radioactivities were measured by scintillation counter.

Spontaneous metastasis assay. Rat prostate tumor cells AT2.1 (0.5 × 10⁶ cells in 0.2 mL of PBS) were injected s.c. in the dorsal flank of 5-week-old severe combined immunodeficiency (SCID) mice (Harlan Sprague-Dawley). Mice were monitored daily and the tumor volume was measured as an index of the growth rate using the equation: volume = (width × length) / 2 × width × length × 0.5236. The doubling time of tumors during the fastest growing period was calculated by measuring the tumor volume every 4 days. Mice were sacrificed 4 weeks after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically.

Immunohistochemical analysis. Formaldehyde-fixed and paraffin embedded tissue specimens from 63 patients with prostate cancer were obtained from surgical pathology archives of the Akita Red Cross Hospital (Akita, Japan). Four-micron-thick sections were cut from the paraffin blocks of prostate tumors and mounted on charged glass slides. The sections were deparaffinized and rehydrated, and antigen retrieval was done by heating the slide in 25 mmol/L of sodium citrate buffer (pH 9.0) at 80°C for 30 min. The slides were incubated overnight at 4°C with anti-RhoC antibody (Santa Cruz Biotechnology) or anti-phospho-Akt (Ser⁴⁷³; Cell Signaling Technology). The sections were then incubated with the horseradish peroxidase-conjugated anti-goat secondary antibody, and 3,3'-diaminobenzidine substrate chromogen solution (Envision Plus kit; DAKO, Corp.) was applied followed by counterstaining with hematoxylin. Immunohistochemical staining conditions with other antibodies (NDRG1, AR, and PTEN) were described previously (17). Results of the immunohistochemistry for RhoC were judged by two independent persons (M. Iizumi and K. Watabe) based on the intensity of staining combined with the percentage of cells with positive staining.

In vitro motility and invasion assay. For the motility assay, 1 × 10⁵ cells were added to the cell culture inserts with microporous membrane without any extracellular matrix coating (Becton Dickinson) and RPMI medium containing 20% fetal bovine serum was added to the bottom chamber. The cells were then incubated for 24 h at 37°C, and the upper chamber was removed. The cells on the bottom of the upper chambers were stained with tetrazolium dye, and the number of cells was counted under a microscope. For the *in vitro* invasion assay, the working method was similar to that described above, except that the inserts of the chambers to which the cells were seeded were coated with Matrigel (Becton Dickinson).

Wound-healing migration assay. Cells were seeded in a 10-cm dish and cultured to confluency. The cell monolayer was then scraped in the form of a cross with a plastic pipette tip. Three "wounded" areas were marked for orientation and photographed by a phase contrast microscopy before and after 24 h of incubation.

Real-time reverse transcription-PCR. Forty-eight hours after transfection of appropriate plasmid DNA to the cells or 48 h after induction by tetracycline, total RNA was isolated from the cells and reverse transcribed using random hexamer and MuLV reverse transcriptase (Applied Biosystems). The cDNA was then amplified with a pair of forward and reverse primers for *RhoC* (5'-TAAGAAGGACCTGAGGCAAG and 5'-ATCTCAGAGAATGGGACAGC), *MMP2* (5'-TGATGGTGTCTGCTGGAAAG and GACACGTGAAAAGTGCCTTG), *MMP9* (5'-GGAGACCTGAGAACCAATCTC and 5'-TCCAATAGGTGATGTTGTGGT), human *β-actin* (5'-TGAGACCTTCAACACCCAGCCATG and 5'-GTAGATGGGCACAGTGTGGGTG), *Pyk2* (5'-GCTAGACGGCAGATGAAAGT and 5'-AAGCAGACCTTGAGGATACG). PCRs were done using the Dyma SYBRGreen qPCR kit (New England Biolabs) and DNA Engine Opticon2 System (MJ Research). The thermal cycling conditions were composed of an initial denaturation step at 95°C for 5 min followed by 30 cycles of PCR using the following profile: 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s.

Gelatin zymography assay. For zymography assay, cells (2.5 × 10⁵) were seeded in 12-well plates and incubated for 48 h. Supernatants were collected and mixed with sample buffer followed by electrophoresis on a 10% SDS-polyacrylamide gel containing 5 mg/mL of gelatin. The gel was washed with 2.5% Triton X solution for 2 h and further incubated in the reaction buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl₂, 1 µmol/L ZnCl₂, and 1% Triton X-100) for an additional 18 h at room temperature. The gel was then stained with 0.5% Coomassie blue for 9 h and subsequently immersed with destaining buffer (30% methanol, 10% acetic acid) for 12 h. The image was photographed and the intensity of each band was digitally quantified.

Antibody microarray. Antibody microarray was performed using a Panorama Antibody Microarray-Cell Signaling kit (Sigma-Aldrich) according to the manufacturer's instructions. Briefly, 1.5 × 10⁷ cells were seeded in T-75 flasks and incubated for 48 h in the medium with or without tetracycline. Cells were collected and protein samples were prepared according to the manufacturer's protocol. These protein samples were labeled with Cy3 or Cy5 (Amersham Biosciences, UK) and subjected to antibody microarray (Sigma-Aldrich) analysis. The array slides were

scanned by GenePix Personal 4100A scanner (Molecular Devices) and the data was analyzed by GenePix Pro 5.0 (Molecular Devices).

Statistical analysis. For *in vitro* experiments and animal studies, *t* test or one-way ANOVA was used to calculate the *P* values. The association between RhoC and other clinical markers was calculated by χ^2 test. The Kaplan-Meier method was used to calculate the overall survival rate, and prognostic significance was evaluated by the log-rank test. Univariate and multivariate analyses for the prognostic value of RhoC was performed by the Cox proportional hazard-regression model. For all of the statistical tests, the significance was defined as *P* < 0.05. SPSS software was used in all cases.

