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Drg-1 as a Differentiation-related, Putative Metastatic Suppressor Gene in Human Colon Cancer[1]

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ABSTRACT

A gene related to cell differentiation was identified by differential display as a candidate suppressor of metastases in colon cancer. This gene, with a full-length cDNA of 3 kb, is expressed in normal colon and primary colon cancer tissues and cell lines but not in their metastatic counterparts. A GenBank search found that it is identical to a recently cloned gene, *differentiation-related gene-1* (*Drg-1*), isolated from differentiated HT-29 colon cancer cells. Stable transfection of the SW620 metastatic colon cancer cell line with *Drg-1* cDNA induced morphological changes consistent with differentiation and up-regulated the expression of several colonic epithelial cell differentiation markers (alkaline phosphatase, carcinoembryonic antigen, and E-cadherin). Moreover, the expression of *Drg-1* is controlled by several known cell differentiation reagents, such as ligands of peroxisome proliferator-activated receptor γ (troglitazone and BRL46593) and of retinoid X receptor (LG268), and histone deacetylase inhibitors (trichostatin A, suberoylanilide hydroxamic acid, and tributyrin). A synergistic induction of *Drg-1* expression was seen with the combination of tributyrin and a low dose of 5′-aza-2′-dexoycytidine (100 nM), an inhibitor of DNA methylation. Functional studies revealed that overexpression of *Drg-1* in metastatic colon cancer cells reduced *in vitro* invasion through Matrigel and suppressed *in vivo* liver metastases in nude mice. We propose that *Drg-1* suppresses colon cancer metastasis by inducing colon cancer cell differentiation and partially reversing the metastatic phenotype.

INTRODUCTION

Metastasis consists of the spreading of tumor cells from the primary neoplasm to distant sites. Despite significant improvements in early diagnosis and surgical ablation, as well as local and systemic adjuvant therapies, the majority of cancer deaths are attributable to metastases that are resistant to conventional therapies. It is believed that the outcome of metastatic diseases is influenced by intrinsic changes of the tumor cell (seed) and by changes in host factors (soil; Ref. 1). The process of metastasis is not random but rather consists of a complex series of linked and interrelated steps involving multiple host-tumor interactions (1). Many proteins including proteases, adhesion molecules, angiogenesis, and growth factors are involved in metastasis. Therefore, understanding the gene expression changes in metastatic cancer cells may aid in early diagnosis and therapeutic intervention. In the last decade, considerable progress has been made in understanding these changes. Yet a sensitive and reliable method for detection of early metastasis in colon cancer is still not available, and clinicians still rely primarily on marginally sensitive pathological findings to predict metastasis (2).

To further define gene expression changes in metastatic colon cancer, we used differential display and identified 19 genes that are expressed differentially between primary and metastatic colon cancer. One of these genes is identical to a gene identified previously named *Drg*[3]*-1,* which was found to be down-regulated in colonic adenomas and primary colon cancer (3). The expression of this gene was also found to be regulated by homocysteine, testosterone, and Ni^{2+} in different cell types (4–6). However, the function of this gene remains unknown. We now report that *Drg-1* is further down-regulated in metastatic colon cancer cells to levels that are nearly undetectable when compared with the primary colon cancer counterparts. We have further demonstrated that stable transfection of a metastatic colon cancer cell line SW620 with *Drg-1* cDNA induced morphological changes indicative of differentiation, up-regulated the expression of several colonic epithelial cell differentiation markers, and reduced *in vitro* invasion through Matrigel and *in vivo* liver metastasis in nude mice. In mechanistic studies, we found that the expression of *Drg-1* was controlled by several differentiation reagents, such as ligands of PPARγ and the retinoid X receptor, as well as by reagents affecting DNA methylation and histone acetylation. These data suggest that *Drg-1* may suppress colon cancer metastasis by inducing cell differentiation and reversing the metastatic phenotype.

MATERIALS AND METHODS

Human Tissues and Cell Culture. Sporadic human colon cancer tissues and their metastatic lesions were randomly obtained from the Pathology Department of Beth Israel Deaconess Medical Center. Tumor tissues were carefully dissected from adjacent normal colon tissues, snap frozen, and stored in liquid nitrogen before analysis. Colon cancer cell lines were purchased from American Type Culture Collection and cultured at 37°C in 5% CO_2 in a mixture of DMEM (½) and RPMI 1640 (½) with 10% fetal bovine serum and antibiotics. *Drg-1* transfectants were maintained in the same culture medium containing 0.2 mg/ml of G418.

Chemical and Biological Reagents. Aza, all-*trans* retinoic acid, tributyrin, and TSA were purchased from Sigma Chemical Co. (St. Louis, MO). LG268, a retinoid X receptor selective ligand, was a gift from Richard A. Heyman (Ligand Pharmaceuticals, San Diego, CA). Troglitazone and BRL49653, ligands of PPARγ, were gifts from Dr. Bruce M. Spiegelman at Dana-Farber Cancer Institute. SAHA, a second-generation hybrid polar cytodifferentiation agent shown to inhibit histone deacetylase and induce terminal differentiation in transformed cells (7), was a gift from Dr. Paul Marks (Sloan-Kettering Cancer Center, New York, NY).

DD. SW480 and SW620 cell lines were both derived from the same colon cancer patient. SW480 was established from a primary colon cancer lesion, and SW620 was from a lymph node metastasis (8). To ensure that the observed differences were not an artifact of long-term cell culture, we also studied freshly isolated primary colon cancer tissue and lymph node metastasis from a single patient. DD was performed with a DD kit purchased from GenHunter Corp. (Nashville, TN), according to the manufacturer's protocol (9). The anchor and arbitrary primers that led to detection of *Drg-1* were 5′-AAGCTTTTTTTTTTTG-3′ and 5′-AAGCTTTGGT-CAG-3′. Band isolation and direct sequencing of the DD band were performed as described (9).

