Oncogene (2003) 22, 4118–4127

www.nature.com/onc

Epigenetic silencing of multiple interferon pathway genes after cellular immortalization

Olga I Kulaeva[1,4], Sorin Draghici[2,4], Lin Tang[1], Janice M Kraniak[1], Susan J Land[3] and Michael A Tainsky*[,1,3]

[1]*Barbara Ann Karmanos Cancer Institute, and Department of Pathology, Wayne State University School of Medicine, 110 Warren Ave., Detroit, MI 48201, USA;* [2]*Wayne State University, 5143 Cass Ave., Rm. 408, Detroit, MI, 48202, USA;* [3]*Molecular Medicine and Genetics, Wayne State University, 5107 Biological Sciences Bldg., Detroit, MI 48202, USA*

Abrogating cellular senescence is a necessary step in the formation of a cancer cell. Promoter hypermethylation is an epigenetic mechanism of gene regulation known to silence gene expression in carcinogenesis. Treatment of spontaneously immortal Li–Fraumeni fibroblasts with 5-aza-2′-deoxycytidine (5AZA-dC), an inhibitor of DNA methyltransferase (DNMT), induces a senescence-like state. We used microarrays containing 12 558 genes to determine the gene expression profile associated with cellular immortalization and also regulated by 5AZA-dC. Remarkably, among 85 genes with methylation-dependent downregulation (silencing) after immortalization, 39 (46%) are known to be regulated during interferon signaling, a known growth-suppressive pathway. This work indicates that gene silencing may be associated with an early event in carcinogenesis, cellular immortalization. *Oncogene* (2003) **22,** 4118–4127. doi:10.1038/sj.onc.1206594

Keywords: senescence; immortalization; interferon gene silencing; LI-Fraumeni syndrome

Introduction

Immortalization is one of the necessary, multiple steps of tumorigenesis. Normal mammalian somatic cells can only divide a limited number of times *in vitro*. The maximum number of divisions is called the 'Hayflick limit' (Hayflick, 1976). This nonproliferative state is also referred to as replicative cellular senescence. Typical characteristics of senescing cells include a large, flat morphology, a high frequency of nuclear abnormalities, and positive staining for β-galactosidase activity specifically at pH 6.0. The counting mechanism for the intrinsic replicative lifespan appears to be the shortening of telomeres with each cell division cycle (Huschtscha and Holliday, 1983). The phenotype of senescence is a dominant trait, and the genes associated with it fall into four complementation groups (Pereira-Smith and Smith, 1983).

Human cells can be immortalized through the transduction of viral and cellular oncogenes (Graham *et al*., 1977; Huschtscha and Holliday, 1983), various human oncogenes such as c-*myc* (Gutman and Wasylyk, 1991), or in some rare cases spontaneously (Bischoff *et al*., 1990; Rogan *et al*., 1995; Shay *et al*., 1995). These mechanisms of immortalization result in abrogation of p53 and pRB/p16^{ink4a}-mediated terminal proliferation arrest and the activation of a telomere maintenance mechanism (Rogan *et al*., 1995; Duncan *et al*., 2000). The activation of human telomerase reverse transcriptase (hTERT) expression is one of the telomere maintenance mechanisms that allow cells to bypass senescence. Certain immortalized human cell lines (Bryan *et al*., 1995) and some tumors (Bryan *et al*., 1997) maintain their telomeres in the absence of detectable telomerase activity by a mechanism, referred to as alternative lengthening of telomeres (ALT), that may involve telomere–telomere recombination (Dunham *et al*., 2000).

Senescence can also be induced in immortal cells by a DNA methyltransferase (DNMT) inhibitor, 5-aza-2′-deoxycytidine (5AZA-dC) (Vogt *et al*., 1998), implying that replicative senescence can result from epigenetic changes in gene expression (Herman and Baylin, 2000; Newell-Price *et al*., 2000; Baylin *et al*., 2001). Genes regulated by DNA methylation usually contain upstream regulatory regions and immediate downstream sequences enriched in CpG dinucleotides (CpG islands). Cytidine residues within CpG islands are methylated by DNMT that can recruit histone deacetylases resulting in the formation of condensed chromatin structures containing hypoacetylated histones. Hypomethylation of CpG islands in oncogenes and hypermethylation of tumor-suppressor genes are important regulatory mechanisms in tumor initiation and progression of cancer (Vogt *et al*., 1998; Baylin *et al*., 2001).

Li–Fraumeni syndrome (LFS) is a familial cancer syndrome that is characterized by multiple primary tumors including soft-tissue sarcomas, osteosarcomas, breast carcinomas, brain tumors, leukemias, adrenal-cortical carcinomas, to a lesser extent melanoma and

*Correspondence: MA Tainsky; E-mail: tainskym@karmanos.org
[4]These two authors contributed substantially to this work and both should be considered as first authors.
Received 14 November 2002; revised 18 March 2003; accepted 20 March 2003

carcinomas of the lung, pancreas, and prostate. Heterozygous germline p53 mutations were found in 75% of families having LFS (Malkin *et al*., 1990; Malkin, 1994). Fibroblast cell lines established from individuals with LFS develop changes in morphology, chromosomal abnormalities, and spontaneously form immortal cell lines (Hayflick, 1976; Bischoff *et al*., 1990; Malkin *et al*., 1990). Vogt *et al*. (1998) demonstrated that the treatment of immortal LFS fibroblasts with 5AZA-dC results in arrest of growth of the fibroblasts and development of a senescent phenotype. We report here that repression of gene expression because of methylation-dependent silencing occurs upon cellular immortalization and a significant proportion of these genes are regulated in the interferon (IFN) pathway. Silencing of this growth-suppressive pathway may be an important early event in the development of cancer, specifically associated with immortalization.

Results

Changes in gene expression after immortalization

We employed preimmortal (PD 11) and immortal (PD 212) fibroblast cells (MDAH041 cell line) from an LFS patient to analyse the changes in gene expression during cellular immortalization. We isolated total RNA from these cells and probes were synthesized for hybridization to microarrays, Affymetrix HGU95Av2 GeneChips. The genes were selected using two different methods: (i) the classical method of selecting the genes with at least a predetermined fold change and (ii) an ANOVA-based noise sampling selection method (Draghici, 2002). All the four possible pairings between preimmortal *vs* immortal cell gene expression comparisons were performed using independent cellular RNAs prepared from these cells. The fold change method was used to select the genes with twofold or greater change in gene expression. There were 169 upregulated and 450 downregulated genes satisfying this condition (Table 1). The noise-sampling selection method is based on ANOVA (Kerr *et al*., 2000) and uses replicate measurements to estimate an empirical distribution of the noise. Given this distribution and a chosen confidence level, one can establish which genes are differentially regulated beyond the influence of the noise. This method identified 76 upregulated and 217 downregulated genes.

