Expression of Activating Transcription Factor 3 in Prostate Cancer

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 promotes 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.

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 thioresterase site, and one phosphoanthotheine 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
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 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 selection (2 μg/mL). Then, the pcDNAs/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'-AGTCCTGCTCAGGCAGAC and 5'-TGCTCCTGCTCTTAG) 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, 10² 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 was 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 χ² 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 the 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 cells 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
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. 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 are shown in Fig. 2.
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, RhoGD12, RKK, 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 (TGACGTCG) 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...
implicated in this process as well. For example, strong nuclear expression of ATF3 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 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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