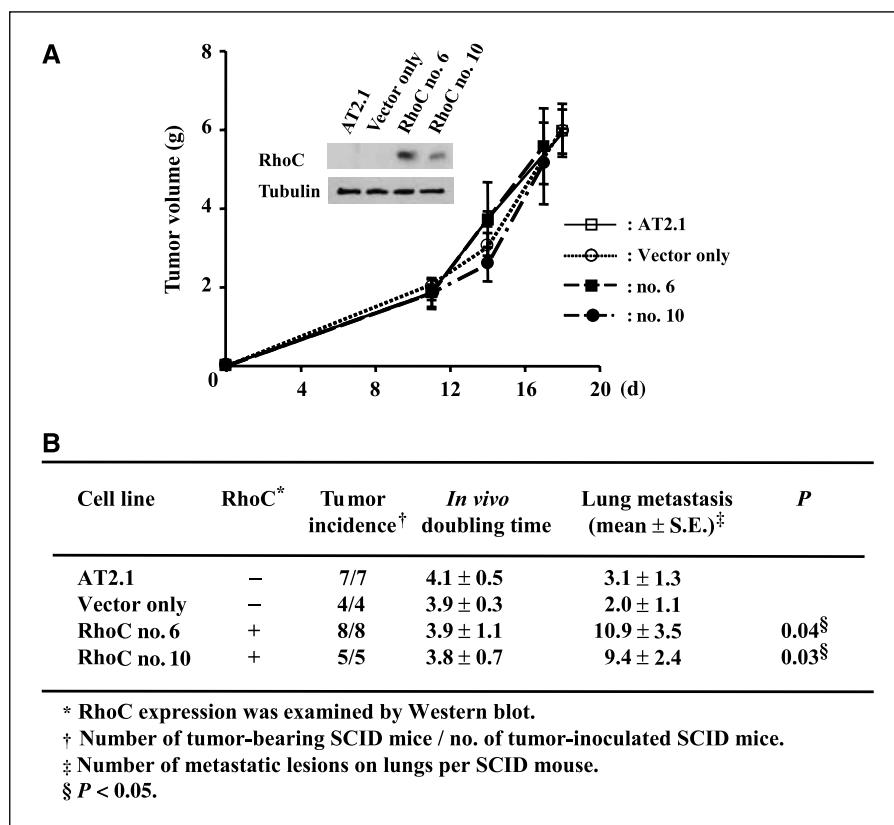
Results

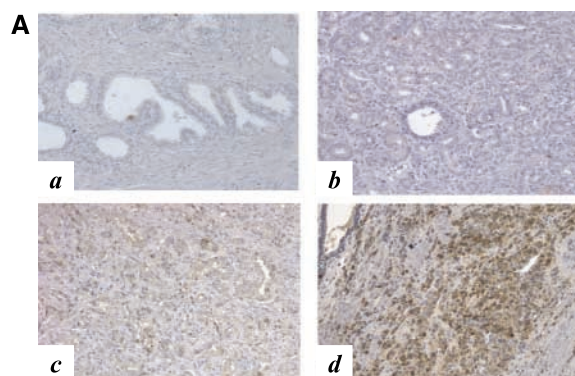
RhoC promotes tumor metastasis, but not cell growth. To understand the role of RhoC in prostate cancer, we first established permanent cell lines expressing RhoC using the rat prostate carcinoma cell line, AT2.1, which has a poor metastatic potential (18). These cell lines expressing RhoC (clone no. 6 and no. 10) and a clone containing only the vector as well as the parental cell line, AT2.1, were individually injected s.c. into SCID mice. The mice were monitored for the formation and the growth rate of tumors and then sacrificed 3 weeks after the inoculation of the cells. As shown in Fig. 1A, all of the clones and the parental cells formed primary tumors in the animals with similar growth rates during the 3-week period, suggesting that RhoC does not have an effect on tumorigenesis or tumor growth. On the other hand, the clones stably expressing RhoC showed a significantly higher incidence of lung metastases compared with the parental cell line and the vector-only clones (Fig. 1B). These results strongly suggest that RhoC can promote the metastatic process of prostate cancer cells without affecting tumorigenicity *in vivo*. We also examined the effect of RhoC on the growth of these cells *in vitro*. The results of a

colorimetric assay after 72 h indicate that there was no significant difference in the growth rate between the cells with and without RhoC (Supplementary Fig. S1A). We then examined the rate of DNA synthesis of the cells with and without the expression of RhoC and found that there was no significant difference between these cells (Supplementary Fig. S1B). Furthermore, we established a human prostate cell line, PC3MM/tet/RhoC, which contains the tetracycline-inducible *RhoC* gene, as well as PC3 cell lines that did or did not ectopically express RhoC. We then examined the rate of cell growth and DNA synthesis of these cells. Again, we found that RhoC did not affect the rate of proliferation of the cells (Supplementary Fig. S1A and B), which further supports our notion that RhoC has no apparent role in the growth of prostate cancer cells, although it significantly promotes tumor metastasis.

RhoC expression is significantly increased with the advancement of human prostate cancer. To further corroborate our results in a clinical setting, we examined the status of RhoC expression and its relationship with different clinicopathologic factors in prostate cancer by immunohistochemical analysis of 63 prostate tumor specimens. They were randomly selected from surgical pathology archives dating from 1988 to 2001. As shown in Fig. 2A and B, the expression of RhoC was found to be strongly elevated in high-grade tumors, particularly in specimens from patients with metastatic disease, compared with normal prostatic tissue or low-grade tumors. The results of our statistical analyses indicate that RhoC is strongly expressed in tumors with higher Gleason grade, although the correlations are not statistically significant (Fig. 2B). Importantly, the RhoC expression showed significant positive correlation with the metastases status of the patients (*P* = 0.028). It was also noted that RhoC expression showed a significant inverse correlation to that of NDRG1 (*P* = 0.02), which

Figure 1. RhoC promotes tumor metastasis without affecting the primary tumor growth *in vivo*. The RhoC expression plasmid was introduced into a low-metastasis rat prostate cell line, AT2.1, and clones (no. 6 and no. 10) that constitutively express RhoC were established. As a control, the original vector was also cloned into AT2.1. These clones, as well as the parental line, were injected s.c. into SCID mice as described previously. The volume of the primary tumor for each clone at the indicated time was measured using the equation: volume = (width + length) / 2 × W × L × 0.5236 (A). *Inset*, results of a Western blot of RhoC expression for each clone. Mice were sacrificed 3 wk after the inoculation of the cells, and metastatic lesions on the lungs were counted macroscopically (B). §, *P* < 0.05, statistically significant difference.



