Pharmacologic unmasking of epigenetically silenced tumor suppressor genes in esophageal squamous cell carcinoma

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Summary

We performed a comprehensive survey of commonly inactivated tumor suppressor genes in esophageal squamous cell carcinoma (ESCC) based on functional reactivation of epigenetically silenced tumor suppressor genes by 5-aza-2′-deoxycytidine and trichostatin A using microarrays containing 12599 genes. Among 58 genes identified by this approach, 44 (76%) harbored dense CpG islands in the promoter regions. Thirteen of twenty-two tested gene promoters were methylated in cell lines, and ten in primary ESCC accompanied by silencing at the mRNA level. Potent growth suppressive activity of three genes including *CRIP-1*, *Apolipoprotein D*, and *Neuromedin U* in ESCC cells was demonstrated by colony focus assays. Pharmacologic reversal of epigenetic silencing is a powerful approach for comprehensive identification of tumor suppressor genes in human cancers.

Introduction

Cancer of the esophagus is the eighth most common malignancy and ranks as the sixth most frequent cause of death worldwide (Pissani et al., 1999). The frequency of different histologic types of esophageal carcinoma varies, but throughout the world, squamous cell carcinoma is the predominant type. There is considerable epidemiological evidence suggesting that alcohol, tobacco, diets deficient in vitamins/protective antioxidants, carcinogens (i.e., frequent consumption of pickled vegetables), and thermal injuries are important in the pathogenesis of esophageal squamous cell carcinoma (ESCC) (Chen et al., 1995; Garidou et al., 1996). Recent advances in molecular biology have revealed common genetic and/or epigenetic alterations of the *p53* and *p16/Rb* tumor suppressor pathways in human ESCC (Xu et al., 2002; Montesano et al., 1996; Mandard et al., 2000). Further identification of molecular targets would enable the prevention, diagnosis, and treatment of ESCC to be approached at the molecular level. However, as in other cancers, a genome-wide comprehensive survey of commonly inactivated tumor suppressor genes (TSGs) in ESCC has remained elusive.

In addition to genetic alterations, alterations in DNA methylation, an epigenetic process present in mammalian cells, are also a hallmark of human cancer (Baylin et al., 2001). The promoter regions of many genes, particularly "housekeeping" genes, are populated by many CpG dinucleotides, which are often underrepresented in the remainder of the genome. These regions have been termed "CpG islands," and, with the exception of genes on the inactive X chromosome and imprinted genes, CpG islands are protected from methylation in normal cells (Bird, 1986; Baylin et al., 2001). This protection is critical since methylation of CpG islands is associated with loss of expression of that particular gene. In carcinogenesis, global hypomethylation (Jones and Laird, 1999; Baylin et al., 2001) is often accompanied by dense hypermethylation of specific promoters (Merlo et al., 1995; Dammann et al., 2000; Yoshikawa et al., 2001; Li et al., 2002). Many studies have demonstrated that the silencing of tumor suppressor genes associated with promoter hypermethylation is a common feature in human cancer, and serves as an alternative mechanism for loss of tumor suppressor gene function. For example, we showed that *p16* hypermethylation is associated with loss of expression and is a common feature

SIGNIFICANCE

Promoter hypermethylation is a common pathway for tumor suppressor gene inactivation. In this study, the authors describe a method to identify novel methylated genes based on pharmacological unmasking of epigenetic silencing in esophageal cancer cell lines. This approach and selection algorithm is robust in identifying a number of novel methylated genes in primary esophageal tumor tissues. These methylated genes provide insight into tumor biology and provide novel diagnostic and therapeutic targets. Three of these genes showed potent tumor suppressive activity when overexpressed in carcinoma cells.

Table 1. Genes upregulated by microarray analysis after treatments

	KYSE30	KYSE410	KYSE520	
1 μM 5Aza-dC	46	15	5	
1 μM 5Aza-dC + 300 nM TSA	57	21	51	
5 μM 5Aza-dC	242	334	149	
Unique gene number	289	363	185	Total unique = 565 genes

of many solid tumor malignancies (Merlo et al., 1995). Hypermethylation is associated with inactivation of the tumor suppressor gene *VHL* and occurs in a subset of clear cell renal cancers without inactivating point mutations (Herman et al., 1994; Meyer et al., 2000), while hypermethylation-associated loss of *p15* expression is a feature of many acute leukemias (Herman et al., 1996). The transcriptional silencing of other tumor suppressor genes, such as the mismatch repair gene *hMLH1* (Kane et al., 1997; Herman et al., 1998), has established hypermethylation as a common mechanism for loss of tumor suppressor function in human cancer. Thus, an increasing number of tumor suppressor genes display both genetic and epigenetic inactivation in human tumors. Because promoter hypermethylation is linked to silencing of gene expression, knowledge of methylation patterns across the genome, sometimes dubbed "the methylome" (Feinberg, 2001), may help to identify key TSGs inactivated during tumor formation.

5-aza-2′-deoxycytidine (5Aza-dC) has been used to unravel epigenetic inactivation. 5Aza-dC is incorporated into genomic DNA where it forms a covalent complex with methyltransferase active sites (Creusot et al., 1982; Wilson et al., 1983; Jones and Taylor, 1980). This suicide inhibition depletes methyltransferase activity resulting in generalized demethylation. However, chromatin is a complex of DNA and histones, and histone acetylation also impairs gene transcription (Pennisi, 1997; Marks et al., 2001); and recently, DNA methylation was demonstrated to help model histone acetylation (Gray and Teh, 2001; Eden et al., 1998). Methyl-CpG binding protein MeCP2 appears to reside in a complex with histone deacetylase activity (Nan et al., 1998), while DNA methyltransferase binds histone deacetylase 2 (HDAC2) and a co-repressor, DMAP1 (Rountree et al., 2000). Thus, densely methylated DNA associates with transcriptionally repressive chromatin characterized by the presence of underacetylated histones. Trichostatin A (TSA), a histone deacetylase inhibitor, reverses formation of transcriptionally repressive chromatin on methylated promoter templates (Yoshida and Horinouchi, 1999). Epigenetic alterations are thus dynamically linked, and synergy between demethylation and histone deacetylase inhibition using TSA was shown to reactivate genes silenced in carcinoma more robustly than 5Aza-dC alone (Cameron et al., 1999; Suzuki et al., 2002).

In this study, we undertook pharmacological unmasking of ESCC carcinoma cells with 5Aza-dC and TSA followed by cRNA microarray analysis to comprehensively identify epigenetically inactivated genes in cancer. This approach identified a large number of genes with dense promoter hypermethylation. Moreover, a more closely examined subset of these genes was frequently inactivated in primary tumors and displayed clear tumor suppressive activity.