RNA Isolations and Northern Blot Analysis. RNA from colon cancer cells was isolated with TRIzol reagent (Life Technologies, Inc., Rockville,

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[3] The abbreviations used are: *Drg,* differentiation-related gene; PPAR, peroxisome proliferator-activated receptor γ; Aza, 5′-aza-2′-deoxycytidine; TSA, trichostatin A; SAHA, *N*-hydroxy-*N*′-phenyl-octane-1,8-diotic acid diamide suberoylanilide hydroxamic acid; DD, differential display; CEA, carcinoembryonic antigen; RXR, retinoid X receptor.

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MD), according to the manufacturer's protocol. RNA from colon cancer tissues was isolated by the guanidinium thiocyanate/CsCl method, as described (10). A multiple-tissue dot blot was obtained from Clontech (Palo Alto, California). Northern and dot blot analysis were performed as described (10), except ExpressHyb hybridization solution from Clontech was used. Nucleotides 4–337 of *Drg-1* were ^{32}P-labeled with a random labeling kit (Boehringer Mannheim, Indianapolis, IN) and used as a probe for Drg-1. Probes for E-cadherin and CEA were produced as described (11, 12). The probe for PPARγ was a gift from Bruce M. Spiegelman at Dana-Farber Cancer Institute (13). Membranes were hybridized in ExpressHyb hybridization solution (Clontech) with ^{32}P-labeled probe, washed, and exposed to a PhosphorImager (Bio-Rad Laboratories, Richmond, CA) or X-ray films. The signal intensities were quantified with Imagequant software (Bio-Rad Laboratories) and normalized to 28S RNA expression.

Generation of *Drg-1* Stable Transfectants. The coding region of *Drg-1* (nucleotides 110-1346) was cloned from a human normal prostate cDNA library (Clontech) by PCR with Advantage cDNA Polymerase Mix from Clontech. The coding region of *Drg-1* cDNA was inserted in-frame into the pcDNA3.1 vector, which contains the cytomegalovirus enhancer-promoter (Invitrogen Corp., Carlsbad, CA). The cDNA was then fully sequenced to ensure that no mutations were introduced during the PCR amplification. SW620 colon cancer cells were seeded in 0.6-cm dishes at 5×10^5 cells/dish and transfected with a pcDNA3.1 vector containing *Drg-1* cDNA or with an empty vector as control using Superfect (Qiagen, Inc., Valencia, CA), according to the manufacturer's protocol. After culturing in medium containing 0.8 mg/ml of G418 (LifeTechnologies, Inc.) for ~2 weeks, individual clones were isolated using cloning cylinders. The cell clones that expressed the 1.2-kb *Drg-1* cDNA coding region (as confirmed by Northern blot) were maintained in medium containing 0.2 mg/ml of G418 and used for further investigation.

Alkaline Phosphatase Assays. Alkaline phosphatase assays were performed as described (14). Control cells and different *Drg-1* expressed cell clones were cultured for 48 h to half confluence and lysed. Alkaline phosphatase activity in cell lysates was determined with *p*-nitrophenyl phosphate disodium hexahydrate (Sigma 104) as a substrate. Synthetic alkaline phosphatase (Life Technologies) was used to construct a standard dilution curve. Each assay was performed in triplicate. The means ± SE from two separate experiments are presented.

***In Vitro* Matrigel Invasion Assay and *in Vivo* Nude Mice Studies.** *In vitro* Matrigel invasion assays were performed as described using 6.5-mm transwell chambers (8-μm pore size; Costar). The transwell filters were coated with 5 μg of Matrigel (15). SW620 cells (1×10^5) overexpressing *Drg-1* or vector control cells were cultured in the upper chamber, and conditioned NIH 3T3 medium was added to the bottom chamber. After 72 h, the cells were fixed and stained, and the number of cells that invaded through the Matrigel was quantified as described (15).

Animal protocols were approved by the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Female BALB/c athymic nude mice (National Cancer Institute Frederick Cancer Research Facility, Rockville, MD), 8 weeks of age, were anesthetized with methoxyfluorane by inhalation, and a small abdominal incision was made under sterile conditions. Viable tumor cells (5×10^5) in 0.02 ml of serum-free medium were injected into the spleen by means of a sterile tuberculin syringe and a 30-gauge needle. During the injection, care was taken to maintain uniform cell suspensions and to avoid injecting clumped cells. The injection site was then dabbed gently with sterile gauze dampened with 95% ethanol to kill tumor cells that may have escaped. The abdomen was closed with a metal surgical clip, and the animals were returned to their cages. After 8 weeks, the animals were euthanized. The spleen and liver were weighed, as well as examined for splenic "primary" tumor and liver metastases by visual inspection. Metastases were confirmed with histological studies as described (16).

Statistical Analysis. Statistical computations were performed using the statistical analysis systems statXact (Statistics Unlimited, Savanna, GA). For the statistical analysis of the difference between control and transfected cells in the Matrigel assay, ANOVA with the Tukey correction for multiple comparisons to provide a nominal significance level (α) of 0.05 was used. For the statistical analysis of the difference between control and transfected cells in the animal study, the Fisher exact test with an appropriate cutoff P of <0.05 was used.