**B****Relationship between RhoC and other clinical variables**

Factor	All (63)	RhoC expression		P
		Positive (33)	Reduced (30)	
Age (y)				
> 71	41	20 (48.78%)	21 (51.22%)	0.605
≤ 70	22	13 (59.09%)	9 (40.91%)	
Gleason grade				
≥ 7	33	21 (63.64%)	12 (36.36%)	0.104
< 7	30	12 (40%)	18 (60%)	
Androgen receptor				
Positive	50	29 (58%)	21 (42%)	0.150
Negative	13	4 (30.77%)	9 (69.23%)	
PTEN				
Positive	39	18 (46.15%)	21 (53.85%)	0.316
Reduced	24	15 (62.5%)	9 (37.5%)	
NDRG1				
Positive	40	16 (40%)	24 (60%)	0.02*
Reduced	23	17 (73.91%)	6 (26.09%)	
Metastasis status				
Organ-confined	34	14 (41.18%)	20 (58.82%)	0.028*
Lym/bone	26	19 (73.08%)	7 (26.92%)	

Figure 2. Immunohistochemical analysis of RhoC in human prostate cancer. Immunohistochemical staining was performed on paraffin-embedded human prostate tissue sections using anti-RhoC antibody and the results were compared with other clinical variables. A, representative field with immunostaining for RhoC in normal prostate tissue (a), low-grade carcinoma (b), high-grade localized carcinoma (c), and high-grade metastatic carcinoma tissue (d). B, association of RhoC with other clinical variables was analyzed by standard χ^2 test using SPSS software. *, $P < 0.05$, statistically significant difference.

has recently been shown to be a tumor metastases suppressor in prostate cancer (19). These results suggest that the expression of RhoC is up-regulated at a relatively late stage and is directly involved in metastatic progression of prostate cancer, which is in good agreement with our *in vivo* data. Furthermore, the results of our survival analyses on 50 patients with prostate cancer over a period of 5 years indicates that patients with positive expression of RhoC had significantly worse overall survival rate than the patients with a reduced expression of the gene ($P = 0.018$, log-rank test; Fig. 3). The results of univariate Cox regression analysis revealed that the death risk of patients with increased RhoC expression was 4.8 times higher than the risk of patients with RhoC negativity. However, when we performed a multivariate analysis for RhoC, Gleason score, and metastasis, only the metastasis status gave a significant value ($P = 0.015$) and other two factors were excluded. The fact that multivariate analyses of these three factors excluded RhoC status indicates that the profiles of the RhoC expression and

metastasis status of patients significantly overlaps and that each factor has enough “power” for predicting patient outcome. In fact, when we did a multivariate analysis for a combination of RhoC status and Gleason score, which is the most widely used pathologic marker for prostate cancer, RhoC status turned out to be a better predicting marker than Gleason score ($P = 0.037$ and $P = 0.237$ for RhoC and Gleason score status, respectively). Although RhoC expression did not significantly and independently predict survival compared with metastasis, increased RhoC correlates with aggressive disease which could account for increased metastatic disease.

RhoC promotes invasiveness and motility of prostate cancer cells *in vitro*. To understand how RhoC contributes to the progression of prostate cancer, we ectopically expressed the *RhoC* gene in the human prostate cancer cell line, PC3, followed by examining the invasiveness and migration of the cells *in vitro*. We found that the expression of RhoC significantly enhanced both cell

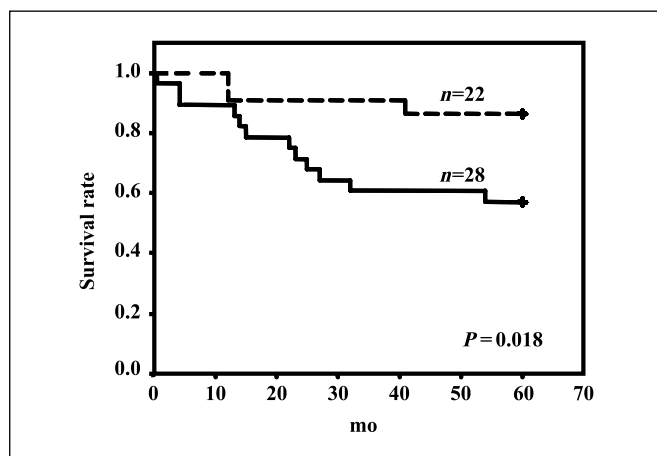


Figure 3. Prognostic value of RhoC expression. Overall survival rate over a period of 5 y was calculated in 50 patients with prostate cancer in relation to the expression of the *RhoC* genes by Kaplan-Meier method. $P = 0.018$ was determined by a log-rank test. RhoC-positive (solid line) patients and patients with reduced expression (dotted line) of RhoC.

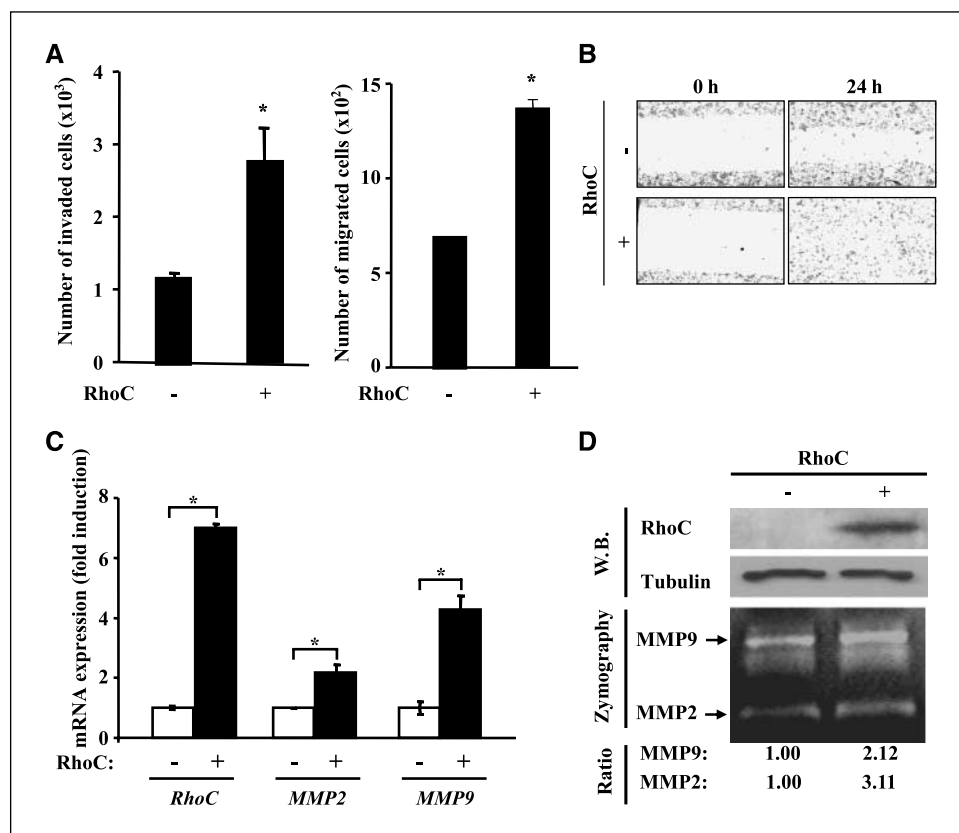
invasiveness and migration ($P = 0.03$ and 0.004 , respectively; Fig. 4A), which is in good agreement with the previous results of Yao and colleagues (20). The effect of RhoC on cell motility was also examined by the “wound healing” assay. As shown in Fig. 4B, cells with ectopically expressing RhoC showed a much higher rate of motility compared with the cells with an empty vector transfectant. These results strongly suggest that RhoC promotes metastasis by enhancing the invasiveness and/or motility of tumor cells. Because the invasive ability of tumor cells is known to often be correlated