Results

Pharmacologic unmasking of transcriptionally repressed genes

We used the demethylating agent 5Aza-dC (1–5 μM) for 3 to 5 days ± the deacetylase inhibitor TSA (300 nM last 24 hr) to reactivate genes epigenetically silenced in three esophageal squamous cell carcinoma (ESCC) cell lines. After treatment, we measured changes in gene expression using microarray chips containing 12599 transcripts (Affymetrix) and confirmed randomly picked genes by reverse transcription-polymerase chain reaction (RT-PCR). As expected, treatment with these agents resulted in upregulation (defined as a 3.0-fold increase) of more than 500 unique genes (Table 1). Almost all of the genes (>80%) were included in the 5 μM 5Aza-dC treatment group, but a few genes were identified at a lower 5Aza-dC concentration or by addition of TSA (see below). We then reasoned that commonly reactivated genes, inactivated in 2 or 3 ESCC cell lines, were more likely to represent frequently inactivated TSGs (120 genes). We further diminished the number of candidate genes by comparing expression patterns (http://cgap.nci.nih.gov) in normal esophagus and carcinoma tissue samples (eliminating those genes not expressed in normal tissue) and excluded unknown genes. Finally, we focused on 58 genes that were commonly upregulated after demethylation treatments (Figure 1; Table 2).

Fifty-three of the fifty-eight genes (91%) harbored CpG sites and forty-four genes (76%) harbored dense CpG islands (GC content > 60% or CpG content > 15%) in the promoter region (Table 2). RT-PCR was performed to confirm upregulation after treatment in 25 randomly selected genes (Table 2) in the three key ESCC cell lines (Figure 2A). All 25 genes demonstrated robust reexpression after 5 μM demethylation treatment. Genes such as *cytokine-like factor-1* (*CLF-1*) and *Hep27* demonstrated synergistic reactivation with 1 μM 5Aza-dC + 300 nM TSA as compared to TSA or 5Aza-dC alone in several cancer cells (Figure 2B).

Expression and promoter hypermethylation in ESCC cell lines

We then investigated a subset of those 58 genes for silencing or downregulation in an additional 12 ESCC cell lines. TE4 and TE5 retained expression of *Apolipoprotein D* (*Apo D*), while all the other remaining cell lines were downregulated at the mRNA level when compared to normal esophagus (Figure 2C). *Neuromedin U* (*NU*) was completely silenced in six cell lines, and other cell lines also showed a marked reduction in expression as compared with normal esophagus (Figure 2C). *Cystein rich intestinal protein 1* (*CRIP-1*) was completely silenced in 8 out of 15 ESCC cell lines (53%), while normal esophageal tissues showed abundant expression of *CRIP-1* (Figure 2C). Several other selected candidate genes (*CLF-1*, *Swiprosin-2*, *cellular*

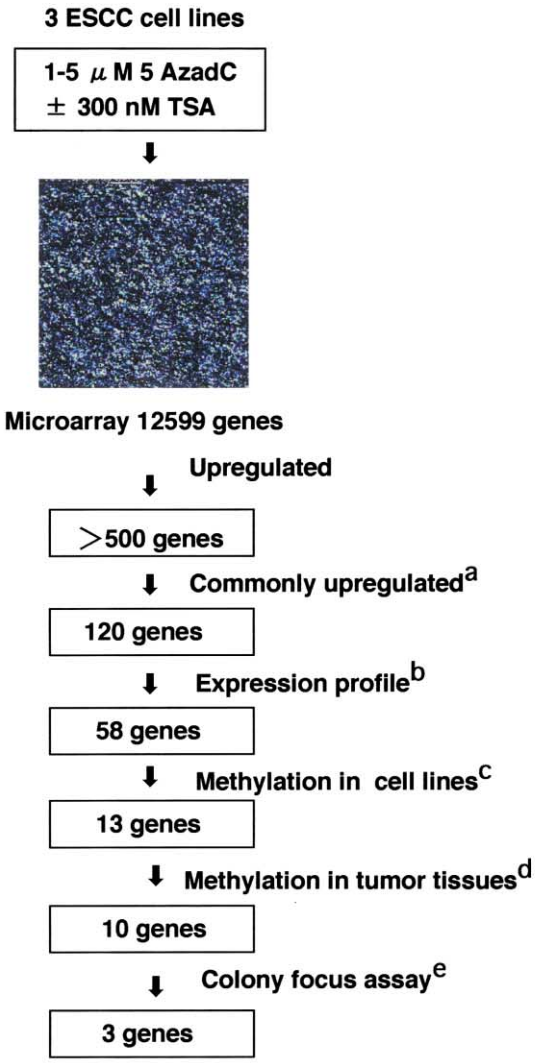

Figure 1. Flowchart for selection of candidate TSGs

We used key three ESCC cell lines to screen for candidate TSGs after 1 to 5 μM 5Aza-dC ± 300 nM TSA treatments followed by cRNA hybridization to a 12,599-oligonucleotide microarray. We obtained over 500 unique genes, which showed ≥3-fold increase after treatments (Table 1). We diminished the number of candidates by selecting genes commonly upregulated (120) and further removed several genes by expression profiling and elimination of unknown genes (58). We selected 22 genes to test for promoter hypermethylation by direct sequence or MSP in ESCC cell lines and 13 were confirmed. Ten of these thirteen genes were found to be methylated in ESCC tissues. Three of these genes were tested and found to possess growth suppression by a colony focus assay.

a. **reexpressed in more than two ESCC cell lines.**

b. **excluded if there was no evidence of expression in normal esophagus.**

c. **22 genes with CpG rich promoter selected to test for methylation in cell lines.**

d. **10/13 genes confirmed to harbor promoter methylation in primary tumor tissues.**

e. **3/10 genes selected and confirmed to possess tumor suppressive activity.**

retinol binding protein [*CRBP*], *Metallothionein 1G*, *Keratin 14*, *Crystallin α2*, and *IL-1 receptor 2*) demonstrated considerable downregulation in ESCC cell lines (Figure 2C).