RESULTS

Down-Regulation of *Drg-1* mRNA Expression in Metastatic Colon Cancer Cell Lines and Tissues. Using DD, we identified 19 genes that are expressed differentially between primary (SW480) and metastatic (SW620) colon cancer cell lines and tissues (data not shown). Fig. 1 illustrates a typical example of DD (Fig. 1*A*) and a Northern blot (Fig. 1*B*), which confirms the existence of 3.0-kb mRNA that is expressed in the SW480 primary colon cancer cells and the primary colon cancer tissues but not in their metastatic counterparts. To determine the identity of this gene, the DD band (Fig. 1*A*) was extracted, reamplified, and sequenced. A BLAST computer database search found that this cDNA was 99% homologous to the 3′ untranslated region of *Drg-1* (3), thus revealing its identity.

The presence of multiple CpG sites in the most 5′ end of the *Drg-1* cDNA implies that this gene may be controlled by DNA methylation (17, 18). The effect of Aza, an inhibitor of DNA methylation (19), on *Drg-1* expression was investigated. Treatment with Aza partially up-regulated the expression of this gene in all colon cancer cell lines tested (Fig. 2*A*). The role of DNA methylation on the expression of this gene will be discussed in detail below. To extend these findings, we studied a total of eight colon cancer cell lines and 10 human colon

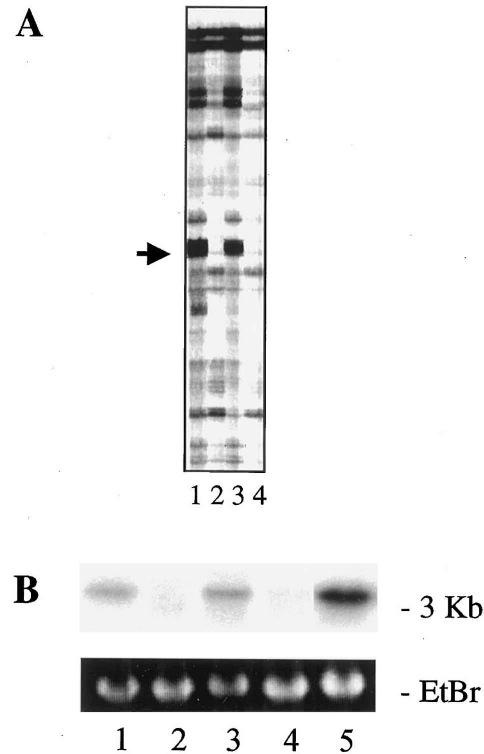

Fig. 1 DD and Northern blot identify decreased expression of *Drg-1* in metastatic colon cancer as opposed to their primary counterparts. RNA isolated from colon cancer cell lines and tissue was used for DD and Northern blot studies. *Lane 1,* a primary colon cancer cell line (SW480); *Lane 2,* a metastatic colon cancer cell line (SW620); *Lane 3,* a primary colon cancer tissue; *Lane 4,* a lymph node metastatic lesion from the same patient; *Lane 5* (Northern blot only), adjacent normal colon tissue from the same patient. *A,* DD demonstrates markedly diminished expression of *Drg-1* (*arrowhead*) in a metastatic colon cancer cell line and tissue when compared with the primary counterpart. *B,* Northern blot using this gene fragment as probe identifies a 3-kb mRNA and confirms that the expression of this gene is down-regulated in metastatic colon cancer cell lines and tissues. Ethidium bromide (*EtBr*) staining of 28S RNA was used to compare loading.

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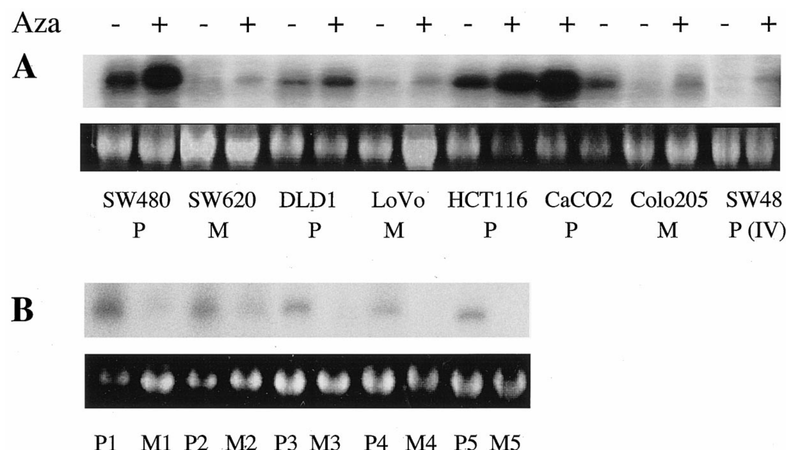

Fig. 2 *Drg-1* is down-regulated in metastatic colon cancer, and its expression can be partially restored by inhibiting DNA methylation. *A,* Northern blot studies demonstrated a marked decrease expression of *Drg-1* in all metastatic colon cancer cell lines. Treatment with Aza (5 μM) for 72 h resulted in an increased level of expression of *Drg-1* in colon cancer cells. *P,* primary colon cancer cell lines; *M,* metastatic colon cancer cell lines; *IV,* grade IV, poorly differentiated colon cancer cells. *B,* Northern blot studies of *Drg-1* expression in pairs of primary and metastatic colon cancer tissues. *P1–P5,* primary colon cancer tissues. *M1, M3, M4,* and *M5,* cancer tissues isolated from lymph nodes metastases. *M2,* from liver metastases. Ethidium bromide staining of 28S RNA was used to compare loading.