with their production of secretory proteases (21), we examined the expression of MMP2 and MMP9 in the cells that overexpressed RhoC. As shown in Fig. 4C, quantitative reverse transcription-PCR (qRT-PCR) analysis for the cell overexpressing RhoC significantly augmented the level of the expression of the *MMP2* and *MMP9* genes ($P = 0.049$ and 0.02 , respectively). These results were further validated by gelatin zymography and Western blot analyses as shown in Fig. 4D. Therefore, our results indicate that the invasiveness of tumor cells induced by RhoC is, at least in part, due to the overexpression of MMP2 and MMP9.

RhoC activates MMP through the Pyk2 signal pathway. To gain further insight into the signaling pathways by which RhoC promotes the invasive phenotype, we prepared cell lysates from PC3MM/tet/RhoC with or without induction of the *RhoC* gene by tetracycline. The lysates were labeled with Cy3 and Cy5 and analyzed on an antibody microarray which contained 224 antibodies for various key molecules of cell signaling and cell cycle, and the results of ratios were rank-ordered. As shown in Fig. 5A (left), ectopic expression of RhoC significantly phosphorylated a series of protein kinases including MAPK, FAK, Akt, and Pyk2. The result of the array analysis was also confirmed by Western blot using the antibodies specific to phosphorylated proteins as well as the antibodies to the total proteins for each signal molecule (Fig. 5A, right; Supplementary Fig. S2A). These results suggest that RhoC can directly activate a cascade of signal pathways involving these key signal molecules that are closely related to cell motility and tumor progression.

Pyk2 is a tyrosine kinase and belongs to a member of the FAK subfamily which plays a critical role in cell migration and motility of various cell types (22, 23). Pyk2 is also known to be able to phosphorylate Akt (23). Therefore, we investigated the possibility

Figure 4. RhoC promotes invasiveness and motility of prostate cancer cells *in vitro*. A, the RhoC expression plasmid (pcDNA3/RhoC) or the vector alone was transfected into the PC3 cell line. After 24 h, cells were collected and subjected to invasion (left) and migration (right) assays. *, $P < 0.05$, statistically significant difference. B, for the motility assay, the PC3 cells stably transfected with the RhoC expression plasmid or an empty vector were cultured to confluency. The monolayer was scratched by drawing lines and photographed under a microscope. After 24 h of incubation, they were photographed again. C, to test the effect of RhoC on MMP2 and MMP9, PC3 cells that have been stably transfected with the RhoC expression plasmid or an empty vector were cultured in 12-well plates. Cells were then collected and their total RNA was treated with DNase. The RNA was then subjected to qRT-PCR using specific primers for the *RhoC*, *MMP2*, and *MMP9* genes. Results were presented as ratios of the expression level of each gene in RhoC-positive and RhoC-negative cells. *, $P < 0.05$, statistically significant difference. D, MMP2 and MMP9 activities in the conditioned medium from the PC3 cells with or without the RhoC expression plasmid as described in C were assayed by gelatin zymography. The image was photographed and the intensity of each band was digitally quantified. The expression of Flag-RhoC was confirmed by Western blot (top).