In order to confirm promoter hypermethylation in reactivated genes, we investigated the promoter region of 22 genes (21 genes harbored dense CpG islands plus *Apo D*) using bisulfite sequencing (Table 2). We noted that most candidate genes (*Tbc1d1*, *lysosomal neuraminidase precursor*, *Apolipoprotein J* [*Apo J*], *KLF6*, *putative cyclin G1 interacting protein*, and *XAP-5*) with moderate basal expression before treatment by RT-PCR were invariably unmethylated (*lysosomal neuraminidase precursor*, *putative cyclin G1 interacting protein*). On the other hand, 13 genes (*Alpha-tubulin*, *Swiprosin-2*, *Insulin-like growth factor binding protein 2* [*IGFBP2*], *CRBP*, *Apo D*, *NU*, *Claudin-3*, *Uncoupling protein-2* [*UCP-2*], *CRIP-1*, *MT 1G*, *Apolipoprotein CI* [*Apo CI*], *CLF-1*, *Transglutaminase-2*) retained high cytosine content in their CpG islands or CpG sites deemed critical for transcription (Apo D) after bisulfite treatment, indicating heavy cytosine methylation (Figures 3A and 3B). In all of these genes except *Swiprosin-2*, methylation status correlated tightly with

Table 2. Identification of candidates of tumor suppressor genes in esophageal carcinoma

Gene bank	Gene Name	Chr.	ESCC cell line CpG[a]	Unmasking[b]	expression[c]	ESCC tissue CpG[d]	expression[e]	CpG[f]	known or proposed function
M21302	Small proline rich protein (sprII)	1	(−)	*	*	*			UV-induced gene
AI813532	TNF receptor 1B	1p36	(++)	*	*	*			cytokine receptor
AI885852	Histone 2A.2	1q21	(++)	*	*	*			nucleosome protein
J05581	MUC1	1q21	(++)	Yes	Yes	U			tumor antigen
L13463	Regulator of G protein signaling 2 (RGS2)	1q31	(++)	*	*	U			G-protein signal
AF010309	Pig3	2p23	(+)	*	*	*			p53-induced gene
X06956	alpha-tubulin (b alpha 1)	2p36	(++)	*	*	M	5/5 (100%)	0/10 (0%)	microtubule
X59770	IL-1 R2	2q12	(−)	Yes	Yes	*			cytokine receptor
AB011103	KIF5C	2q23	(?)	Yes	*	*			cytoplasmic transport
X63368	HSP40 homolog (HSJ1)	2q32	(+)	*	*	*			stress-induced gene
W27472	Swiprosin-2	2q36	(++)	Yes	Yes	M	5/5 (100%)	10/10 (100%)	growth arrest?
S37730	IGFBP2	2q36	(++)	*	*	M	1/5 (20%)	0/10 (0%)	IGF signal
M11433	Cellular retinol-binding protein (CRBP)	3q21	(++)	*	Yes	M	5/5 (100%)	6/10 (60%)	retinol-binding protein
J02611	Apolipoprotein D	3q26	(+)	Yes	Yes	M	3/5 (60%)	8/10 (80%)	growth arrest
AB029031	Tbc1d1	4p14	(++)	Yes	No	*			oncosis
AF084481	Transmembrane protein (WFS1)	4p16	(++)	*	*	*			Wolfram syndrome
X76029	Neuromedin U	4q12	(++)	Yes	Yes	M	3/5 (60%)	3/10 (30%)	G-protein receptor ligand
X57522	TAP1	6p21	(++)	*	No	U			tumor antigen processing
AF040958	Lysosomal neuraminidase precursor	6p21	(++)	Yes	No	U			enzyme
X70683	Sry	6p22	(++)	*	*	*			sex-determining gene
AB000714	Claudin-3	7q11	(++)	*	*	M	4/5 (80%)	6/10 (60%)	tight junction
M25915	Apolipoprotein J	8p21	(++)	Yes	No	*			apoptosis
M22488	Bone morphogenic protein 1 (BMP-1)	8p21	(++)	*	*	U			TGF-beta superfamily
AF001461	KLF6 (Zf9)	10p15	(++)	Yes	No	*			tumor suppressor gene
M13755	Interferon-induced 17-kDa/15-kDa	11p36	(++)	*	*	*			Interferon-induced gene
U94592	Uncoupling protein 2	11q13	(++)	Yes	Yes	M	1/5 (20%)	1/10 (10%)	apoptosis
W68521	Cystatin E/M (6)	11q13	(++)	*	*	*			protease inhibitor
AF001436	Cdc42 effector protein 2 (CEP2)	11q13	(++)	*	*	*			motility inhibition
X15675	pTR7	11q13	(++)	*	*	*			repetitive sequence gene
AL038340	Crystallin alpha B	11q22	(++)	Yes	Yes	*			stress-induced gene
AJ001684	NKG2C	12p13	(−)	*	*	*			NK cell recognition
AJ001685	NKG2E	12p13	(−)	Yes	*	*			NK cell recognition
M20681	GLUT3	12p13	(+)	*	*	*			glucose transport
U31875	Hep27	14q11	(+)	Yes	*	*			growh arrest?
X81637	Clathrin light chain b	14q21	(++)	*	*	*			membrane recycling
X67325	Interferon stimulatory gene 12 (p27)	14q32	(+)	Yes	*	*			IFN-induced gene
AI017574	Cystein rich protein with LIM (CRIP1)	14q32	(++)	Yes	Yes	M	1/5 (20%)	2/10 (20%)	LIM protein
AI985272	Neuromedin B	15q22	(++)	*	*	*			G-protein receptor ligand
NM_002201	HEM45 (interferon stimulatory gene 20)	15q26	(++)	Yes	*	*			IFN-induced gene
J03910	Metallothionein-1G	16q	(++)	*	Yes	M	5/5 (100%)	4/10 (40%)	apoptosis-related gene
NM_004165	Rad	16q22	(++)	Yes	Yes	U			motility inhibition
AA976838	Apolipoprotein CI	16q22	(++)	*	*	M	3/5 (60%)	8/10 (80%)	growth promotion?
J00124	Keratin 14	17q12	(+)	Yes	*	*			intermediate filament
U20982	IGFBP4	17q12	(++)	Yes	*	*			IGF signal
Z19574	Cytokeratin 17	17q12	(++)	*	*	*			intermediate filament
AF059293	Cytokine-like factor-1 (CLF-1)	19p13	(++)	Yes	Yes	M	3/5 (60%)	5/10 (50%)	cytokine receptor homolog
D12620	Cytochrome P-450LTBV	19p13	(+)	*	*	*			electron transmission
AF061812	Keratin 16	19q12	(++)	*	*	*			intermediate filament
U76702	Follistatin-related protein FLRG	19q13	(++)	Yes	*	U			TGF-beta related gene
AF044968	Nectin 2	19q13	(++)	*	*	*			tight junction
AA1524 06	"Cytochrome oxidase c, type VIIa"	19q13	(++)	*	*	*			electron transmission
M37457	"Na+, K+-ATPase catalytic subunit, alpha-III"	19q13	(++)	*	*	*			ion transport
U61837	Putative cyclin G1 interacting protein	20p13	(++)	Yes	No	U			cell cycle (G2/M)
M55153	Transglutaminase 2 (tissue glutaminase)	20q11	(++)	Yes	Yes	M	3/5 (60%)	4/10 (40%)	apoptosis
W72816	Cleavage stimulation factor	20q13	(++)	*	*	U			BRCA1-related gene
X75315	Seb4B (RPM type RNA binding protein)	20q13	(++)	*	*	*			RNA binding protein
X91817	Transketorase 2	Xq28	(+)	*	*	*			metabolite cofactor
AD001530	XAP-5	Xq28	(++)	Yes	No	*			repetitive sequence gene