cancer specimens (5 primary tumors paired with 5 metastatic colon cancer lesions; Fig. 2). Of the four cell lines with the lowest level of *Drg-1* expression (Fig. 2*A*), three (SW620, LoVo, and Colo205) were derived from metastatic lesions of colon cancer, and the fourth, SW48, was derived from a poorly differentiated primary colon cancer. In contrast, the other four cell lines (SW480, DLD-1, HCT116, and $CaCO_2$) were derived from primary colon cancer lesions (20–22). Similarly, the expression of this gene was also found to be substantially down-regulated in two and completely undetectable in three metastatic lesions (Fig. 2*B*) when compared with the paired primary colon cancer lesions. In preliminary Northern blot studies of 36 clinical specimens of breast cancers (data not shown), the level of *Drg-1* expression did not differ appreciably between primary breast cancers and metastatic lesions. These data suggest that *Drg-1* may be specific for colon cancers.

To determine the pattern of expression of *Drg-1* in normal human tissue, a master dot blot containing a total of 50 normal human tissues was probed with *Drg-1*. As shown in Fig. 3, the expression of *Drg-1* was found in all tissues tested with a slightly higher expression level in the brain, prostate, and adult and fetal kidney, as well as placenta. The same blot was stripped and reprobed with ubiquitin to ensure equal loading (data not shown). The absence of signals in bacterial or yeast cDNA samples demonstrated the specificity of the probing. The presence of signals in human DNA (Fig. 3, *right lower corner*) suggests that *Drg-1* is highly abundant in human tissue or belongs to a multigene family. The ubiquitous expression of *Drg-1* suggests that this gene may function as a housekeeping gene.

Overexpression of *Drg-1* Induces Metastatic Colon Cancer Cell Differentiation. To further investigate the function of *Drg-1,* we transfected the metastatic colon cancer cell line (SW620) with a pcDNA3.1 vector containing the 1.2-kb coding region of *Drg-1* under the control of the cytomegalovirus promoter. Multiple SW620/T clones stably expressing transfected *Drg-1* mRNA were selected for the subsequent studies. An *in vitro* translational study with pcDNA3.1/*Drg-1* plasmid yielded a protein with a molecular weight of M_r 43,000. This matches the predicted molecular weight of *Drg-1,* indicating that this plasmid construct functions *in vitro* (data not shown).

Although the growth rate of the transfected cells was very similar to that of the *neo* controls and parental nontransfected cells (data not shown), distinct morphological changes were seen in the *Drg-1*-transfected cells (Fig. 4). The transfected cells were larger, flatter, and spindle shaped (Fig. 4*B*), in contrast to the smaller, more round *neo* control cells (Fig. 4*A*). These morphological changes were consistently observed in multiple transfected clones

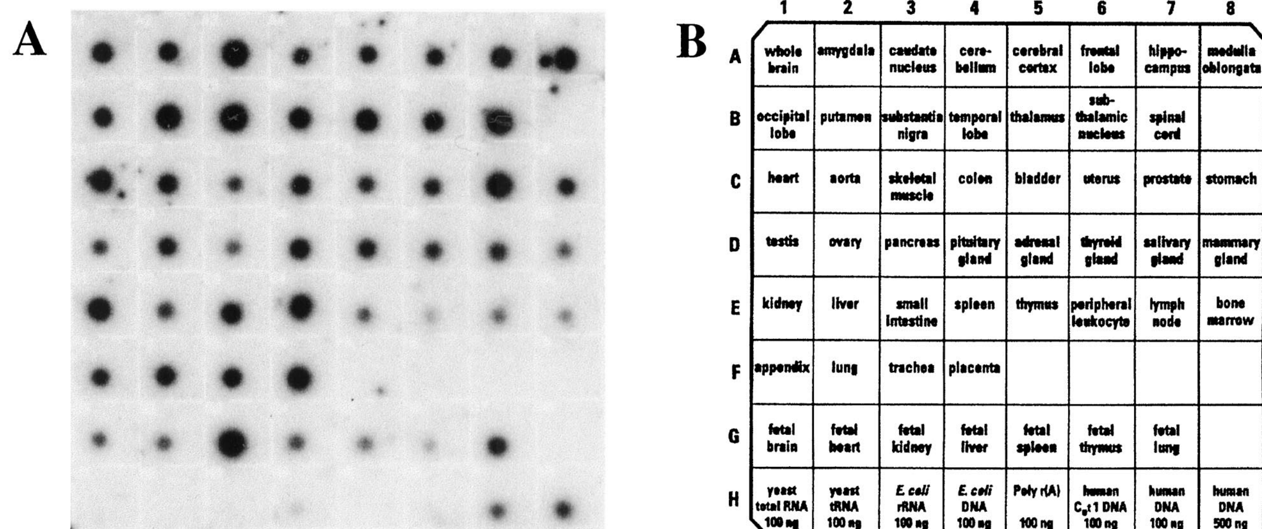

Fig. 3 *Drg-1* expression in normal human tissues. A human RNA master blot from Clontech was probed with a ^{32}P-labeled *Drg-1* probe. *A, Drg-1* expression pattern in normal human tissues. *B,* RNA samples included on the blot.