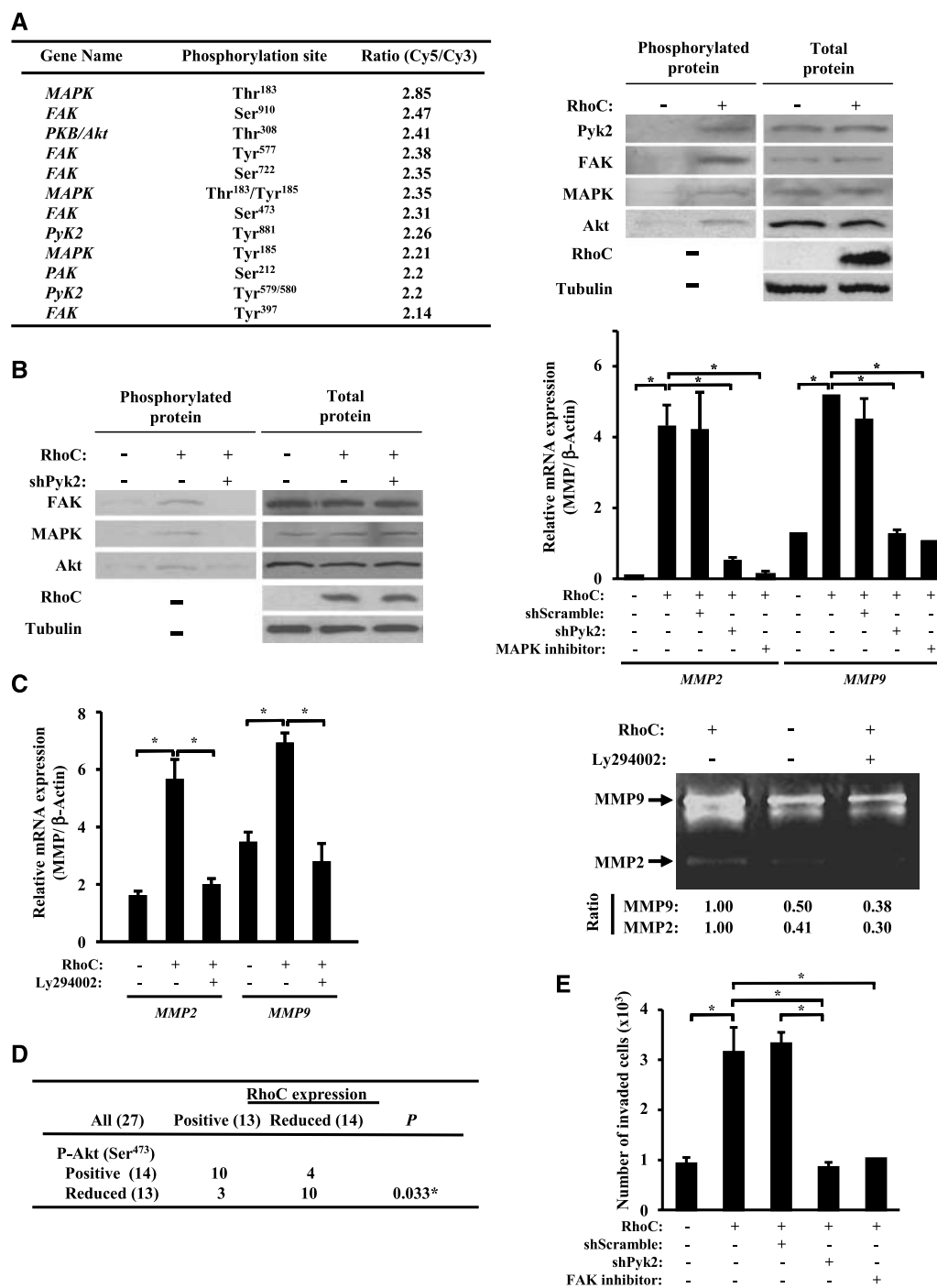


Figure 5. RhoC activates MMPs through the Pyk2/FAK pathway. **A**, for antibody array analysis, cell lysates were prepared from the PC3MM/tet cells containing the tetracycline-inducible *RhoC* gene with or without induction of *RhoC*. The proteins were labeled with Cy3 or Cy5 and subjected to antibody microarray (Sigma-Aldrich) analysis. The scanned data was analyzed by GenePix Pro 5.0 (Axon Instrument). The result of the antibody array data was confirmed by Western blot using phosphospecific antibodies to Pyk2, FAK, MAPK, and Akt as well as using antibodies to the total protein of each corresponding gene. **B**, PC3 cells stably transfected with the *RhoC*-expression plasmid or an empty vector were transfected with the expression plasmid of shRNA for *Pyk2* or a scrambled sequence. After 48 h, cells were collected and subjected to Western blot analysis using phospho-specific antibodies (left). To examine the effect of Pyk2 and MAPK on the MMP expression, the same set of cells were treated with or without the MAPK inhibitor, PD98059 (100 μ mol/L) for 48 h. RNA was extracted from each sample (in triplicate) and subjected to qRT-PCR using specific primers for *MMP2* and *MMP9* (right). **C**, the effect of Akt phosphorylation on MMP expression was examined. Cells with or without expression of *RhoC* were treated with or without PI3K/Akt inhibitor, Ly294002 (100 nmol/L), for 48 h. The cells were then collected and RNA was extracted followed by qRT-PCR analysis for *MMP2* and *MMP9* expression (left). The conditioned culture mediums of the same set of samples were subjected to zymography assay for *MMP2* and *MMP9* (right). The image was photographed and the intensity of each band was digitally quantified. **D**, to examine the clinical status of *RhoC* and p-Akt expression, 27 samples from patients with prostate cancer were analyzed by immunohistochemistry using antibodies to *RhoC* and p-Akt. The result was analyzed by χ^2 test. **E**, PC3 cells with or without *RhoC* expression were treated with shPyk2 or the FAK-specific inhibitor, TAE226, for 48 h. The cells were then assayed for their invasiveness by using a Matrigel invasion assay as described in Materials and Methods.

that Pyk2 is an immediate effector of the RhoC signal and that it controls the downstream pathways. PC3/RhoC cells were transfected with the expression vector of shRNA targeted to *Pyk2*. After 48 h of incubation, cell lysates were prepared and subjected to Western blot analysis using antibodies to RhoC, p-FAK, p-MAPK, and p-Akt. As shown in Fig. 5B (left) and Supplementary Fig. S2B, induction of RhoC strongly phosphorylated FAK, MAPK, and Akt, and this RhoC-dependent phosphorylation of these molecules was strongly blocked by the addition of shRNA to the *Pyk2* gene, suggesting that RhoC first activates Pyk2, which then phosphorylates FAK, MAPK, and Akt. We then examined whether MMP2 and MMP9 are indeed activated by Pyk2 and MAPK in a RhoC-dependent manner. RNA was prepared from PC3/RhoC cells that were cultured in the presence or absence of shRNA for *Pyk2* and the MAPK inhibitor, PD98059. RNAs were then examined for the expression of *MMP2* and *MMP9* by qRT-PCR. As shown in Fig. 5B (right) and Supplementary Fig. S2C (left), RhoC-dependent activation of both MMP2 and MMP9 was significantly abrogated in the presence of shRNA for *Pyk2* or the MAPK inhibitor, suggesting that the activation of MMP2 and MMP9 by RhoC is at least partly due to the phosphorylation of Pyk2 followed by the activation of MAPK. Because our results indicate that Akt is also phosphorylated at Ser⁴⁷³ by RhoC in a Pyk2-dependent fashion, we examined whether Akt is also involved in the activation of MMP2 and MMP9 in the RhoC signal pathway. As shown in Fig. 5C (left) and Supplementary Fig. S2C (right), we found that the RhoC-dependent induction of *MMP2* and *MMP9* was indeed significantly blocked by PI3K/Akt inhibitor, Ly294002. This result was further confirmed by gelatin zymography analysis as shown in Fig. 5C (right). To further corroborate the *in vitro* results, we examined 27 clinical specimens from patients with prostate cancer by conducting immunohistochemistry using anti-RhoC and anti-phospho-Akt (Ser⁴⁷³) antibodies. As shown in Fig. 5D, we found that RhoC expression was significantly correlated with the expression of phospho-Akt in these tumor tissues. Therefore, these clinical data as well as the *in vitro* results strongly suggest that Akt is part of the downstream effectors of RhoC signals and plays an important role in RhoC-dependent activation of MMP2 and MMP9. To further validate the role of Pyk2 and FAK in the RhoC-induced signal, we treated the PC3 cells that do or do not express RhoC with shPyk2 or the FAK-specific inhibitor, TAE226, followed by measuring the invasiveness of these cells using the Matrigel invasion chamber assay. As shown in Fig. 5E, we found that inhibition of Pyk2 and FAK indeed significantly blocked the RhoC-induced invasiveness of the prostate tumor cells, which strongly suggests the functional involvement of Pyk2 and FAK in the RhoC signaling pathway.