[a] CpG sites. (++) dense CpG islands, (+) CpG sites, (−) no CpG sites in the promoter region.
[b] RT-PCR results of the experiment after treatments. *: not done. Yes: confirmed silencing in three key cells and reactivation after treatments.
[c] Gene expression by RT-PCR in extended panel of ESCC cells. *: not done. Yes: silencing confirmed in several cell lines. No: ubiquitously expressed in all investigated cell lines.
[d] CpG status of ESCC cells. M: methylated, U: unmethylated, *: not done.
[e] Gene expression by RT-PCR in ESCC tissues. Silenced tumors/tumors tested (% silenced).
[f] Promoter methylation by direct sequence in ESCC tissues. Methylated tumors/tumors tested (% in methylation).

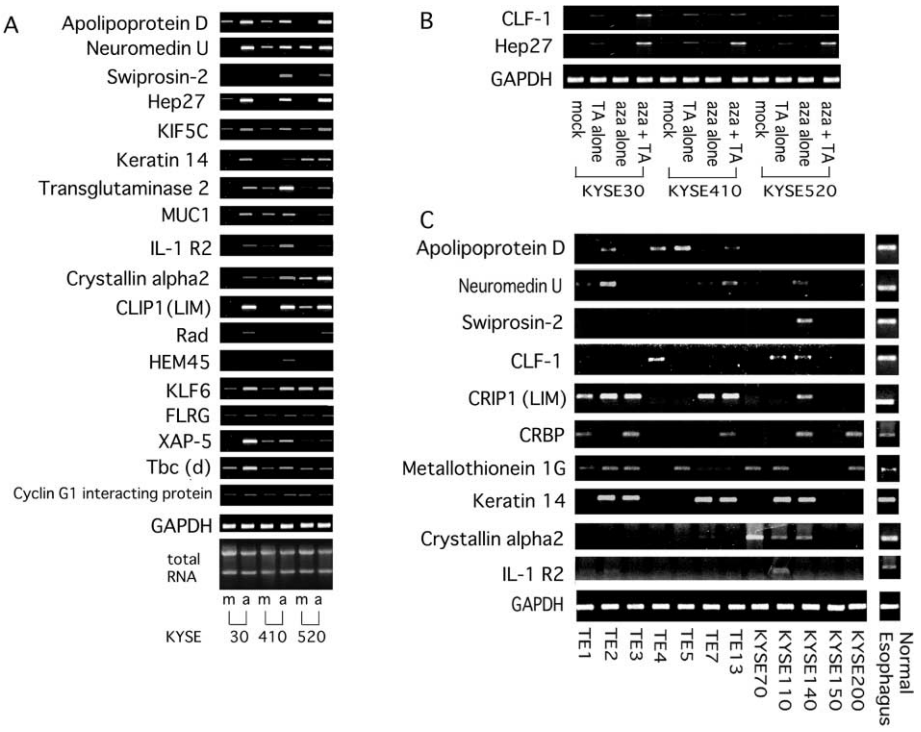

Figure 2. Genes silenced in carcinoma cell lines (KYSE30, KYSE410, and KYSE520) and reactivated by 5Aza-dC and/or TSA treatments

A: Five and/or 1 μM 5Aza-dC treatment reactivated genes identified by microarray hybridization and selected by the algorithm in Figure 1. *GAPDH* expression was used for equal loading into each lane. a: 5Aza-dC in solution of acetic acid/PBS. m: mock including the same volume of acetic acid.

B: One μM 5Aza-dC and 300 nM TSA treatment showed synergistic reactivation of several genes (*CLF-1* and *Hep27*). Treatment with both showed more potent induction than 5Aza-dC or TSA alone. TA: Trichostatin A aza: 5Aza-dC.

C: Gene expression of *Apo D* and other genes listed on the left in ESCC cell lines. ESCC cell lines showed marked reduction in expression of these genes compared to robust expression in normal esophagus (right). *GAPDH* expression is shown as a loading control at the bottom.

expression status. For example, KYSE30 was silenced for *NU* expression and harbored dense methylation of the promoter, while KYSE410 and KYSE520 both expressed *NU* and were free of promoter methylation (Figure 3A). For *Apo D*, all ESCC cell lines except TE5 demonstrated methylation of the promoter, while TE5, which expressed *Apo D* abundantly, did not harbor any methylation (Figure 3A). TE2, TE4, and TE13 showed weak expression of *Apo D* mRNA (Figure 2C) and approximately 50% of the cells harbored unmethylated alleles by direct sequence of bisulfite-treated DNA (data not shown).

Expression and promoter hypermethylation in primary ESCC tumors

We then investigated these 13 methylated genes for promoter methylation in ESCC tissues by MSP or direct sequence analysis (Figure 3C). Ten of the thirteen genes harbored tumor-specific promoter methylation (Figure 4A). The frequency of tumor methylation ranged from 10% (*UCP-2*) to 80% (*Apo C1*). Apo D was methylated in almost all primary ESCC tissues (80%), and showed low-level methylation in some normal tissues by MSP (Figure 4B). *Swiprosin-2* and *Alpha-tubulin* showed methylation in normal esophageal mucosa specimens, suggesting tissue-specific, but not tumor-specific, hypermethylation (data not shown). None of the 10 primary tumors tested harbored *IGFBP2* methylation (data not shown).

We then investigated expression of these 13 genes in five primary ESCC tissues and found them to be reduced in expression in the primary cancers compared to the corresponding normal tissues (Table 2 and Figure 4C). *NU* and *Apo D* were markedly repressed at the mRNA level in several primary tumors as compared to the corresponding normal tissues (Figure 4C). Every gene (except *Swisprosin-2*) methylated in primary tumors demonstrated a marked decrease in expression at the RNA level. For *Swisprosin-2*, methylation did not correlate with decreased expression in primary tumors or cell lines (Table 2). A few tumors did not harbor methylation, but still demonstrated occasional downregulation of a particular gene (Table 2), presumably due to other mechanisms.