The two methods are in some sense complementary. The noise-sampling method selects those genes that have reproducible changes higher than the noise threshold at some confidence level, whereas the *N*-fold method selects those genes that have a minimal fold change that can be confirmed with other assays such as quantitative real time PCR (Q-RT–PCR). The intersection of the subsets of genes reported as differentially regulated by both methods identified 59 upregulated genes and 192 downregulated genes after immortalization (Table 1). Using a representative set of the genes satisfying both conditions (for both downregulated and upregulated genes), the microarray data were confirmed using Q-RT–PCR (Table 2). Comparison of the levels of gene expression after immortalization obtained by using

Table 2 Comparison of expression levels of genes differentially regulated after immortalization[a] by Affymetrix microarray technology and quantitative real-time PCR

Gene	*Microarray, fold change*	*Q-RT–PCR, fold change*
Downregulated genes		
MIF	277	65
MGSA	145	6700
IFN-inducible protein p78	75.7	2700
NDN	60	2790
CD24	45	7450
IP-30	27.8	12
CYP1B1	20	45
OAS-1	19	160
CIG49	13	36
IFNα-inducible protein, p27	13	70
IFITM1	12	8
Dermatopontin	10	13
IFN-inducible 56 kDa protein	8.6	108
IFN-regulatory factor 7	6	2683
AIM2	5.3	165
MRP3	5	17
IFN-induced 17 kDa/15 kDa protein	4.5	43
GST4A	4	8
OAS-2	2.7	142
STAT1, 91kDa	1.9	8.5
Upregulated genes		
WISP	8	20
SNF2A	6	3
ERCC2	5	4
RAGE3	7	10
hTERT	1.6	486

[a]Fold change of gene expression level in the immortal cells (MDAH041 high passage) relative to nonimmortal cells (MDAH041 low passage)

Table 1 Summary of differentially regulated genes in Li–Fraumeni cells: immortalization and gene silencing

Comparison	*Number of genes*		
	Twofold change	*ANOVA*	*Common*
A. MDAH041LP *vs* MDAH041HP upregulated	169	76	59
B. MDAH041LP *vs* MDAH041HP downregulated	450	217	192
C. MDAH041HP *vs* MDAH041HP 5azaCdR, downregulated	48	150	1
D. MDAH041HP *vs* MDAH041HP 5azaCdR, upregulated	190	328	81
Genes in Sets B and D[a]	78	36	30
Genes in Sets A and C[a]	2	1	0

[a]The consensus set of MDAH041LP *vs* MDAH041HP downregulated with MDAH041HP *vs* MDAH041HP 5AZA-dC upregulated genes. The common set of MDAH041LP *vs* MDAH041HP upregulated with MDAH041HP *vs* MDAH041HP 5AZA-dC, downregulated did not contain any genes

both microarray hybridization and Q-RT–PCR revealed outstanding accuracy of the data. Since Q-RT–PCR data can cover a larger range of expression levels, the data obtained using microarrays and Q-RT–PCR differed quantitatively.

Effect of 5AZA-dC gene expression in immortal LFS fibroblasts

As was first shown by Fairweather *et al.* (1987), *in vitro* lifespan of normal human fibroblasts could be shortened by exposure of the cells to the demethylating agent 5AZA-dC. In agreement with this, Vogt *et al.* (1998) have shown that treatment of LFS immortal fibroblasts with 5AZA-dC results in growth arrest and senescence. Thus, there is a possibility that development of immortalization is related to methylation-induced silencing of gene expression. To address this issue, the immortal cells (MDAH041 high passage cell culture) were treated with 5AZA-dC to induce gene demethylation. Treated MDAH041 cells had flat morphology, contained lipofuscin granules, and showed senescence-associated β-galactosidase activity at pH 6 (data not shown), typical for the senescent cells (Dimri *et al.*, 1995).

Total RNA was prepared from MDAH041, high-passage (HP) treated or untreated with 5AZA-dC, and used to prepare probes for the microarray hybridizations. Affymetrix HGU95Av2 GeneChips were again used and the data were analysed as described above for the comparison of preimmortal and immortal MDAH041 cells. The comparison of treated and untreated HP cells identified 48 5AZA-dC upregulated and 190 5AZA-dC downregulated genes with at least a twofold change and 150 upregulated and 328 downregulated genes selected by ANOVA (Table 1). There were 81 upregulated genes and only one downregulated gene that satisfied both conditions ($P<\alpha$ and fold change >2) (Table 1). A sampling of genes covering a range of gene expression changes was chosen and confirmed using Q-RT–PCR (Table 3).

We next determined whether changes in gene expression using Q-RT–PCR after 5AZA-dC treatment were specific to cells undergoing senescence by comparing gene expression changes induced by 5AZA-dC treatment in normal mortal human fibroblasts with those in the immortal MDAH041 cells. The expression levels of 15 of these genes were analysed in preimmortal low-passage (LP) MDAH041 and normal mortal fibroblast cells (CRL-1502) untreated or treated with 5AZA-dC using Q-RT–PCR (Table 4). The vast majority of the 5AZA-dC-dependent changes in expression found in the immortal MDAH041 cells were not induced by 5AZA-dC treatment of the normal human fibroblasts or preimmortal MDAH041 LFS fibroblasts. The exception, IFNα-inducible protein, p27, is found in a known imprinted region on chromosome 14q32 and its induction by 5AZA-dC in all cells therefore was not surprising. In summary, while treatment with 5AZA-dC strongly induces expression of many genes silenced in immortal cells, the expression levels of the same genes were not significantly affected by 5AZA-dC treatment of mortal fibroblasts.

Table 3 Comparison of expression levels of genes differentially regulated during demethylation[a] by Affymetrix microarray technology and quantitative RT–PCR

Gene	*Microarray, fold change*	*Q-RT–PCR, fold change*
Upregulated genes		
IFNα-inducible protein, p27	482	1320
IFN-inducible protein p78	162	478
OAS-1	92	4379
CIG49	70	204
MGSA	65	839
MIF	42	128
IFN-inducible 56 kDa protein	36.6	1807
IP-30	31.2	7
AIM2	21.6	686
OAS-2	19.6	231
IFN regulatory factor 7	17.5	2003
MRP3	14	54
IFN-induced 17 kDa/15 kDa protein	14	228
IFITM1	9	278
STAT1, 91 kDa	7.6	158
CYP1B1	7	77
hTERT	1.65	17

[a]Fold change of gene expression level in the immortal cells (MDAH041 high passage) treated with 5AZA-dC relative to nontreated immortal cells

Table 4 Comparison of expression level using quantitative-RT–PCR of genes differentially regulated in normal[a], preimmortal[b], and immortal[c] cells after 5AZA-dC-induced DNA demethylation[d]

Gene name	*1502* vs *1502 5aza*[a]	*041LP* vs *041LP 5aza*[b]	*041HP* vs *041HP 5aza*[c]
OAS1	2↑	5↑	4379↑
IRF7	1.5↑	1.7↑	2003↑
IFN-inducible 56 kDa protein	1.2↓	1.2↓	1807↑
IFNα-inducible protein, p27	84↑	16↑	1320↑
AIM2	2.6↑	22↑	1094↑
MGSA	3.2↓	1.1↑	839↑
IFN-inducible protein p78	1.8↑	3↑	478↑
IFITM1	1.8↓	1.25↓	278↑
OAS2	1.3↓	1.5↓	231↑
IFN-induced 17 kDa/15 kDa	1.8↑	1.4↓	228↑
CIG49	1.1↑	1.1↑	204↑
STAT1	1.8↑	4↑	158↑
MIF	1.7↓	2↑	128↑
NDN	1.4↓	1.6↑	10↑
IP30	5↑	1.6↑	7↑