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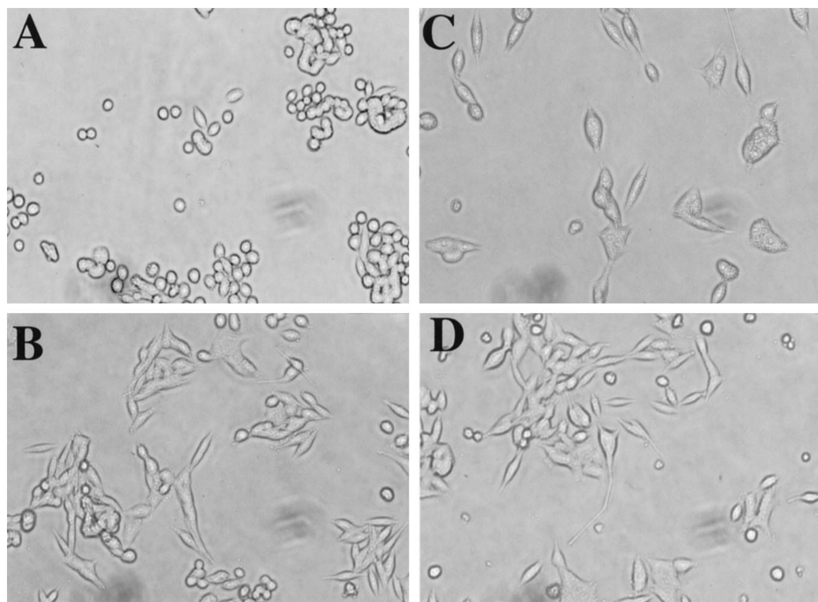

Fig. 4 *Drg-1* expression induces morphological changes that are similar to differentiation-specific changes induced by tributyrin and LG268. *A,* SW620 cells transfected with an empty vector. *B,* SW620 cells transfected with *Drg-1* cDNA. *C,* SW620 nontransfected parental cells treated with 1 mM tributyrin for 72 h. *D,* SW620 nontransfected parental cells treated with 0.1 μM LG268 for 72 h.

expressing *Drg-1* but not in those clones that did not express *Drg-1*. Similar morphological changes were seen when differentiation was induced in parental SW620 by known differentiation reagents such as tributyrin, a prodrug of butyrate (Fig. 4*C*), LG268, a ligand of RXR (Fig. 4*D*), and all-*trans* retinoic acid, a ligand of retinoic acid receptor (not shown). These findings suggest that the expression of *Drg-1* induces changes characteristic of cell differentiation in colon cancer cells.

To substantiate this finding, the expression level of several colonic epithelial cell differentiation markers (14), including alkaline phosphatase, CEA, and E-cadherin, were determined. As shown in Fig. 5*A,* the activity of alkaline phosphatase was 2–3-fold higher in all five transfected cell clones (620/T) compared with the *neo* control cells (620/V). Similarly, the expression of E-cadherin and CEA was also up-regulated in all transfected cell clones, although the levels of expression varied among individual clones (Fig. 5*B*). Together, these findings suggest that the expression of *Drg-1* induces colon cancer cell differentiation.

Regulation of *Drg-1* Expression by Ligands of PPARγ, RXR, DNA Methylation, and Histone Acetylation. Because of the potential role of *Drg-1* in the regulation of colonic epithelial cell differentiation, the effects of several known differentiation reagents on expression of the *Drg-1* gene were sought. As shown in Figs. 2 and 6*B,* treatment with Aza, an inhibitor of DNA methylation and a known differentiation inducer (19), partially up-regulated the expression of *Drg-1* in all colon cancer cell lines tested, suggesting that the expression of *Drg-1* is controlled by DNA methylation. PPARγ is a ligand-inducible transcription factor

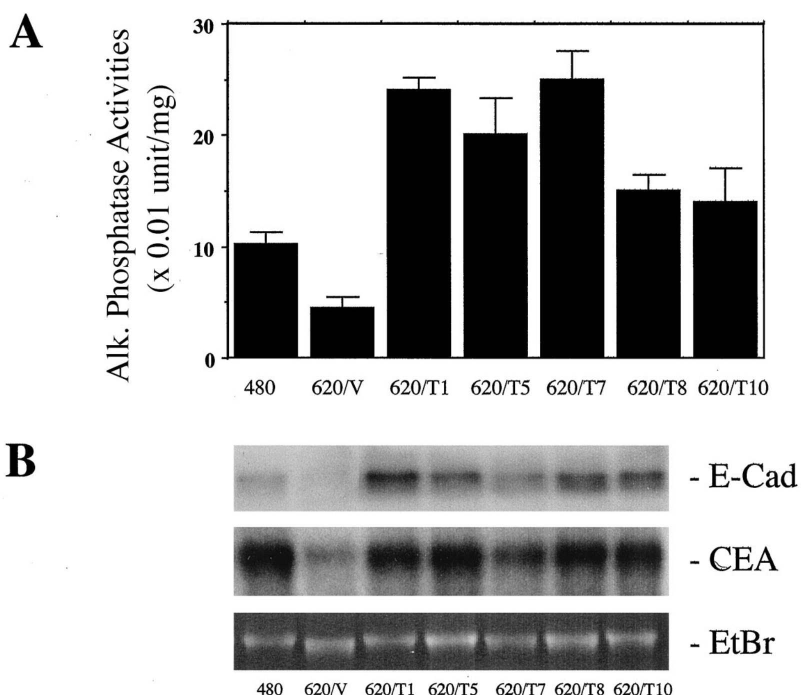

Fig. 5 Overexpression of *Drg-1* induces expression of colonic epithelial cell differentiation markers. *A,* increased alkaline phosphatase activity in colon cancer cells transfected with *Drg-1*. Transfected and control colon cancer cells were cultured for 72 h and lysed. Alkaline phosphatase activity was measured in cell extracts by a standard spectrophotometric method as described in "Materials and Methods." Enzyme activities were calculated by interpolation against simultaneously assayed alkaline (*Alk.*) phosphatase enzyme standards. *Columns,* means of triplicate values of two separate experiments; *bars,* SE. *B,* expression of E-cadherin and CEA in *Drg-1*-transfected and control colon cancer cells. Northern blot analysis using probes specific for human E-cadherin (*E-Cad*) and CEA were performed. The expression of E-cadherin and CEA were up-regulated in all transfected cell clones (620/T), although the level of expression varied slightly among individual clones. *480,* SW480 cells; *620/V,* SW620 cells transfected with an empty vector. Ethidium bromide (*EtBr*) staining of 28S RNA was used to compare loading.