Tumor suppressor activity

We then focused on three methylated genes and their ability to function as suppressors of tumor growth (CRIP-1, Apo D, and NU) in KYSE30, which was silenced for all three genes. We tested these three genes by a colony focus assay using G418 selection after transfection with each gene or control vector (pcDNA3). STAT3 is an oncogenic protein and STAT3C is a constitutive active form (Bromberg et al., 1999). STAT3 expression did not change after 5Aza-dC treatments, and thus it was used as a negative control of tumor suppressive activity. In the case of NU, we used the actual protein at a concentration of 100 μM. All three genes demonstrated potent tumor-suppressing activity with a marked reduction of colony forming ability after transfection (Apo D: 61.3% ± 15.0%, CRIP-1: 18.3% ± 15.7%) or addition of protein (NU: 33.6% ± 12.3%) in three independent experiments (Figures 5A–5C). To further confirm a role for CRIP-1 in expression, we transiently transfected IRES-*CRIP-1* into KYSE30 cells. Cells expressing high GFP protein (and thus CRIP-1 simultaneously) showed classic morphological changes of apoptosis including rounding, apoptotic body appearance, and nuclear shrinkage. A TUNEL assay (Figure 5D) confirmed DNA fragmentation in CRIP-1-expressing cells.

Discussion

Pharmacologic reversal of epigenetic silencing uncovered a myriad of transcriptionally repressed genes in ESCC. We identi-

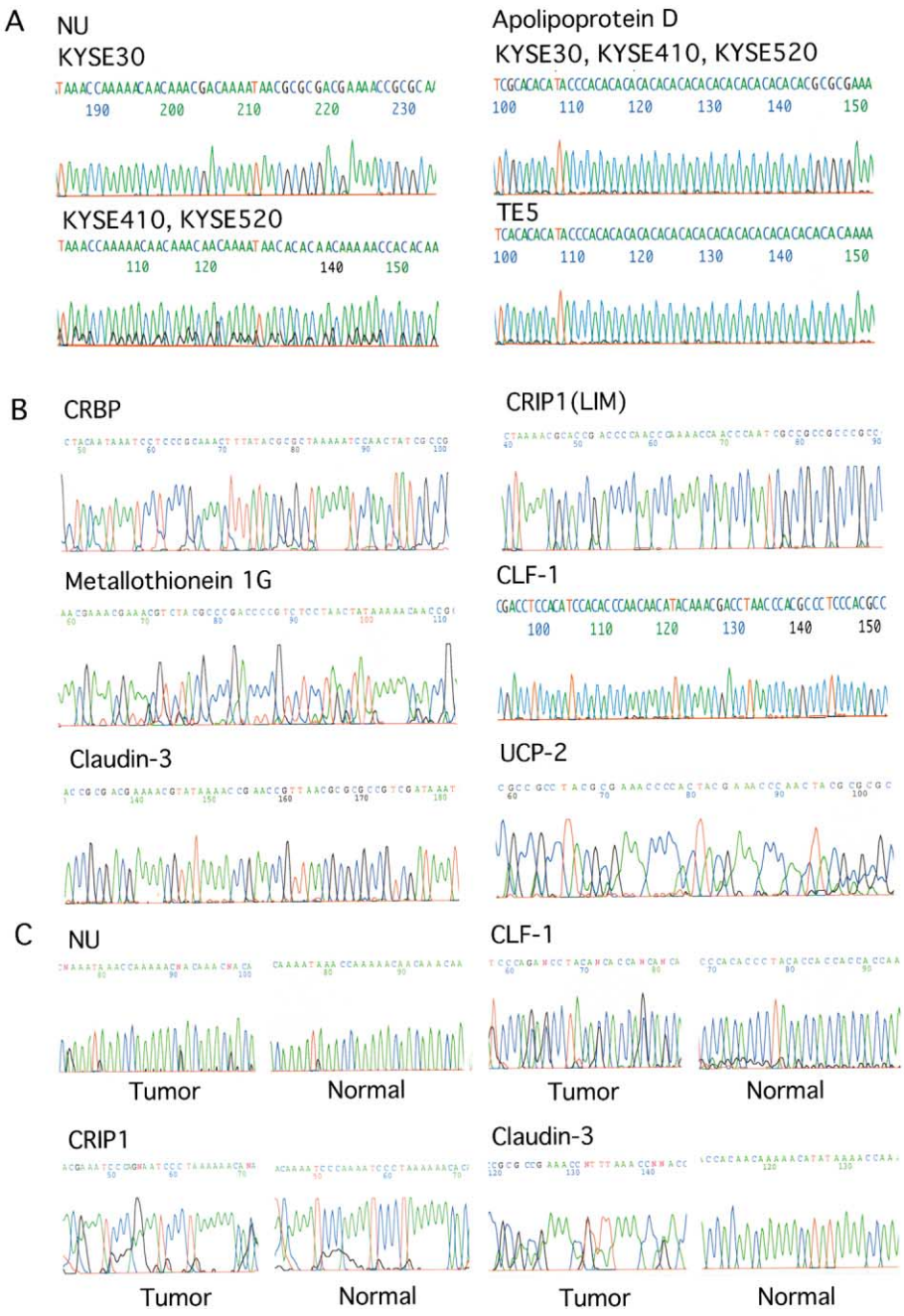

Figure 3. Promoter methylation of representative candidate suppressor genes

A: Direct sequence of *NU* and *Apo D* promoters. All guanines present after sequencing are derived from methyl cytosines on the complementary strand. In cell lines that showed marked reduction of expression, expression levels correlated tightly with methylation status.

B: Bisulfite direct sequencing of the gene promoters in representative ESCC cell lines. *CRBP* (KYSE30), *CRIP-1* (KYSE410), *MT1G* (KYSE30), *CLF-1* (KYSE30), *Claudin-3* (KYSE30), and *UCP-2* (KYSE30).

C: Representative cases of methylation for *NU*, *CLF-1*, *CRIP-1*, and *Claudin-3* in primary ESCC as compared to normal esophageal tissues. Normal tissues were obtained from normal distal mucosa in patients with ESCC.

fied several unknown TSG candidates, using a cutoff of a 3-fold increase by microarray analysis and an intuitive algorithm. The majority of genes were identified by high dose 5Aza-dC treatment, but a subset of genes were reactivated by synergistic treatment with low dose 5Aza-dC and TSA as demonstrated previously in colon cancer cell lines (Cameron et al., 1999; Suzuki et al., 2002). This survey may represent a minimal number of upregulated genes since reversal of epigenetic silencing is likely to occur in a subset of cells with variable reexpression (Cameron et al., 1999). A more complex approach involving selective cloning identified many methylated targets in colorectal cancer and could be used to identify more subtle targets (Suzuki et al., 2002). Our use of high dose 5Aza-dC likely induced reexpression in a higher number of cells, facilitating our direct hybridization approaches. Multiple approaches to reverse epigenetic silencing are likely to yield the most comprehensive gene surveys. It is likely that other algorithms and better demethylation or HDAC inhibition could also improve the yield by further unmasking of methylated targets.