[a]Fold change of gene expression level in a nonimmortal fibroblast cell line 1502 MDAH041 before and after treatment with 5AZA-dC; [b]Fold change of gene expression level in a nonimmortal fibroblast cell line MDAH041 low passage before and after treatment with 5AZA-dC; [c]Fold change of gene expression level in an immortal fibroblast cell line (MDAH041, high passage) before and after treatment with 5AZA-dC; [d]↑ and ↓ indicate increase and decrease in gene expression, respectively

Genes downregulated after immortalization and silenced by gene methylation

Since 5AZA-dC-induced gene expression results in the reversal of immortal phenotype and the induction of a senescent-like state, we investigated whether inhibition of DNMT by 5AZA-dC upregulates genes repressed after immortalization. Table 5 shows the list of 85 genes selected by either or both selection methods as silenced

Table 5 Genes differentially regulated after immortalization and demethylation[a]

Accession no.	*Gene name*	*IMMORT*	*5AZA*	*Gene selection method*
L19686	Macrophage migration inhibitory factor (MIF)	−278.0	42.1	N/ANOVA
X54489	Melanoma growth-stimulatory activity (MGSA) (GRO-1)	−146.3	64.6	N
AI017574	Cysteine-rich heart protein	−85.0	8.8	N
M33882	Interferon-induced p78, Mx1	−75.7	162	N/ANOVA[b]
U66711	Ly-6-related protein (9804) gene (responsive to IFNs)	−73.7	34.1	N/ANOVA
X82494	Fibulin-2	−70.1	21.8	N/ANOVA
AF054825	VAMP5 (vesicle-associated membrane protein 5)	−69.1	9.1	N
AL049946	Adlican	−60.2	17.6	N/ANOVA[b]
M55153	Transglutaminase (TGase)	−50.9	144.6	N
AF037335	Carbonic anhydrase precursor (CA 12)	−47.4	8.9	N
L24564	Rad (Ras associated with diabetes)	−35.9	19.7	N/ANOVA[b]
AA631972	NK4 protein (natural killer cell transcript 4)	−35.0	20.2	N/ANOVA
U20982	Insulin-like growth factor binding protein-4	−33.3	3.8	N[b]
AF039103	Tat-interacting protein TIP30	−30.3	8.9	N
J03909	Gamma-interferon-inducible protein (IP-30)	−27.8	31.2	N/ANOVA
AF053944	Aortic carboxypeptidase-like protein	−27.4	12.3	N/ANOVA
AL080059	cDNA DKFZp564H142	−23.4	9.5	N
U88964	HEM45 (interferon-stimulated gene, 20 kDa; ISG20)	−21.7	50.2	N/ANOVA
U03688	Dioxin-inducible cytochrome *P*450 (CYP1B1)	−20.6	6.9	N
U59185	Putative monocarboxylate transporter	−19.5	6.3	N
AB029000	KIAA1077 protein Sulfatase FP	−18.6	10.9	N
U45878	Inhibitor of apoptosis protein 1	−18.4	13.1	N/ANOVA
M28130	Interleukin 8	−15.5	92.7	N/ANOVA
X04371	(2′–5′) oligoadenylate synthetase E OAS-1	−15.4	76.5	N/ANOVA[b]
X02419	uPA gene (urokinase-plasminogen activator gene)	−14.6	4.4	N
M13509	Skin collagenase MMP1	−14.4	4.8	N
AF026941	CIG5 (cytomegalovirus induces interferon-responsive)	−13.9	66.6	N
AB025254	PCTAIRE 2 (pctaire protein kinase)	−13.7	19.3	N/ANOVA
X67325	Interferon α stimulated inducible protein, p27 mRNA	−13.0	482.0	N
M36820	Cytokine (GRO-beta, GRO-2)	−12.9	29.6	N/ANOVA
AF026939	CIG49 (cytomegalovirus induces interferon-responsive)	−12.8	70.2	N/ANOVA
M90657	Tumor antigen (L6)	−12.6	17.5	N/ANOVA[b]
AF060228	Retinoic acid receptor responder 3	−12.2	7.1	N/ANOVA
J04164	Interferon-inducible protein 9-27 (IFITM1)	−11.8	8.8	N
AI885852	Similar to gb:L19779 HISTONE H2A.1	−7.3	10.3	N/ANOVA[b]
M36821	Cytokine (GRO-gamma)	−11.1	56.8	N
AL050162	TESTIN 3 testis derived transcript (3 LIM domains)	−10.7	8.1	N
U77643	K12 protein precursor (SECTM1)	−10.7	19.6	N/ANOVA
D28137	BST-2 (bone marrow stroma cell surface gene)	−9.9	38.7	N/ANOVA
M17017	Beta-thromboglobulin-like protein	−9.7	17.5	N/ANOVA
AC004142	BAC clone RG118D07 from 7q31	−9.4	5.5	N
M24283	Major group rhinovirus receptor (HRV)	−9.3	28.9	N
AL022723	HLA-F, gene for major histocompatibility complex class I F	−8.9	29.4	N/ANOVA[b]
U15932	DUSP5 (dual specificity MAP kinase-phosphatase)	−8.9	12.6	N/ANOVA
M24594	Interferon-inducible 56 kDa protein	−8.6	36.6	N/ANOVA[b]
AB020315	Dickkopf-1 (hdkk-1)	−8.3	14.4	ANOVA
J02931	Placental tissue factor (two forms)	−8.0	8.7	N
X86163	B2-bradykinin receptor, 3	−7.9	3.8	N
M13755	Interferon-induced 17 kDa/15 kDa protein	−7.6	17.0	N/ANOVA[b]
L20817	Tyrosine protein kinase (CAK) gene	−7.5	5.7	N
AJ225089	(2′–5′) Oligoadenylate synthetase 59 kDa OAS-L	−7.4	40.0	N/ANOVA
AF085692	Multidrug resistance-associated protein 3B	−7.3	13.9	N
M26326	Keratin 18	−6.9	13.5	N
M22489	Bone morphogenetic protein 2A	−6.8	9.9	N
U37518	TNF-related apoptosis inducing ligand TRAIL	−6.7	42.2	N
M92357	B94 protein (tumor necrosis factor-alpha-inducible)	−6.7	5.5	N
X07523	Complement factor H	−6.6	6.8	N
AB018287	KIAA0744 protein	−6.5	8.6	N
U53831	Interferon regulatory factor 7	−6.3	17.5	N/ANOVA
X55110	Neurite outgrowth-promoting protein	−6.2	7.1	N
AL039458	Integral membrane glycoprotein LIG-1 (TM4SF1)	−6.1	5.2	N
AL021977	Transcription factor MAFF	−6.1	8.1	ANOVA
M65292	Factor H homolog	−5.8	7.5	N
D29992	Placental protein 5 (PP5)	−5.7	29.3	N
AF070533	Optineurin-like protein	−5.7	4.2	N[b]
AF052135	Associated molecule with the SH3 domain of STAM	−5.6	7.6	ANOVA
D28915	Microtubular protein p44	−5.5	17.8	N/ANOVA
M62402	Insulin-like growth factor binding protein 6	−5.5	5.9	N/ANOVA
AF024714	Interferon-inducible protein AIM2 (absent in melanoma)	−5.3	21.6	N
M31165	Tumor necrosis factor-inducible (TSG-6)	−5.2	10.3	N