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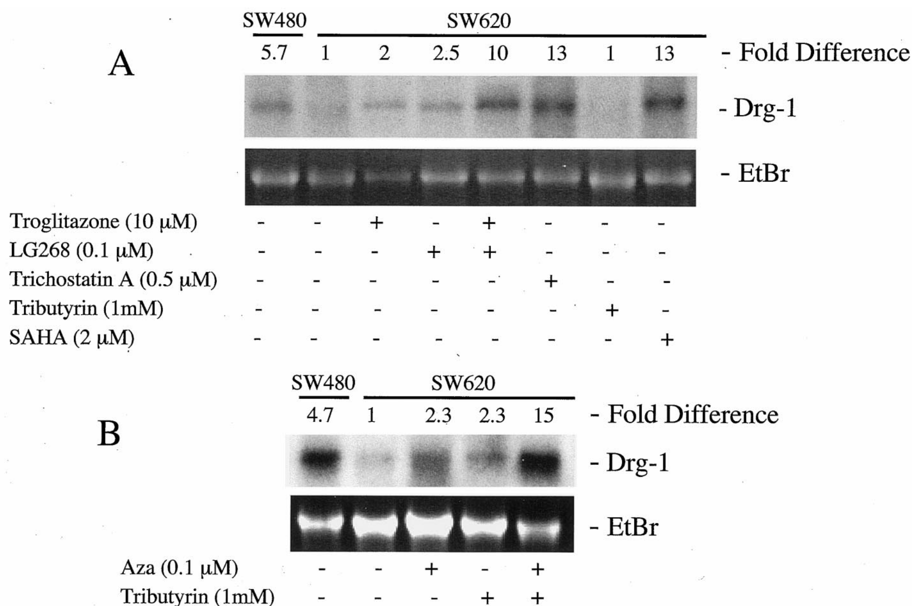

Fig. 6 Regulation of *Drg-1* expression by ligands of PPARγ, RXR, DNA methylation, and histone acetylation. Colon cancer cells (SW480 and SW620) were treated with different drugs for 3 days (*A*; with the exception of TSA, which is toxic and required a short treatment of 48 h) and 5 days (*B*). RNA was isolated, and Northern blots were performed. Ethidium bromide (*EtBr*) staining of 28S RNA was used to compare loading. The signal intensities of Northern blots were analyzed by densitometry and normalized for 28S RNA level. The changes of *Drg-1* expression level are represented as fold difference with respect to the expression level in the nontreated controls (the average of two individual experiments).

known to control differentiation of a variety of cells including adipocytes and colonic epithelial cells (13). To investigate the potential functional association between the PPARγ and *Drg-1,* we first studied the expression of PPARγ in *Drg-1*-transfected and *neo* control SW620 cells. Northern blot studies with a probe specific for PPARγ revealed that its expression level in both transfected and *neo* control SW620 cells is nearly identical (data not shown), suggesting that the expression of PPARγ is independent of *Drg-1*. On the other hand, the expression of *Drg-1* was up-regulated by troglitazone (Fig. 6*A*) and BRL46593 (not shown), two synthetic ligands of PPARγ (13), suggesting that *Drg-1* is actually downstream of PPARγ. In addition, LG268, a synthetic ligand specific for RXR (23) also up-regulated *Drg-1* (Fig. 6*A*). A combination of troglitazone and LG268 induced *Drg-1* expression by 10-fold, suggesting a possible synergistic effect from these two drugs. Moreover, the expression of *Drg-1* was also markedly up-regulated by two histone deacetylase inhibitors, TSA and SAHA (7, 24, 25). The effect of another histone deacetylase inhibitor, tributyrin (a prodrug of butyrate), was only seen after 5 days of treatment (Fig. 6*B*). However, when cells were treated with tributyrin plus a low dose of Aza (0.1 μM), a marked increase in *Drg-1* expression was seen (Fig. 6*B*), suggesting a synergistic effect from these two drugs. Together, these data suggest that Drg-1 may be a downstream element of the PPARγ transcriptional pathway and is controlled by both DNA methylation and histone acetylation, two global mechanisms of gene regulation (26). We suggest that *Drg-1* may suppress colon cancer metastasis by inducing cell differentiation and reversing the metastatic phenotype.

Overexpression of *Drg-1* Inhibits *in Vitro* Invasion through Matrigel and *in Vivo* Liver Metastasis in Nude Mice. To test the hypothesis that *Drg-1* may suppress colon cancer metastasis, we used an *in vitro* Matrigel assay (15) to examine the invasive capabilities of metastatic colon cancer cell lines transfected with the *Drg-1* cDNA (SW620/T) or with an empty vector as a control (SW620/V). As shown in Fig. 7, the metastatic colon cancer cell line (SW620) migrated through the Matrigel at levels about five times greater than the primary colon cancer cell line (SW480), in agreement with data published previously (27). Expression of *Drg-1* cDNA in SW620 cells (T1 and T5) reduced Matrigel invasion by up to 70% ($P < 0.0001$). Expression of the *neo* control vector had little effect on Matrigel invasion. These data suggest that the overexpression of *Drg-1* inhibits the *in vitro* invasion ability of metastatic colon cancer cells.