Discussion

RhoC has been shown to be involved in various types of tumors (9–11). However, the exact role of RhoC in tumor progression and its underlying mechanism are unclear, and the previous results from different groups have presented an apparently contradictory picture of the function of this gene (12–15). In this study, we have integrated multiple approaches, both *in vitro* and *in vivo*, to clarify the functional role of RhoC in prostate cancer progression. The results of our animal experiments clearly indicate that RhoC plays a critical role in the metastatic progression of prostate tumor but it is not essential for tumor cell growth. The results of immunohistochemical analysis of human prostate cancer specimens also

indicates that RhoC expression is significantly correlated with the metastatic status of the patients but not with Gleason grade, which strongly supports our notion that RhoC is implicated mainly in the metastatic process but not in tumorigenesis. Importantly, RhoC expression is inversely correlated with patient survival, suggesting that RhoC can serve as a prognostic marker as well as a potential therapeutic target for prostate cancer.

The molecular mechanism by which RhoC promotes tumor progression is an intriguing question. We have constructed a RhoC-inducible cell line and examined its protein expression profile using an antibody array to clarify the signal pathway. The results of the array analysis revealed that Pyk2, FAK, MAPK, and Akt were all phosphorylated upon induction of the RhoC expression, and the knockdown of *Pyk2* resulted in significant reduction in phosphorylation of FAK, MAPK, and Akt, suggesting that Pyk2 is the upstream effector and plays a central role in the RhoC signal pathway. Pyk2 belongs to the subfamily of focal adhesion protein tyrosine kinases and it has been shown to be involved in cell migration, invasion, and proliferation (24–28). It was reported that in the *in vitro* model of transforming growth factor- β -induced epithelial to mesenchymal transition, Pyk2 was strongly phosphorylated at Tyr⁸⁸¹ whereas during migration, Pyk2 was strongly phosphorylated at Tyr⁵⁸⁰ (22). It should be noted that, in our antibody array analyses, both of these sites were found to be phosphorylated (Fig. 5A). Pyk2 is capable of transducing signals via several known pathways, and one of the effectors is FAK which has been shown to be phosphorylated by Pyk2 at Tyr³⁹⁷, Tyr^{576/577}, and Tyr⁹²⁵ (29). The results of our antibody array data also revealed that both of these sites were indeed phosphorylated upon induction of RhoC. These results suggest that RhoC activates FAK via phosphorylation of Pyk2. FAK is a focal-adhesion kinase and plays a critical role in cell migration and motility (30–32). The enhanced expression of FAK has been documented in a number of different types of human cancers (33–41). The phosphorylation of FAK is known to be linked to the activation of several downstream signals including ERK and JNK/MAPK as well as PI3K/Akt (42, 43). Furthermore, it was previously shown that the invasive ability of RhoC was significantly attenuated by a MAPK inhibitor *in vitro* (44). Notably, the results of our knockdown experiments using *Pyk2*-specific shRNA has shown that the RhoC-dependent phosphorylation of both ERK/MAPK and Akt was significantly blocked by knockdown of *Pyk2*, suggesting that MAPK and Akt are activated by RhoC via phosphorylation of Pyk2 and FAK.

We have shown that RhoC promotes metastasis by augmenting the motility and invasion of tumor cells (Figs. 4 and 5) via activation of MMP2 and MMP9, two key proteases for the invasion of tumor cells. It should be noted that the expression of both MMP2 and MMP9 was previously shown to be modulated by the activation of Akt and MAPK (45–47). We have indeed shown that inhibitors of both molecules significantly blocked the RhoC-dependent activation of MMP2 and MMP9. In this context, it should be noted that Ruth and colleagues have recently shown that RhoC promoted the invasion of human melanoma cells in a PI3K/Akt-dependent manner (48). Our results also indicate that Akt was significantly phosphorylated at Ser⁴⁷³ by RhoC, and that the phosphorylation of this serine residue has previously been found to be involved in the motility and invasiveness of tumor cells (45, 46, 49). The activation of Akt has also been shown to be clinically associated with aggressiveness and earlier recurrence of prostate cancer (50). Collectively, our results indicate that RhoC enhances the invasiveness and metastatic ability of tumor cells by