Table 5 Continued

Accession no.	*Gene name*	*IMMORT*	*5AZA*	*Gene selection method*
U81607	Gravin	−5.1	12.2	ANOVA
M30818	Interferon-inducible protein, myxovirus resistance, Mx2	−5.0	42	N
M25915	Complement cytolysis inhibitor (CLI)	−4.9	5.1	N
AB013382	DUSP6 (dual specificity MAP kinase-phosphatase)	−4.7	4.6	N
AB000115	mRNA expressed in osteoblast	−4.3	32.5	N/ANOVA
D50919	Tripartite motif-containing protein 14, TRIM14	−4.3	6.2	N
X58536	HLA class I locus C heavy chain	−4.1	5.7	N
AF010312	Pig 7	−4	9.4	ANOVA
AI985272	Neuromedin B precursor	−3.9	5.6	N
X57985	Genes for histones H2B.1 and H2A	−3.6	4.3	N
U07919	Aldehyde dehydrogenase 6	−3.5	4.1	N
AA883502	Ubiquitin-conjugating enzyme E2L 6 (UBE2L6)	−3.4	5.9	N
U22970	Interferon-inducible peptide (6-16)	−3.3	10.2	ANOVA
M87434	(2′–5′) oligoadenylate synthetase 69/71 kDa OAS-2	−2.7	19.6	N
M97935	Transcription factor ISGF-3 (Stat 1)	−1.9	7.6	N[b]

[a]Common set of genes downregulated with a fold change >2 in the immortal cells relative to nonimmortal cells and upregulated genes in the immortal MDAH041 cells treated with 5AZA-dC *vs* untreated. Negative numbers indicate fold change of downregulated gene expression and positive numbers indicate fold change of upregulated gene expression. Data shown were obtained from Affymetrix HU95Av2 microarrays. Gene selection method: indicates the selection method that identified the particular change in gene expression; *N* (*N*-fold change, $N=2$); ANOVA (99.99% confidence). Gene downregulation after immortalization indicated in column IMMORT and by demethylation indicated in column 5AZA; [b]The fold changes for genes with multiple probe set ID's were averaged

after immortalization due to methylation. Interestingly, when the 'reverse' identification of genes was attempted (i.e. genes, both upregulated after immortalization but repressed by 5AZA-dC), no common genes were identified using the dual selection method approach (Table 1, comparison of A and C). In view of the fact that the numbers of genes identified in these comparisons (comparisons B and D (85 genes), and A and C (three genes)) were so vastly different, these suggested that methylation-dependent gene silencing is mechanistically significant to the process of immortalization. Microarray analysis of MDAH041 cells containing a tetracycline-modulated p53 gene revealed that none of these 85 genes were regulated by p53 in these cells (data not shown). Analysis of the functional annotations of the genes downregulated in immortalization (Table 5), because of methylation-dependent silencing, revealed that a significant fraction, 39 out of 85 genes, are known to be regulated by the IFN pathway, with 19 of the 39 genes containing CpG islands identified using CpGPlot software (Table 6).

Hierarchical clustering

The hierarchical map of the silenced gene expression set and two subsets of genes (identified by both software methods) that are repressed after immortalization by methylation-dependent silencing is shown in Figures 1a,b. In these figures, the height of each bridge between members of a cluster is proportional to the average squared distance of each leaf in the subtree from that subtree's centroid (or mean). These data indicate that the level of expression of the same set of genes that are downregulated during immortalization is also stimulated by 5AZA-dC-induced DNA demethylation. Interestingly, the approach showed that the total pattern of gene expression (12 558 genes) in preimmortal MDAH041 cells is similar to the 5AZA-dC-treated immortal MDAH041 cells as compared to the untreated immortal cells (data not shown). In Figure 1a, the set of 85 genes silenced by methylation show a pattern of low expression in the immortal fibroblasts (indicated by the green color) and higher expression in the preimmortal MDAH041 cells and in the 5AZA-dC-treated immortal cells (indicated by the red color). Figure 1b similarly shows the pattern of gene expression in the group of 30 genes selected by 99.99% confidence and >twofold change in expression.

Discussion

The indefinite lifespan necessary for the formation of a cancer cell appears to be a complex genetic trait with four complementation groups of recessive genes (Pereira-Smith and Smith, 1983, 1988; Berube *et al*., 1998). Since treatment of spontaneously immortalized Li–Fraumeni cells, MDAH041, with the DNMT inhibitor, 5AZA-dC, results in a replicative senescent state (Baylin *et al*., 2001), epigenetic control of immortalization needed to be considered in these cells. We employed Affymetrix microarrays to profile gene expression changes associated with immortalization and determined which of those genes were also regulated by DNA demethylation. We hypothesized that genes downregulated after immortalization (493 genes) would fit the pattern of recessive senescence genes predicted by the somatic cell genetics experiments (Pereira-Smith and Smith, 1988). Consistent with this hypothesis, we reasoned that those in common with the 190 genes upregulated after the 5AZA-dC treatment would focus the gene set on those involved in replicative senescence. This gene set included a total of 85 genes from those available on the microarrays used. One of these genes is known to be maternally imprinted in the Prader–Willi

Table 6 Interferon pathway genes regulated by demethylation and immortalization

Gene	*IMMORT*	*5AZA*	*CpG*	*Locus*
Interferon-induced 17 kDa/15 kDa	−4.2	13.9	+	1p36.33
Interferon-inducible peptide (6–16)	−3.3	10.2	−	1p36
mRNA expressed in osteoblast	−4.3	32.5	−	1p31
Microtubular protein p44 (IFI44)	−5.5	17.8	−	1p31.1
Interferon-inducible protein (absent in melanoma 2, AIM2)	−5.3	21.6	−	1q22
Complement factor H	−6.6	6.8	−	1q32
CIG5 vipirin; similar to inflammatory response protein 6	−13.9	67.0	−	2p25.3
Signal transducer activator of transcription 1 STAT1 91 kDa	−1.9	7.6	+	2q32.2
TNF-related apoptosis-inducing ligand, TRAIL	−6.7	42.2	−	3q26
Cytokine (GRO-beta, GRO-2)	−12.9	29.6	+	4q12-13
Interleukin 8	−15.5	92.7	−	4q12-13
Melanoma growth-stimulatory activity (MGSA) (GRO-1)	−146.3	64.6	+	4q21
HLA class I locus C heavy chain	−4.1	5.7	+	6p21
HLA-F, gene for major histocompatibility complex class I F[a]	−8.9	29.4	+	6q21.3
uPA gene (urokinase-plasminogen activator gene)	−14.6	4.4	+	8p12
Ly-6-related protein (9804) gene	−73.7	34.1	+	8q24.3
Tripartite motif-containing protein 14, TRIM14	−4.3	6.2	+	9q22-q31
PCTAIRE 2 (pctaire protein kinase)[a]	−13.7	19.3	+	9q22.33
Dickkopf-1 (hdkk-1)[a]	−8.3	14.4	−	10q11.2
CIG49 interferon-induced protein with tetratricopeptide repeats 4	−12.8	70.2	−	10q24
DUSP5 (dual specificity MAP kinase-phosphatase)[a]	−8.9	12.6	+	10q25
Interferon-inducible 56 kDa protein	−8.6	36.6	−	10q25-q26
Interferon-inducible membrane protein 9–27 (IFITM1)	−11.8	8.8	−	11p15.5
Interferon regulatory factor 7	−6.3	17.5	+	11p15.5
DUSP6 (dual specificity MAP kinase-phosphatase) α	−4.7	4.6	+	12q22-q23
(2–5′) oligoadenylate synthetase p46/p42 E gene, OAS-1	−15.4	76.5	−	12q24.1
(2–5′) oligoadenylate synthetase 59 kDa isoform, OAS-L	−7.4	40.0	−	12q24.2
(2–5′) oligoadenylate synthetase 69/71 kDa isoform, OAS-2	−2.7	19.6	−	12q24.2
Interferon α-inducible protein, p27	−13.0	482.0	−	14q32
HEM45, ISG-20	−21.7	50.2	−	15q26
NK4 protein (natural killer cell transcript 4)	−35.0	20.2	−	16p13.3
Insulin-like growth factor binding protein-4	−33.3	3.8	−	17q12-q21
K12 protein precursor (SECTM1)[a]	−10.7	19.6	+	17q25
Gamma-interferon-inducible protein (IP-30)	−27.8	31.2	+	19p13.1
BST-2 (bone marrow stroma cell surface gene)	−9.9	38.7	−	19p13.2
Major group rhinovirus receptor (HRV) ICAM	−9.3	28.9	+	19p13.3
Transglutaminase (TGase)	−50.9	144.6	+	20q12
Interferon-inducible protein p78, MX1	−75.7	162	+	21q22.3
Interferon-inducible protein MX2	−5	42	−	21q22.3