NU is proposed to be involved in normal esophageal mucosa integrity, and NU as well as the recently identified cognate receptor (Hedrick et al., 2000) are abundantly expressed in normal esophageal mucosa (Hedrick et al., 2000; Lynch et al., 2000) (Figure 4C). The *NU* receptor (*FM3*) is a G protein-coupled receptor that can signal through PI3 kinase γ, recently confirmed to block growth of human colon cancer cells (Sasaki et al., 2000).

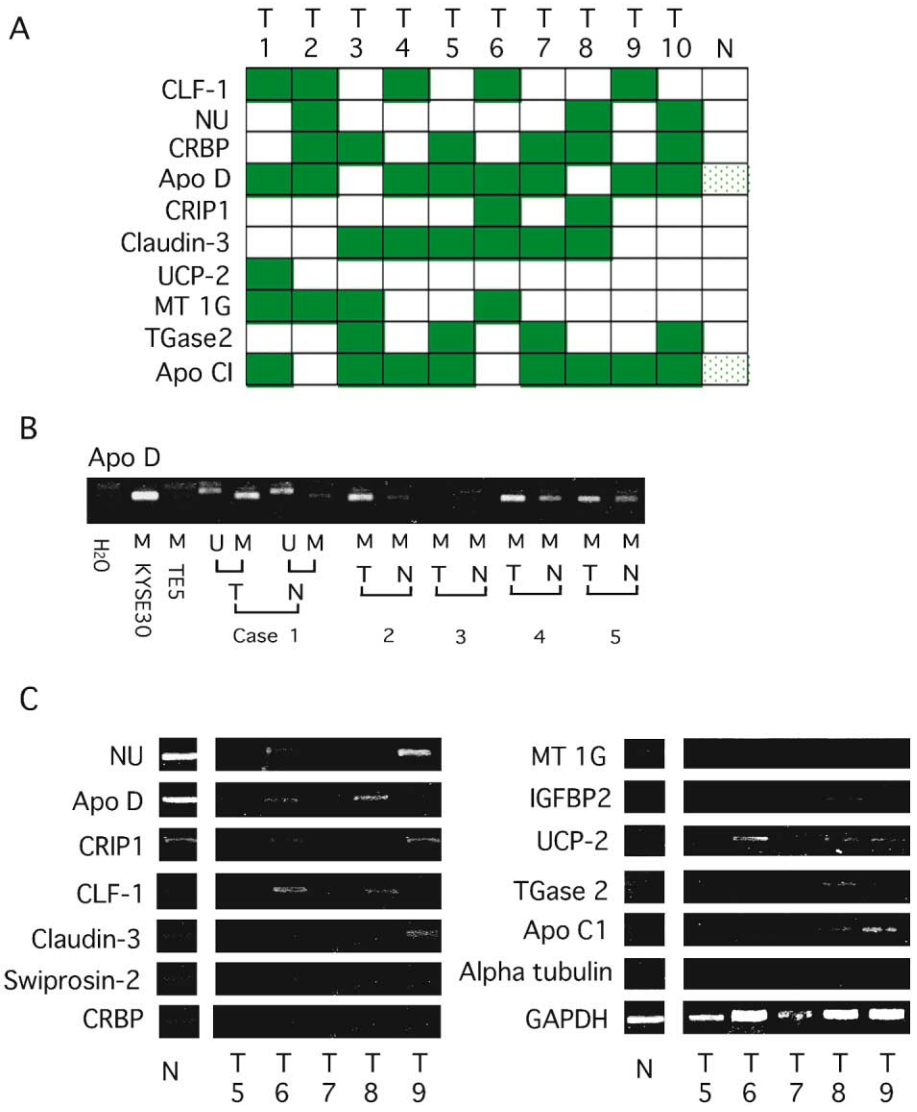

Figure 4. Methylation and expression in primary tumors

A: Methylation in primary tumors for 13 tested genes that showed methylation in ESCC cell lines. Green boxes indicate methylation of the promoter region. Light speckled green indicates partial (low-level) methylation by direct sequencing. T: Tumor tissues, N: normal esophageal mucosa.

B: MSP analysis of ESCC tissue samples for *Apo D*. Representative cases of tumor (T) and corresponding normal esophageal mucosa (N) in primary tumors. Low-level methylation in some distal normal tissues is seen.

C: Representative gene expression in ESCC tumor tissues by RT-PCR. *NU* and *Apo D* in tumors (T) showed marked reduction in expression as compared to normal mucosa (N). The low-level or minimal expression in tumors is probably due to normal cell contamination in the primary tissue samples. *GAPDH* expression is shown as a loading control.

Moreover, in our present study, the list of candidate TSGs includes *Neuromedin B* and *RSG2*, which are thought to be involved on the same pathway.

CRIP-1 has a LIM domain (Tsui et al., 1994) recently confirmed to be involved in carcinogenesis (e.g., *Lmo2* and *Lmo4*). *Lmo2* is a transcriptional factor proposed to play an oncogenic role in T cell leukemogenesis (Grutz et al., 1998), perhaps by enhancing angiogenesis (Yamada et al., 2002). *Lmo4* is overexpressed in breast cancer cells and binds with BRCA1, resulting in suppression of BRCA1 transcriptional activity (Visvander et al., 2001; Sum et al., 2002). Paxillin is a focal adhesion-associated adaptor protein with multiple LIM domains involved in cell spreading and motility (Schaller, 2001). Paxillin LIM binds with α-tubulin (Herreros et al., 2000), a known partner for other critical tumor suppressors including APC and Fhit. Our identified LIM gene (CRIP-1) is a very small molecule (open reading frame is 273 bp) that could modulate other LIM proteins in a dominant-negative manner and potentially modulate or affect many pathways in carcinogenesis. Moreover, our yeast two-hybrid screen (D.S. and K.Y., unpublished data) identified Ubc13 as a binding partner, implicating CRIP-1 in NF-kappa B and JNK pathways critical for apoptosis (Wang et al., 2001).

Apo D is well known to be associated with cell growth arrest (Do Carmo et al., 2002), but its underlying mechanism is unknown. Other apolipoproteins (*Apo CI* and *Apo J*) are also included in our list of 58 genes. *Apo J* was also demonstrated to possess a potent ability to induce cell death (Han et al., 2001). Recently, *Apo D* was demonstrated to bind to a cytokine type receptor that mediates MAP kinase signaling (Liu et al., 2001). This cytokine signaling pathway may also be used by recognized tumor suppressors like *SOCS-1* (Yoshikawa et al., 2001), and our study suggests that the *IL-1 receptor antagonist* (*IL-1 R2*) (Colotta et al., 1993) and *CLF-1* (Elson et al., 1998) may also be involved.