To further investigate the role of *Drg-1* in invasion and metastasis, three *Drg-1*-transfected cell clones (620/T1, 620/T5, and 620/T7) were each injected into the spleen of athymic nude mice. Two *neo* stably transfected cells (620/V and 620/V1) were injected to serve as controls. As shown in Table 1, 12 of 14 mice injected with *neo* control cells and 13 of 15 mice injected with transfected cells developed "primary" tumors in the spleen. The tumor burdens of the splenic primaries, as judged by their weights, were very similar between the transfected and the *neo* control group (data not shown). However, in the mice that developed "primary" tumors, 9 of 12 mice (75%) developed liver metastases in the neo control group, whereas only 3 of 13 mice (23%) had liver metastases in the *Drg-1*-transfected group. Statistical analysis using the Fisher exact

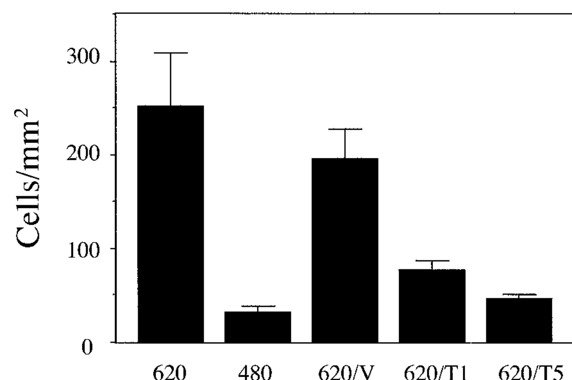

Fig. 7 Overexpression of *Drg-1* inhibits the *in vitro* invasion through Matrigel. A Matrigel gel assay was used to assess the invasive capacity of *Drg-1*-transfected colon cancer cells *versus* controls. Colon cancer cells (1×10^5) were added to the upper chambers. Conditioned NIH-3T3 medium was added to the bottom chambers. After 72 h, the cells that had not migrated were removed, and the cells that had migrated to the lower chamber of the filter were fixed, stained, and quantified by counting the total number of cells in a 1-mm^2 grid area. Five randomly selected fields were counted on each filter. The data shown are the means from two experiments done in triplicate; *bars,* SE. *620,* SW620 metastatic colon cancer cell line; *480,* SW480 primary colon cancer cell line; *620/V,* SW620 cells transfected with an empty vector; *620/T,* SW620 cells transfected with *Drg-1* cDNA. Statistical analysis of the difference between control and transfected cells (*620/T1* and *620/T5*) using Tukey correction test revealed both *P*s were <0.0001.

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Table 1 *Overexpression of Drg-1 suppresses in vivo liver metastases in nude mice*[a]

Cells	Total no. of mice[b]	Mice with splenic primaries[c]	Mice with splenic primaries and liver metastases[c]
620/V	4	3	3
620/V1	10	9	6
Subtotal	14	12	9
620/T1	5	4	1
620/T5	5	5	1
620/T7	5	4	1
Subtotal	15	13	3

[a] Viable colon cancer cells (5×10^5) were injected into the spleen of athymic nude mice. After 8 weeks, mice were euthanized, and the presence of tumors in the spleen and their liver metastases were determined as described in "Materials and Methods."

[b] For the control group, a total of 14 mice were used. Twelve of 14 mice developed tumors; 1 had no tumor, and 1 mouse died right after injection. For the transfected group, a total of 15 mice were used. Thirteen of 15 mice developed tumors; 1 had no tumor, and 1 died right after injection.

[c] The number of mice that developed splenic primaries or splenic primaries and liver metastases in the control and transfected groups was used for statistical analysis using Fisher's exact test. *P* was equal to 0.0169.

test revealed a *P* of 0.0169, suggesting that the differences in liver metastases observed between the control and transfected groups are unlikely attributable to chance alone. Therefore, these findings suggest that *Drg-1* may function as a suppressor of colon cancer metastasis. These results also indicate that *Drg-1* did not alter the ability of cancer cells to form primary tumors.

DISCUSSION

Neoplastic transformation arises from multiple defects in cell growth and differentiation (28). Gene expression changes and/or genomic DNA mutations play a crucial role in the pathogenesis of cancer formation and in its progression (29, 30). Because the dispensable nature of the colon allows removal of the primary tumor, the prognosis of colon cancer directly correlates with the extent of tumor invasion and metastases (2). Molecules involved in cancer metastasis may serve as markers for early detection of metastasis and/or as targets for therapeutic intervention.

Using DD, we have identified 19 genes expressed differentially between primary and metastatic colon cancer. One of these genes, *Drg-1,* was found to be down-regulated in metastatic colon cancer tissues and cell lines. Overexpression of *Drg-1* induced morphological and molecular changes consistent with colon cancer cell differentiation and suppressed *in vitro* invasion and *in vivo* liver metastases in nude mice. *Drg-1* was initially identified by comparing gene expressions between undifferentiated and well-differentiated HT-29 colon cancer cell lines (3). Simultaneously, others found that *Drg-1* was regulated by homocysteine in endothelial cells (4), testosterone in T-cell hybridoma 312.13 cells (5), and Ni^{2+} in human and rodent cell lines (6), implying that *Drg-1* may be a housekeeping gene (4). In fact, a GenBank search revealed that the murine homologue of *Drg-1* (named *Ndr1,* accession no. U60593) is a downstream target of N-*myc*, first suggesting that *Drg-1* may be involved in cell growth and differentiation.