Data were processed in Affymetrix Data Mining Tool (fold change); 5AZA: upregulation in 5AZA-dC-treated HP MDAH041 cells *vs* untreated HP MDAH041 cells; IMMORT: downregulation in HP MDAH041 cells *vs* LP MDAH041 cells; CpG: + indicates that a CpG island within an interval of −500 to +200 bp around the transcription starting site (TSS) of a length of at least 200 bp using CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/); Locus: indicates the chromosome position within the human genome; [a]IFN regulation identified by Dr D Leaman, University of Toledo, personal communication

Syndrome, NDN (Jay *et al*., 1997) (Table 5). The protein encoded by this gene, Necdin, is a growth suppressor expressed in postmitotic neurons of the brain (Nakada *et al*., 1998). We found that this RNA is silenced during immortalization and activated by 5AZA-dC treatment of the immortal MDAH041 cells but not normal fibroblasts or preimmortal MDAH041 (Table 4). Interestingly, this gene was found to undergo loss of heterozygosity in the MDAH041 immortal cells (data not shown).

We observed downregulation in immortal MDAH041 cells of some genes (collagenase, cathepsin O, uPA) (data not shown) that have been detected by others as upregulated genes during replicative senescence in dermal fibroblasts (Shelton *et al*., 1999). We also observed downregulation of DOC1, IGFBP4 and IGFBP6 in immortal cells (data not shown) that is correlated with the published data before of Schwarze *et al*. (2002) who found upregulation of DOC1 and IGFBP3 in human prostate epithelial cells when passaged to senescence.

Remarkably, 39 of these 85 genes were also known to be regulated in the IFN pathway and represent candidate regulatory genes in cellular immortalization. These data are in agreement with others who observed 5AZA-dC upregulation of IFN pathway genes in colon tumor cells (Karpf *et al*., 1999) and human bladder cancer cells (Liang *et al*., 2002). To calculate the significance of this observation, we used the UniGene clusters in order to eliminate overcounting genes with several accession numbers and/or Affymetrix probes. Currently, the 12 558 probes on the array correspond to 8628 Unigene clusters. Among these, there are 137 genes, or 0.015%, known to be IFN-regulated. Thus, we expect that a list of 85 random genes will contain about $85 \times 0.015\%$ or approximately zero IFN-regulated

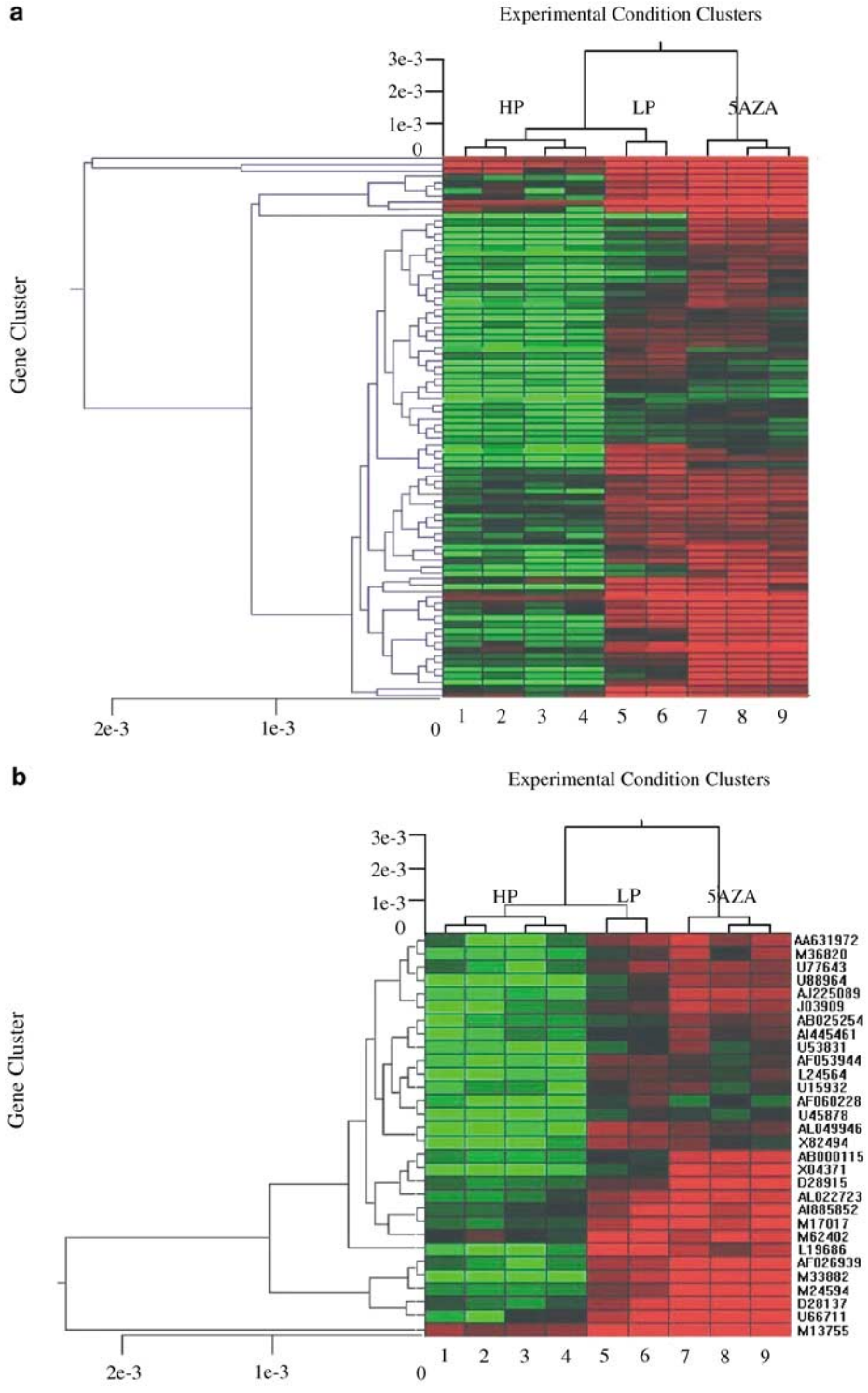

Figure 1 Hierarchical clustering of gene expression. Gene expression in MDAH041 fibroblast culture, LP (preimmortal), HP (immortal) and HP treated with 5AZA-dC (5AZA) was analysed. The graph includes only those genes whose expression was decreased during transition from preimmortal to immortal cell state, and was upregulated to the high level after treatment with 5AZA-dC. Each row represents a gene and each column represents an independent microarray experiment of different RNA preparations. (**a**) 85 genes reported by either analysis methods: twofold and ANOVA; (**b**) 30 genes reported by both twofold and ANOVA methods