The chromosomal localization of the 58 candidate genes is listed in Table 2. We found that many TSGs candidates were clustered in specific chromosomal regions (Table 2), suggesting the presence of methylated chromosomal regions with highly dense methylation and reduced gene expression in tumors that can be unmasked after pharmacological demethylation treat-

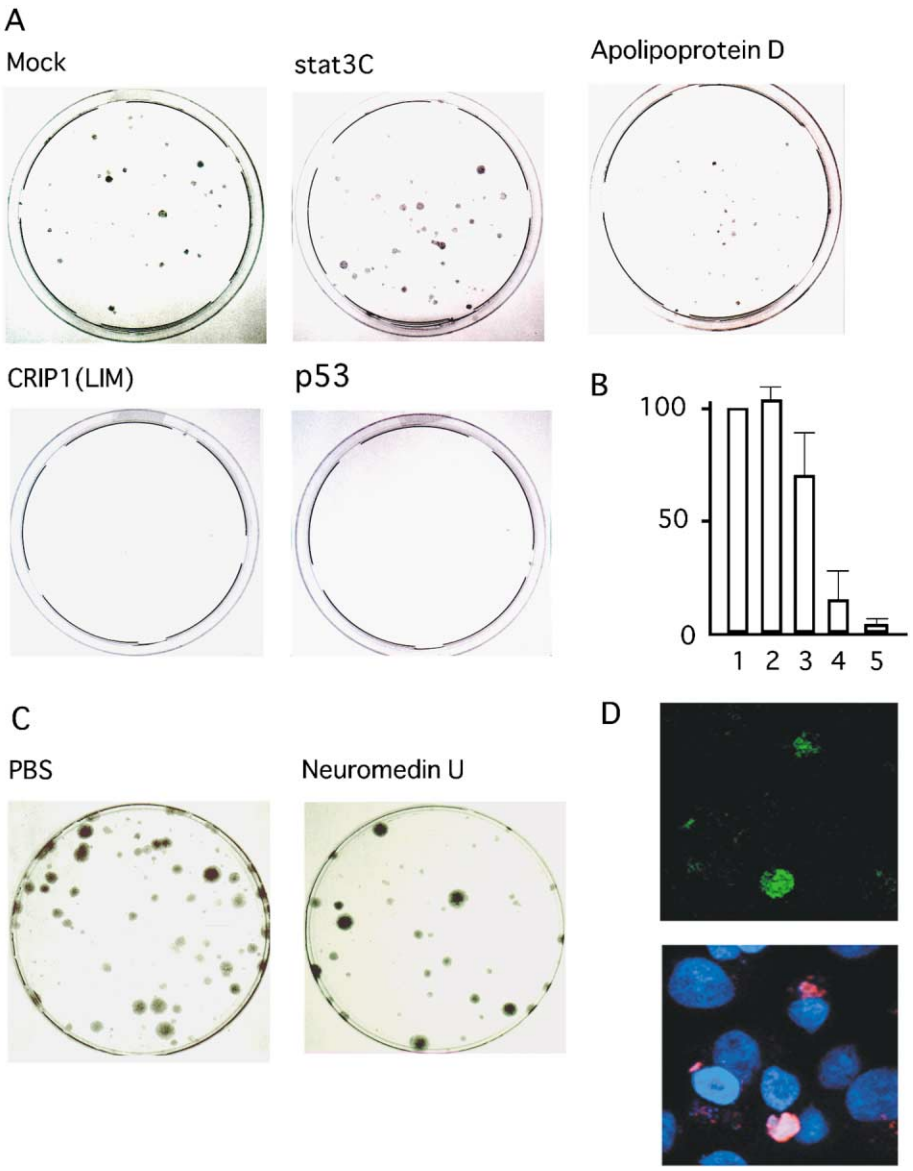

Figure 5. Colony focus assay in KYSE30

All results are an average of three independent experiments.

A: Colony formation after 2 week selection of G418 with pcDNA3 (mock), pcDNA3-*Apo D* (Apo D), pcDNA3-*CRIP-1* (CRIP-1), pcDNA3-*p53* (p53), and pcDNA3-*stat3C* (stat3C).

B: Colony formation efficiency of each gene of stat3C (2), Apo D (3), CRIP-1 (4), and p53 (5) as compared to mock (1). Mock colony number is arbitrarily set at 100% colony formation with Apo D at 61% and 18% for CRIP-1.

C: Colony formation after 2 week selection with G418 using pcDNA3 (mock) with PBS or NU (final concentration, 100 μM). Colony formation efficiency is markedly reduced after NU treatment (43%).

D: Upper box indicates concomitant IRES-GFP and *CRIP-1* expression. These cells showed morphological evidence of apoptosis with rounding, apoptotic body appearance, and nuclear shrinkage. The lower box shows the merged image of rhodamine-TUNEL and Hoechst staining of the nucleus. Only *CRIP-1*-transfected cells demonstrated apoptosis (red color), while cells transformed with IRES alone showed no alterations (data not shown).

ment. *Apo D*, *NU*, and *CRIP-1* are localized at 3q26, 4q12, and 14q24, respectively. All of these loci have been shown to harbor chromosomal deletions or LOH in various cancer (Kruzelock et al., 1997; Burger et al., 1998; Wo et al., 2000; Simon et al., 1995; Herbers et al., 1997) and 3q26 has been reported as a fragile site (Murano et al., 1989). We identified ten genes methylated in a tumor-specific pattern, but, whether methylated or not, all of these genes remain candidates in TSG pathways. *MUC2* was identified in our survey and knockout mice, for example, were recently shown to be predisposed to tumor formation (Velcich et al., 2002). It is likely that many genes in our survey represent TSGs candidates that have not been previously tested for genetic or epigenetic inactivation in cancers.

We have identified candidate genes and pathways epigenetically regulated in ESCC using functional reversal of methylation and deacetylation followed by hybridization on microarrays. This approach is rapid, robust, and can be easily repeated in other cancer cell lines to comprehensively search for epigenetically silenced suppressor genes. Complementary genomic array approaches that search for methylated CpG islands can be compared to functional reactivation surveys (Gitan et al., 2002; Adorjan et al., 2002; Shi et al., 2002). Genes that harbor CpG islands in the promoter regions and methylation in tumor tissue are likely to be bonafide TSGs (Table 2). Our approach thus yielded three new TSG's and many more remain to be explored. It is clear, however, that not all genes identified by this approach are upregulated due to promoter demethylation. Such genes still provide important insights into biologic mechanisms elucidated by activating gene promoters upstream in a given pathway.