activating the Pyk2/FAK pathway followed by phosphorylation of Akt and MAPK, which in turn, activate MMP2 and MMP9. RhoC is considered to serve as an independent prognostic marker to predict patient outcome, and an intervention of the RhoC signal may be an effective therapeutic strategy for prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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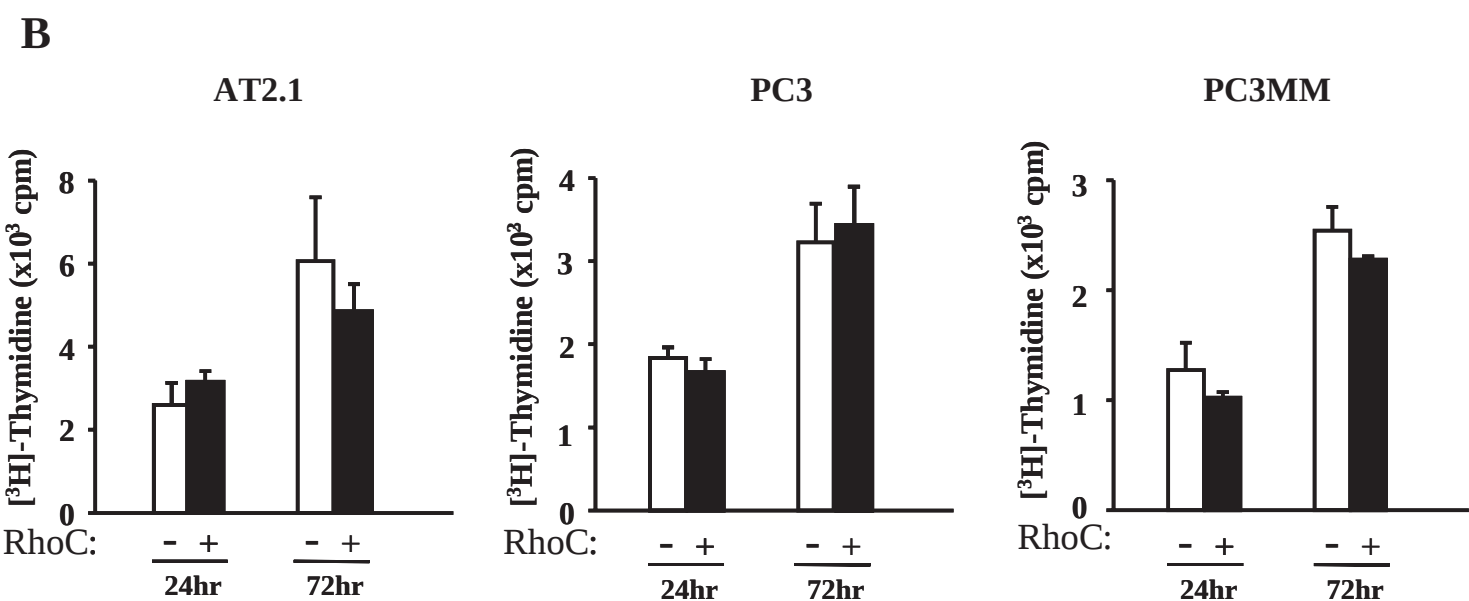
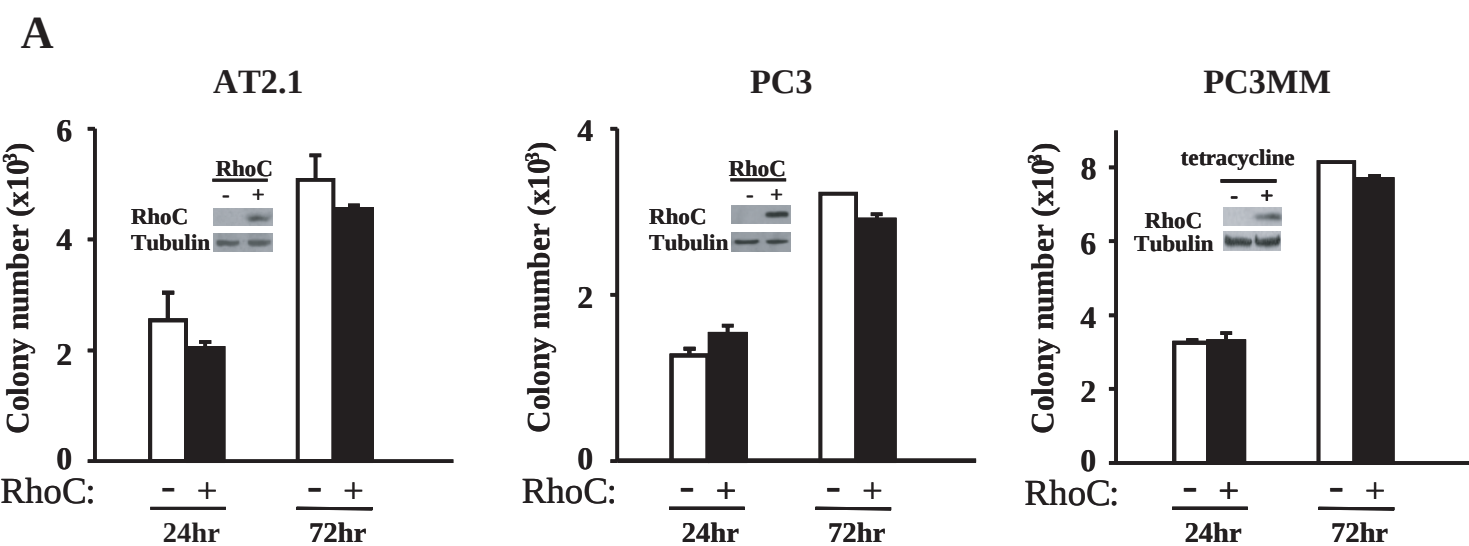
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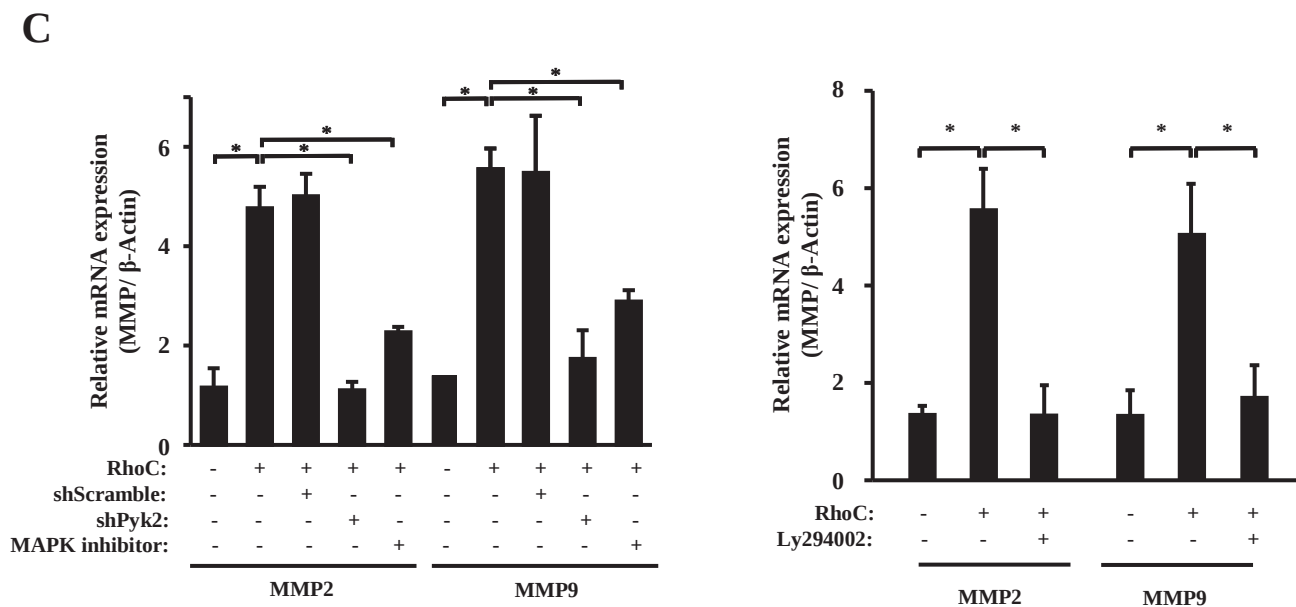
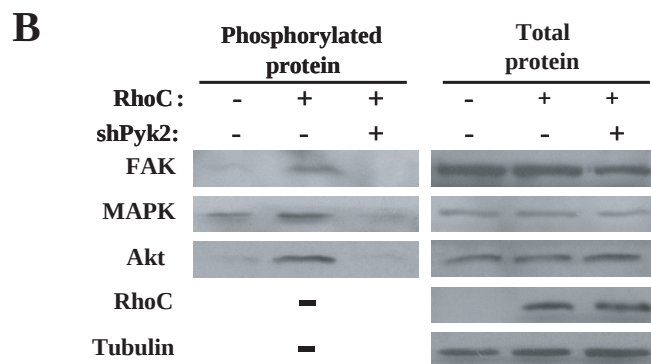
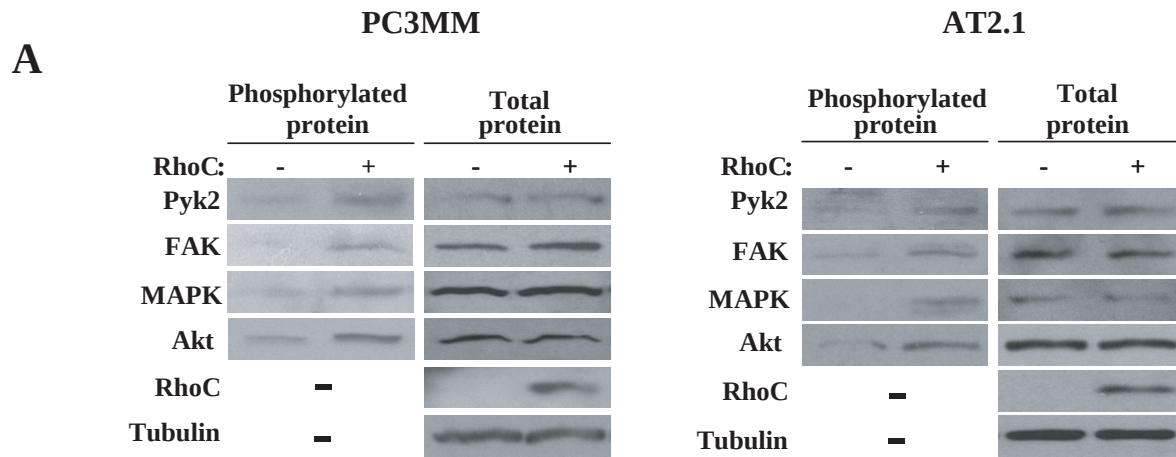
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Schedule