genes due to random chance. In fact, our list of 85 genes silenced in immortalization contained 39 IFN-regulated genes. The probability of this happening by chance is approximately 10^{-47} which shows that the silencing of the IFN-pathway genes is highly significant to the mechanism of cellular immortalization.

Table 7 Oligonucleotide sequences used in Q-RT–PCR

Gene	*Forward primer*	*Reverse primer*
NDN	AGCTCTGCGGGAGGCTAATC[a]	TGGTGAGGGTCAGAAACCATTC
MIF	CCCGCAGGGTCTACATCAA	CGCAGACAGCGTGGGTC
CYP1B1	GGCAGAATTGGATCAGGTCG	AGCGCATGGCTTCATAAAGG
SNF2A	CATGCTTCTCTGTCACAACGC	TCTGCCGGGCACTCTTAAAC
MRP3	GGGTCCTTGTCCCTCCTTTC	TCCGATGCTCAGCCACCTAT
CD24	GAGAATCTACCCCAGATCCAAGC	GCTCTTGTTGAATGGAAACAGGT
MGSA	CAACCCCAAGTTAGTTCAATCTGG	TGTTGCAGGCTCCTCAGAAAT
WISP	AGACAGGGAAAGGTCAGCCC	AAGGAAGAGCCTGGCCAACT
Dermatopontin	TCCAGAGCCGCTACTTCGAG	TTCTGTTGTTAGCCAGCAGGAA
p78	GCCACTGGACTGACGACTTGA	ACTGCTCTCACAGCTTCCTGC
GSTA4	ACCAGATGATCAGCAAAAGGAAG	AGCTTTGTCCGTGACCCCT
IRF7	GCAGCGTGAGGGTGTGTCTT	GCTCCATAAGGAAGCACTCGAT
CIG49	CTTGCCAAACAGATGTCCTCC	TGTGGATTCCAACACCCGTT
OAS-1	TGAGGCCTGGCTGAATTACC	TGAATGGCAGGGAGGAAGC
IFITM1	GACAGGAAGATGGTTGGCGA	ATGGTCATGAGGATGCCCA
p17/p15	CAAATGCGACGAACCTCTGAG	GCTGCTTCAGGTGGGCC
p56	GCAGCCTAGAGGGCAGAACA	CCACCTCAAATGTGGGCTTTT
STAT1	CTGCTGCGGTTCAGTGAGAG	GGTTCAACCGCATGGAAGTC
hTERT	CTACTCCAGCTATGCCCGGA	GCAAGACCCCAAAGAGTTTGC
AIM2	AGCGCCTCACGTGTGTTAGA	TGAAGCGTGTTGATCTTCGG
p27	TCCTCCATAGCAGCCAAGATG	GTTGCTCCCAGTGACTGCAG
OAS-2	GCCAACGTGACATCCTCGAT	CCCATCAAGGGACTTCTGGA

[a]All primers have 5′–3′ orientation

Some IFN-regulated genes have previously been shown to be silenced by DNA methylation and reactivated by 5AZA-dC treatment (Liang *et al*., 2002). Consistent with this observation and the growth-inhibitory effect of IFNs, 5AZA-dC treatment has been shown to inhibit the growth of human tumor cell lines (Bender *et al*., 1998) and our data indicate that gene silencing may be an early event in cancer development. The IFN-regulated RNaseL gene is known to inhibit cell proliferation and induce apoptosis through the IFN-regulated (2′-5′) oligoadenylate synthetase pathway. RNaseL is a candidate tumor-suppressor gene that has been shown to be mutated in the germ line of hereditary prostate cancer patients (Carpten *et al*., 2002). This candidate tumor-suppressor gene, RNaseL, is activated by (2′-5′) oligoadenylate synthetase proteins and therefore it is noteworthy that in MDAH041 cells, three out of four of the isoforms of the (2′-5′) oligoadenylate synthetase are downregulated after immortalization because of methylation-dependent silencing (Table 6). In addition, IRF-1 has been shown to be a tumor-suppressor gene in human leukemias (Harada *et al*., 1993; Willman *et al*., 1993). The double-stranded RNA-activated protein kinase (PKR) has been shown to induce apoptosis, implying that its inactivation would be a procarcinogenic event (Jagus *et al*., 1999). The IFN-inducible proteins of the 'HIN-200 gene family' have been demonstrated to be growth inhibitory, have antitumor activity (Wen *et al*., 2001; Xin *et al*., 2001), and are able to bind to the Rb1 and p53 tumor-suppressor proteins (Choubey and Lengyel, 1995). We found that one of the three members of this gene family, AIM2, is downregulated in MDAH041 cells and silenced by methylation (Table 6). AIM2 functions as a tumor suppressor for a melanoma cell line (DeYoung *et al*., 1997) and a T-cell tumor antigen in neuroectodermal tumors, as well as breast, ovarian, and colon carcinomas (Harada *et al*., 2001). The AIM2 gene contains a site of microsatellite instability (MSI) that results in gene inactivation in 47% of colorectal tumors analysed with high MSI (Mori *et al*., 2001). Interestingly, p202, a member of the murine '200 gene family', is a negative regulator of p53 whose gene expression is controlled by p53 as well (D'Souza *et al*., 2001).

MDAH041 LFS cells contain significant telomerase activity after immortalization (Gollahon *et al*., 1998). Although in microarray analysis, the hTERT gene for the protein of enzymatic subunit of telomerase was not significantly upregulated after immortalization of MDAH041 cells, 1.6-fold, we found using Q-RT–PCR that there was a significant increase in hTERT expression, 486-fold (Table 2). This is consistent with our experience that genes with low basal expression levels are difficult to quantitate accurately using microarrays alone. We found that 5AZA-dC treatment resulted in an additional 17-fold increase in hTERT RNA expression (Table 3). Interestingly, the promoter of the hTERT gene has been shown to be regulated by methylation at CpG islands (Dessain *et al*., 2000; Bechter *et al*., 2002). Using CpGPlot, we performed an analysis for the presence of CpG islands in the 39 interferon-regulated genes that we identified. In all, 19 of those genes contained CpG islands (Table 6). We expect that a subset of these 19 genes represents the primary inducers of cellular senescence and/or aging.