The use of MSP allows rapid detection of the frequency of inactivation in primary tumors by methylation, while functional analysis allows rapid assessment of suppressor activity. These identified genes will further contribute to our understanding of the biologic progression of ESCC and thus represent important therapeutic targets. Moreover, the rapid development of MSP

assays after gene identification allows robust analysis of primary tumors and other clinical samples for implementation of promising molecular detection approaches (Esteller et al., 1999; Kawakami et al., 2000; Sanchez-Cespedes et al., 2000; Jeronimo et al., 2001; Usadel et al., 2002).

Experimental procedures

Cell lines and tissue samples

ESCC lines TE1, TE2, TE3, TE4, TE5, TE7, TE13, KYSE30, KYSE70, KYSE110, KYSE140, KYSE150, KYSE200, KYSE410, and KYSE520 were obtained from the Cell Response Center for Biomedical Research Institute of Department, Aging and Cancer, Tohoku University (TE series) and kindly provided by Dr. Shimada in the Department of Surgery, Kyoto University (KYSE series). Cell lines were grown in RPMI1640 supplemented with 10% fetal bovine serum for isolation of DNA and RNA. Primary ESCC tumors and corresponding adjacent normal tissues were obtained from the Gastroenterology Division, the Department of Medicine, and the University of Maryland School of Medicine.

5Aza-dC and TSA treatment of cells

Cells were split to low density (5×10^5 per T-25 flask) 12–24 hr before treatment. Cells were then treated for 3, 4, or 5 days with 1 or 5 μM 5Aza-dC (Sigma) from 100 mM 50% acetic acid dissolved stock or were mock-treated with the same volume of phosphate-buffered saline (PBS) including the same acetic acid. Following an initial incubation (48 hr) of 5Aza-dC, a final concentration of 300 nM TSA (Sigma) was added to the media from a 5 mM ethanol dissolved stock or cells were mock-treated with an identical volume of ethanol.

Microarray and RT-PCR analysis

We performed oligonucleotide microarray analysis on the GeneChip Human Genome U95Av2 Array (Affymetrix) containing 12599 genes as per the manufacturer's instruction, and identified genes upregulated by pharmacologic treatment according to the manufacturer's algorithm. We isolated RNA using Trizol (Invitrogen) and reverse-transcribed total RNA (8 μg) with M-MLV (Invitrogen) and one hundredth of the DNA was used as a template for PCR. RT-PCR was performed at 24 to 30 cycles: 95°C for 1 min, 54°C or 56°C for 1 min, and 72°C for 1 min. As a control, no amplification of PCR products was seen without reverse transcription (-RT).

Preliminary analysis with a 2-fold increase as a cutoff line yielded many additional genes. However, few of these were upregulated in more than one cell line and several genes like *BIN1*, *BRCA-associated protein 1* (*BAP-1*), and *MAP kinase 8 interacting protein 3* (JNK proteins scaffolding protein) harbored no methylation in the promoter region. Such genes could be on a pathway regulated by more upstream genes epigenetically regulated in our cell lines. A cutoff at a 3-fold increase routinely identified epigenetically silenced genes with promoter hypermethylation and was thus used in our extended study.

Sequencing analysis

We extracted genomic DNA from Trizol (Invitrogen) and carried out bisulfite modification of genomic DNA as described (Merlo et al., 1995). Bisulfite-treated DNA was amplified for the 5′ region that included the ATG start sites or proposed transcriptional start sites (200~500 bp) using primer sets made for the 25 genes (22 listed here and MAPK8IP3, BIN1, and BAP-1). All the PCR products were gel-extracted (Qiagen) and applied for the Applied Biosystems 3700 DNA analyzer using BD terminator dye (Applied Biosystems) and nested primers or forward primers.

Methylation-specific PCR

Bisulfite-treated DNA was amplified with either a methylation-specific or unmethylation-specific primer set for *Apo D* at 33 cycles: 96°C for 30 s, 59°C (methylated) and 55°C (unmethylated) for 30 s, and 72°C for 30 s. The methylation-specific primer sequences for *Apo D* were designed using 5′-CACACGCGCGAAAACAATAT-3′ as the forward primer and 5′-TATGTATG TTACGTTCGTCG-3′ as the reverse primer. The unmethylated-specific primer sequences were 5′-CACACAAAAACAATATCTCATTTCT-3′ and 5′-TTTTTTATGTATGTTATGTTTGTTG-3′.

Construction of human expression vectors

A full-length *CRIP-1* cDNA was isolated from TE2 using PCR with the primer sets 5′-CAGAAGCTTCCACCATGCCCAAGTGTCCCAAGTGC-3′ and 5′-CTCTCGGTGTGAAAGTTCATTAGATCTGAC-3′. The PCR product with HindIII and XbaI was cut out from a gel and ligated to HindIII-XbaI digested pcDNA3 (Invitrogen), which harbors a CMV promoter. One clone, pcDNA3-*CRIP-1*, harbored an insertion with a sense orientation and a correct sequence. *p53* was amplified as a template of pRCC-*p53* (Osada et al., 1998), subcloned into pcDNA3 in HindIII and Xba sites, and then sequenced. Dr. Gregor, Department of Molecular Biology at The Scripps Research Institute kindly provided *Apo D* cDNA inserted into pcDNA3. Dr. Darnell from the Department of Oncology of the University of Rockefeller kindly provided *STAT3C* cDNA inserted into pcDNA3 (Bromberg et al., 1999).

Transfection and colony formation assay

Colony formation assays were performed in monolayer culture (Yoshikawa et al., 2001). Cells were plated at 2×10^4 per well using 6-well plates, and transfected with either pcDNA3-*p53*, pcDNA3-*STAT3C*, pcDNA3-*CRIP-1*, pcDNA3-*Apo D*, or no insert of pcDNA3-mock (1 μg) using Lipofectamine Plus reagents (Invitrogen) according to the manufacturer's protocol. The cells were then detached and plated on 100 mm tissue culture dishes at 24 to 48 hr post-transfection, and simultaneously harvested at 48 hr after transfection to confirm their expression at the mRNA level (RT-PCR) for *Apo D*. Cells transfected with *CRIP-1* died rapidly by 48 hr after transfection, and thus mRNA levels were evaluated 24 hr after transfection. *Apo D* and *CRIP-1* RNA levels in the cells were between 1.5-fold and 2.0-fold higher than basal expression of unmethylated cell lines. Cells were selected with G418 (1 mg/ml), and colonies were counted 2 weeks after transfection. For treatment with NU (Phoenix Pharmaceutical), the colony focus assay included 100 μM NU in control PBS or the medium.

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