Opening Remarks, 8:15 A.M.

Marsha Rosner, Ph.D.

Michelle LeBeau, Ph.D.

Session I, 8:30 A.M. - 11:15 A.M.

Session Chair: Carrie Rinker-Schaffer, Ph.D.

8:30 - 9:15, Danny Welch, Ph.D.

9:15 - 10:00, Patricia Steeg, Ph.D.

10:00 - 10:30, *Break*

10:30 - 11:15, John Isaacs, Ph.D.

Session II, 11:15 A.M. - 3:30 P.M.

Session Chair: Kay Macleod, Ph.D. 11:15 - Noon, Ann Chambers, Ph.D.

Noon - 1:30, *Lunch*

1:30 - 2:15, Chand Khanna, DVM, Ph.D.

2:15 - 3:00, John Condeelis, Ph.D.

3:00 - 3:30 P.M., *Break*

Session III, 3:30 - 5:00 P.M.

Session Chair: Geoffrey Greene, Ph.D.

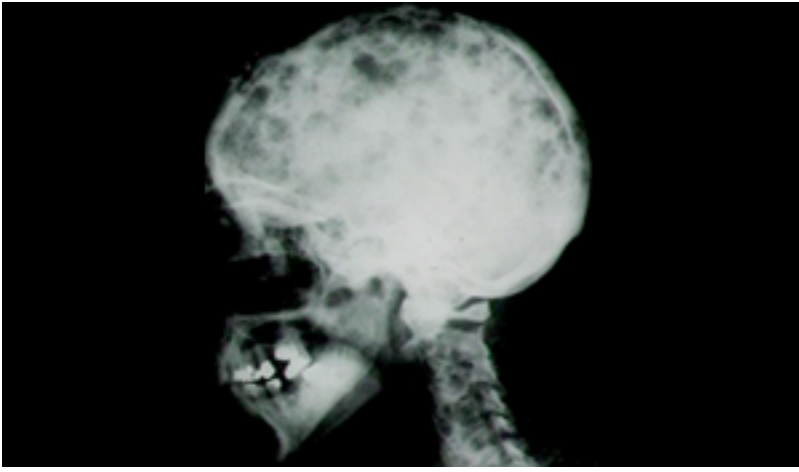
3:30 - 4:15, David Lyden, M.D., Ph.D.

4:15 - 5:00, Kounosuke Watabe, Ph.D.

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