$p16^{INK4a}$ is one of the tumor-suppressor genes whose expression is repressed by methylation, which permits cells to bypass early mortality check points. In our study, we confirmed downregulation of p16 mRNA in immortal cells and upregulation by demethylation using RT–PCR (data not shown). When we tested the level of protein expression using Western blots, we found that

p16^{INK4a} protein was much less abundant in immortal cells and upregulated approximately 500-fold by 5AZA-dC treatment (data not shown). The 5AZA-dC-dependent upregulation of p16^{INK4a} protein in immortal MDAH041 cells was observed by us and by Vogt *et al*. (1998), who demonstrated that retroviral transduction of a *p16*^{INK4a} cDNA was able to induce senescence in MDAH041 cells. Although retroviral transduction of a *p21* cDNA was also able to induce senescence in MDAH041 cells (Vogt *et al*., 1998), p21 protein levels were not regulated by 5AZA-dC treatment of immortal MDAH041 (data not shown) cells. It is noteworthy that p21$^{cip/waf}$ was also identified as *sdi1* because of its high levels of expression in senescing mortal fibroblasts (Noda *et al*., 1994) and is regulated transcriptionally by DNMT (Young and Smith, 2001). p21 can also be regulated by STAT1 which is also a major transcriptional effector of the IFN pathway (Agrawal *et al*., 2002). The level of STAT1 protein is two-fold downregulated after immortalization and 4.7-fold upregulated in immortal cells by 5AZA-dC treatment (data not shown). Therefore, STAT1 is silenced by methylation in immortal MDAH041 cells (Tables 5 and 6) and may be a key regulator of immortalization by controlling the interferon-regulated gene expression pathway and its growth-suppressive effectors. As these mechanisms become better understood, specific demethylation or deacetylation agents currently in preclinical evaluation and clinical trials in cancer patients will provide another approach to control cancer (Brown and Strathdee, 2002).

Materials and methods

Cell culture and p53 genotypin

The MDAH041 (p53 frameshift mutation) cell line was derived from primary fibroblasts obtained by skin biopsy from patients with LFS. Characterization and immortalization of these cells was performed by Bischoff *et al*. (1990). All cells were grown in modified Eagles medium (MEM, Gibco BRL, MD, USA) with 10% fetal calf serum and antibiotics. The CRL1502 cell line was derived from primary fibroblasts obtained by skin biopsy from a normal donor (ATCC 1502, Rockville, MD, USA). The region containing the frameshift mutation in gene encoding p53 from LP preimmortal and HP immortal cells was sequenced to confirm the heterozygosity in LP preimmortal MDAH041 cells.

Treatment of cells with 5AZA-dC

Fibroblast cell cultures were seeded 3×10^5 per plate in MEM medium with 10% fetal calf serum and antibiotics. Cell cultures were treated with 1 μM 5AZA-dC on days 1, 3, and 5 each time with a full media change. After day 6, the cells were returned to regular medium without 5AZA-dC. Total RNA preparation was performed on day 8.

RNA isolation and the Affymetrix microarray assays

The cells were grown to 80% confluence, the medium was changed, and after 16 h the cells were washed with PBS, trypsinized, and pelleted at 300 g for 5 min. Total RNA was isolated using RNeasy kit (Qiagen Inc., Valencia, CA, USA). 1.5×10^7 cells yielded $\sim$200 μg total RNA. The RNA targets (biotin-labeled RNA fragments) were synthesized from 5 μg of total RNA by first synthesizing double-stranded cDNA followed by standard Affymetrix protocols (Affymetrix, Santa Clara, CA, USA).

Quantization of gene expression by Q-RT–PCR

Total RNA (1 μg) was reverse transcribed into cDNA using Superscript II (Life Technologies, Gaithersburg, MD, USA). All methods for reactions were performed as recommended by the manufacturer. The ABI 5700 Sequence Detection System was used for Q-RT–PCR. The protocols and analysis of data are identical to that of the ABI 7700 Sequence Detection System (ABISYBR). All methods for reactions and quantitation were performed as recommended by the manufacturer. An extensive explanation and derivation of the calculations involved can be found in the ABI User Bulletin #2 and also in the manual accompanying the SYBR Green PCR core kit. Primers used in Q-RT–PCR are shown in Table 7.

Analysis of microarray data

Microarray experiments were performed using the Affymetrix HG-U95A chip containing 12 558 probes. Two RNA preparations from immortal cells (HP) were compared with two RNA preparations from preimmortal cells (LP). In addition, two RNA preparations from immortal cells (HP) were compared with three total RNA preparations from immortal cells treated with 5AZA-dC using the HG-U95A chips.

Two analysis methods were used to select differentially regulated genes: fold change and noise sampling method (ANOVA). The fold change method was used to select the genes with at least a twofold change in expression. This was done using the Affymetrix Data Mining Tool (DMT), version 3, *N*-fold method (Affymetrix, Santa Clara, CA, USA). For the control *vs* experiment comparisons, all possible pairings between the two controls and the two experiments were considered.

The noise sampling method is a variation of the ANOVA model proposed by Kerr and Churchill (Kerr *et al*., 2000; Draghici, 2002). The noise sampling method was implemented in GeneSight, version 3.2.21 (Biodiscovery, Los Angeles, CA, USA). In order to apply the noise sampling method, the intensities, obtained from each chip, were normalized by dividing by the mean intensity. Four ratios were formed by taking all possible combinations of experiments and controls. We looked for genes differentially regulated with a 99.99% confidence ($P < 0.0001$).

CpG island analysis

First, we retrieved the genome sequence of each IFN-regulated RNA from UCSD Genome Browser (http://genome.ucsc.edu/). Then we tested the CpG islands within an interval of -500 to $+200$ bp around the transcription starting site (TSS) using CpGPlot program (http://www.ebi.ac.uk/emboss/cpgplot/). The discrimination for CpG islands is based on the formal definition of CpG islands (Gardiner-Garden and Frommer, 1987) (length is over 200 bp, G + C content is greater than 50%, statistical expectation is greater than 0.6).

Acknowledgements
This work was supported by the Barbara and Fred Erb Endowed Chair in Cancer Genetics to MAT, funds from the Karmanos Cancer Institute, the State of Michigan Life Sciences Corridor, the Molecular Medicine and Genetics

Applied Genomics Technology Center at WSU, and the Genomics Core of the Karmanos Cancer Institute, P30CA022453. We acknowledge helpful comments on this manuscript from Vasily Studitsky, Aviva Fridman, George Brush; the assistance of Tara Twomey and Purvesh Khatri is greatly appreciated (Wayne State University). Also, we acknowledge Dr Bruce Hoff for helpful advice (BioDiscovery) and Dr Douglas Leaman for providing interferon gene expression data prior to publication. This work has been supported in part by grants to SD from the National Science Foundation grant number 0234806 and Department of Defense grant number DAMD17-03-2-0035.

References

Agrawal S, Agarwal ML, Chatterjee-Kishore M, Stark GR and Chisolm GM. (2002). *Mol. Cell. Biol.,* **22,** 1981–1992.

Baylin SB, Esteller M, Rountree MR, Bachman KE, Schuebel K and Herman JG. (2001). *Hum. Mol. Genet.,* **10,** 687–692.

Bechter OE, Eisterer W, Dlaska M, Kuhr T and Thaler J. (2002). *Exp. Hematol.,* **30,** 26–33.

Bender CM, Pao MM and Jones PA. (1998). *Cancer Res.,* **58,** 95–101.