In the present study, we demonstrated that overexpression of *Drg-1* induced distinct morphological changes similar to those observed during colonic epithelial cell differentiation. These morphological changes are associated with increased expression of several cell differentiation markers, suggesting that *Drg-1* may function as a promoter of colonic epithelial cell differentiation. Moreover, the expression of *Drg-1* is controlled by several known cell differentiation reagents. These results further support the notion that *Drg-1* may be a key element in colonic epithelial cell differentiation. In addition, we have demonstrated that overexpression of *Drg-1* in metastatic colon cancer cells suppress liver metastases in nude mice but do not alter the ability to form primary tumors. Together, these results suggest that induction of *Drg-1* expression is capable of overriding the existing genetic defects and partially reversing the metastatic phenotype.

Our results indicate that the expression of *Drg-1* is controlled by at least three mechanisms:

(*a*) PPARγ/RXR transcriptional factor pathway. PPARγ is a member of the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid (31). Ligands of PPARγ include polyunsaturated fatty acids such as linoleic, PGJ2, and the synthetic antidiabetic thiazolidinedione drugs, troglitazone and BRL 49653 (32, 33). Although dimerizing with the RXR receptor, PPARγ functions as a transcription factor, controlling differentiation of a variety of cells including adipocytes and colonic epithelial cells (34, 35). Therefore, the finding that the ligands of PPARγ and RXR activate *Drg-1* suggests that *Drg-1* may be a downstream target of the PPARγ/RXR differentiation pathway.

(*b*) DNA methylation pathway. It is well known that methylation of CpG islands in promoter sequences suppresses gene expression. Inhibition of DNA methylation by Aza induces differentiation of many cell types including colon cancer cells (36, 37). The 5′ end of the *Drg-1* cDNA contains multiple CpG sites, which first suggested that *Drg-1* may be controlled by DNA methylation. We have now cloned and sequenced 800 bp of the *Drg-1* promoter region (data not shown). Analysis of this sequence reveals that there are multiple CpG sites, sufficient to comprise a CpG island (17). Additional studies to compare the promoter activity of *Drg-1* with its methylation status will determine the role of DNA methylation in the regulation of *Drg-1* expression. Our data also indicate that the expression of *Drg-1* is only partially regulated by DNA methylation, implying that other mechanisms are involved in the down-regulation of *Drg-1* in metastatic colon cancer cells.

(*c*) Histone deacetylation pathway. Inhibition of histone deacetylase by reagents such as butyrate and trichostatin has been shown to induce differentiation of many different cell types (7, 38). Our data demonstrate that inhibition of histone deacetylase induces the expression of *Drg-1*.

The synergistic effect of Aza and tributyrin on *Drg-1* expression is of interest. The similar effect between an inhibitor of DNA methylation (Aza) and an inhibitor of histone deacetylation (TSA) also resulted in reexpression of genes such as *p16* and *MLH1,* which are silenced in cancers (39). Together, these findings suggest that DNA methylation and histone acetylation, two key processes controlling gene regulation, cell growth, and cell differentiation, may be functionally linked. Because the degree of histone acetylation depends on the balance of acetylation and deacetylation, demethylated DNA may be a prerequisite condition for recruitment of acetyltransferase enzyme and histone acetylation. In this regard, recent studies by two independent groups have reported that MeCP2, a methyl-CpG-binding protein, interacts with histone deacetylase and induces transcriptional silencing by inducing histone deacetylation (40, 41). Additional studies of the regulatory mechanism of *Drg-1* may provide insight about the interaction among transcription factors such as PPARγ as gene-specific regulatory mechanisms, as well as more global regulations such as DNA methylation and histone acetylation (42).

Cytodifferentiation therapies have been used in the treatment of human malignancies for decades (43). The fundamental mechanism of this approach is to "push" poorly differentiated tumor cells back into a genetic pathway of maturation/differentiation and, therefore, to reverse the malignant phenotype of tumor cells. The execution of this therapy, however, is only possible with an understanding of the relevant molecules that control cell differentiation and a realistic approach to manipulate the function of such molecules. Results from the present studies suggest that *Drg-1* may be one of the molecules

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that plays a key role in controlling colonic epithelial cell differentiation. The fact that overexpression of *Drg-1* induced expression of E-cadherin and two other cell differentiation markers, as well as induced morphological changes typical of differentiated cells, strongly suggests that SW620 metastatic colon cancer cells were "pushed" back into the differentiation pathway. Alterations of cell surface molecules, such as E-cadherin and possibly other cell surface molecules, may change the adhesion properties of cancer cells and result in the suppression of their *in vitro* and *in vivo* invasion capabilities (44, 45).

From a clinical point of view, decreased expression of *Drg-1* in colon cancer cells may be used as a potential genetic marker to predict early metastasis. This can be achieved by analyzing the expression of *Drg-1* in primary colon cancer using *in situ* hybridization or immunochemical studies, techniques that allow the identification of *Drg-1* expression in individual colon cancer cells as compared with normal adjacent tissue. Moreover, ligands of PPARγ, RXR, or histone deacetylase inhibitors might be used as pharmacological agents to induce the expression of *Drg-1* and thereby possibly reduce the invasion and metastatic abilities of colon cancer cells. Specifically targeting and manipulating the function of *Drg-1* may offer a novel approach to the differentiation therapy of colon cancer.

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