Berube NG, Smith JR and Pereira-Smith OM. (1998). *Am. J. Hum. Genet.,* **62,** 1015–1019.

Bischoff FZ, Yim SO, Pathak S, Grant G, Siciliano MJ, Giovanella BC, Strong LC and Tainsky MA. (1990). *Cancer Res.,* **50,** 7979–7984.

Brown R and Strathdee G. (2002). *Expert Opin. Invest. Drugs,* **11,** 747–754.

Bryan TM, Englezou A, Dalla-Pozza L, Dunham MA and Reddel RR. (1997). *Nat. Med.,* **3,** 1271–1274.

Bryan TM, Englezou A, Gupta J, Bacchetti S and Reddel RR. (1995). *EMBO J.,* **14,** 4240–4248.

Carpten J, Nupponen N, Isaacs S, Sood R, Robbins C, Xu J, Faruque M, Moses T, Ewing C, Gillanders E, Hu P, Bujnovszky P, Makalowska I, Baffoe-Bonnie A, Faith D, Smith J, Stephan D, Wiley K, Brownstein M, Gildea D, Kelly B, Jenkins R, Hostetter G, Matikainen M, Schleutker J, Klinger K, Connors T, Xiang Y, Wang Z, De Marzo A, Papadopoulos N, Kallioniemi OP, Burk R, Meyers D, Gronberg H, Meltzer P, Silverman R, Bailey-Wilson J, Walsh P, Isaacs W and Trent J. (2002). *Nat. Genet.,* **30,** 181–184.

Choubey D and Lengyel P. (1995). *J. Biol. Chem.,* **270,** 6134–6140.

Dessain SK, Yu H, Reddel RR, Beijersbergen RL and Weinberg RA. (2000). *Cancer Res.,* **60,** 537–541.

DeYoung KL, Ray ME, Su YA, Anzick SL, Johnstone RW, Trapani JA, Meltzer PS and Trent JM. (1997). *Oncogene,* **15,** 453–457.

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith, Peacocke M and Campisi J. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 9363–9367.

Draghici S. (2002). *Drug Discov. Today,* **7,** S55–S63.

D'Souza S, Xin H, Walter S and Choubey D. (2001). *J. Biol. Chem.,* **276,** 298–305.

Duncan EL, Wadhwa R and Kaul SC. (2000). *Biogerontology,* **1,** 103–121.

Dunham MA, Neumann AA, Fasching CL and Reddel RR. (2000). *Nat. Genet.,* **26,** 447–450.

Fairweather NF, Lyness VA and Maskell DJ. (1987). *Infect. Immun.,* **55,** 2541–2545.

Gardiner-Garden M and Frommer M. (1987). *J. Mol. Biol.,* **196,** 261–282.

Gollahon LS, Kraus E, Wu TA, Yim SO, Strong LC, Shay JW and Tainsky MA. (1998). *Oncogene,* **17,** 709–717.

Graham FL, Smiley J, Russell WC and Nairn R. (1977). *J. Gen. Virol.,* **36,** 59–74.

Gutman A and Wasylyk B. (1991). *Trends Genet.,* **7,** 49–54.

Harada H, Kitagawa M, Tanaka N, Yamamoto H, Harada K, Ishihara M and Taniguchi T. (1993). *Science,* **259,** 971–974.

Harada M, Li YF, El-Gamil M, Ohnmacht GA, Rosenberg SA and Robbins PF. (2001). *J. Immunother.,* **24,** 323–333.

Hayflick L. (1976). *N. Engl. J. Med.,* **295,** 1302–1308.

Herman JG and Baylin SB. (2000). *Curr. Top. Microbiol. Immunol.,* **249,** 35–54.

Huschtscha LI and Holliday R. (1983). *J. Cell. Sci.,* **63,** 77–99.

Jagus R, Joshi B and Barber GN. (1999). *Int. J. Biochem. Cell. Biol.,* **31,** 123–138.

Jay P, Rougeulle C, Massacrier A, Moncla A, Mattei MG, Malzac P, Roeckel N, Taviaux S, Lefranc JL, Cau P, Berta P, Lalande M and Muscatelli F. (1997). *Nat. Genet.,* **17,** 357–361.

Karpf AR, Peterson PW, Rawlins JT, Dalley BK, Yang Q, Albertsen H and Jones DA. (1999). *Proc. Natl. Acad. Sci. USA,* **96,** 14007–14012.

Kerr MK, Martin M and Churchill GA. (2000). *J. Comput. Biol.,* **7,** 819–837.

Liang G, Gonzales FA, Jones PA, Orntoft TF and Thykjaer T. (2002). *Cancer Res.,* **62,** 961–966.

Malkin D. (1994). *Biochim. Biophys. Acta,* **1198,** 197–213.

Malkin D, Li FP, Strong LC, Fraumeni Jr JF, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA and Friend SH. (1990). *Science,* **250,** 1233–1238.

Mori Y, Yin J, Rashid A, Leggett BA, Young J, Simms L, Kuehl PM, Langenberg P, Meltzer SJ and Stine OC. (2001). *Cancer Res.,* **61,** 6046–6049.

Nakada Y, Taniura H, Uetsuki T, Inazawa J and Yoshikawa K. (1998). *Gene,* **213,** 65–72.

Newell-Price J, Clark AJ and King P. (2000). *Trends Endocrinol. Metab.,* **11,** 142–148.

Noda A, Ning Y, Venable SF, Pereira-Smith OM and Smith JR. (1994). *Exp. Cell. Res.,* **211,** 90–98.

Pereira-Smith OM and Smith JR. (1983). *Science,* **221,** 964–966.

Pereira-Smith OM and Smith JR. (1988). *Proc. Natl. Acad. Sci. USA,* **85,** 6042–6046.

Rogan EM, Bryan TM, Hukku B, Maclean K, Chang AC, Moy EL, Englezou A, Warneford SG, Dalla-Pozza L and Reddel RR. (1995). *Mol. Cell. Biol.,* **15,** 4745–4753.

Schwarze SR, DePrimo SE, Grabert LM, Fu VX, Brooks JD and Jarrard DF. (2002). *J. Biol. Chem.,* **277,** 14877–14883.

Shay JW, Tomlinson G, Piatyszek MA and Gollahon LS. (1995). *Mol. Cell. Biol.,* **15,** 425–432.

Shelton DN, Chang E, Whittier PS, Choi D and Funk WD. (1999). *Curr. Biol.,* **9,** 939–945.

Vogt M, Haggblom C, Yeargin J, Christiansen-Weber T and Haas M. (1998). *Cell. Growth Differ.,* **9,** 139–146.

Wen Y, Yan DH, Wang B, Spohn B, Ding Y, Shao R, Zou Y, Xie K and Hung MC. (2001). *Cancer Res.,* **61,** 7142–7147.

Willman CL, Sever CE, Pallavicini MG, Harada H, Tanaka N, Slovak ML, Yamamoto H, Harada K, Meeker TC, List AF and Taniguchi T. (1993). *Science,* **259,** 968–971.

Xin H, D'Souza S, Fang L, Lengyel P and Choubey D. (2001). *Oncogene,* **20,** 6828–6839.

Young JI and Smith JR. (2001). *J. Biol. Chem.,* **276,** 19610–19616.