A systematic search for downstream mediators of tumor suppressor function of p53 reveals a major role of BTG2 in suppression of Ras-induced transformation

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Factors that mediate p53 tumor suppressor activity remain largely unknown. In this study we describe a systematic approach to identify downstream mediators of tumor suppressor function of p53, consisting of global gene expression profiling, focused short hairpin RNA (shRNA) library creation, and functional selection of genetic elements cooperating with oncogenic Ras in cell transformation. This approach is based on our finding that repression of gene expression is a major event, occurring in response to p53 inactivation during transformation and immortalization of primary cells. Functional analysis of the subset of genes universally down-regulated in the cells that lacked functional p53 revealed BTG2 as a major downstream effector of p53-dependent proliferation arrest of mouse and human fibroblasts transduced with oncogenic Ras. shRNA-mediated knockdown of BTG2 cooperates with oncogenic Ras to transform primary mouse fibroblasts containing wild-type transcriptionally active p53. Repression of BTG2 results in up-regulation of cyclins D1 and E1 and phosphorylation of Rb and, in cooperation with other oncogenic elements, induces neoplastic transformation of primary human fibroblasts. BTG2 expression was found to be significantly reduced in a large proportion of human kidney and breast carcinomas, suggesting that BTG2 is a tumor suppressor that links p53 and Rb pathways in human tumorigenesis.

[**Keywords:** BTG2; Rb; oncogenic Ras; p53; shRNA library; tumor suppressor gene]

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Malignant transformation results from the gradual acquisition of mutations deregulating two categories of genes: proto-oncogenes and tumor suppressor genes, which are normally involved in positive and negative control of cell proliferation, respectively (Hunter 1997). Frequently, activation of an oncogene requires acquisition of additional genetic alteration of another gene that naturally serves to prevent expansion of such cell, a phenomenon known as cooperation of genetic events in malignant transformation (Land et al. 1983; Newbold and Overell 1983). This concept, originally based on the studies of primary rodent fibroblasts transformed by the combination of oncogenes *Ras* and *Myc* (Land et al. 1983;

Ruley 1983), which were thought to induce transformation and immortalization, respectively, was then extended to several combinations of genetic events, some of which are sufficient to transform human fibroblasts (Hahn and Weinberg 2002). All of them, however, involve activation of Ras and inactivation of p53 pathways, thus reflecting the critical importance of these two regulatory networks for cell proliferation and the tight interconnection between them. It is therefore no surprise that the inactivation of p53 and mutations in members of the *Ras* family of proto-oncogenes are among the most common events found in human cancers (Bos 1989; Levine 1997).

In fact, oncogenic Ras is incapable of transforming primary fibroblasts by itself, instead inducing growth arrest and premature senescence (Franza et al. 1986; Serrano et al. 1997). This arrest results from Ras-dependent transcriptional activation of p19^{ARF} that disrupts p53:MDM2

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interaction, leading to stabilization and accumulation of transcriptionally active p53, which blocks cell proliferation (Palmero et al. 1998; Sherr 1998). Hence, during Ras-mediated transformation of mouse or rat fibroblasts, senescence-like growth arrest can be overcome by inactivation of p19^{ARF} or p53 (Finlay et al. 1989; Kamijo et al. 1997). However, what is sufficient for the acquisition of a fully transformed tumorigenic phenotype in rodent cells is not paralleled by human counterparts, which in addition to p53 inactivation must overcome Rb-mediated arrest of proliferation during the process of complete neoplastic transformation (Hahn et al. 1999).

Since functional inactivation of p53 is essential for Ras-mediated transformation in both cell systems, identification of downstream executors of p53-mediated growth arrest in response to activated Ras is critically important to understanding the mechanisms of tumor suppression. Stabilization and activation of p53 in response to oncogenic signaling leads to growth arrest (Serrano et al. 1997; Lin et al. 1998) or induction of apoptosis (Debbas and White 1993; Hermeking and Eick 1994). While molecular events acting upstream of p53, including those that are involved in regulation of p19^{ARF}, are quite well studied and their direct role in cell transformation is now established (Jacobs et al. 1999; Inoue et al. 2000), downstream factors mediating p53 tumor suppressive function remain largely unknown. Recently, it has been suggested that inhibition of proapoptotic function of p53 through reduced expression of its downstream targets, such as *bax* and *puma*, or overexpression of *bcl-2* and *bcl_{XL}* can promote tumorigenesis in vivo (Yin et al. 1997; Hemann et al. 2004; Lowe et al. 2004); however, deregulation of any of those genes cannot substitute for p53 loss in their ability to cooperate directly with Ras in transformation of primary cells.

In the current study we approached this problem by combining comprehensive microarray profiling of Ras-induced transformation with short hairpin RNA (shRNA) library screening to identify the genes that are responsible for p53-mediated suppression of transformation. As a result, we found that suppression of immediate early growth response gene, *BTG2* (Bradbury et al. 1991; Varnum et al. 1991) was sufficient for Ras-induced oncogenic transformation of primary murine fibroblasts possessing transcriptionally active p53. shRNA-mediated knockdown of *BTG2* results in hyperphosphorylation of Rb and cooperates with p53 inhibitor GSE56, small t-antigen of SV40, and catalytic subunit of H-TERT in transformation of human primary fibroblasts. Additionally, we determined that *BTG2* expression is reduced in significant proportion of patients with kidney, breast, and to a lesser extent, stomach carcinomas.

Results

Establishment of experimental system

The approach we used to identify candidate genes mediating p53 tumor suppressor activity is outlined in Figure 1:

Inactivation of p53 makes mouse embryo fibroblasts (MEFs) fully permissive for Ras-induced transformation (Finlay et al. 1989); thus, we presumed that inactivation of critical components of the p53 pathway, acting downstream of p53 in response to oncogenic Ras, should be functionally equivalent to inactivation of p53 itself and permit full transformation of rodent fibroblasts. To identify such genes, we have compared microarray profiles of p53-deficient immortalized and Ras-transformed MEFs. After determining that repression, rather than activation, was a governing factor regulating expression of the candidate genes, we created a set of shRNAs specific for the selected group of these genes and screened it in combination with oncogenic Ras for the clones capable of transforming primary MEFs. At this stage of the screening, we considered mouse cells as a simplified experimental model of oncogenic transformation where the number of required cooperating factors is minimal in contrast with the human cells, which possess multiple tumor suppressive pathways to prevent tumorigenesis during much longer life span. Therefore, the next step of our study was to determine the involvement of the identified factors in human transformation and their expression in human tumors.

Figure 1. General outline of the study. See the explanation in the text.

In principle, to identify genetic alterations underlying the effect of p53 inhibition in murine cells, one could compare gene expression profiles of MEFs from wild-type and p53-deficient mice. However, the major disadvantages of such a system involve possible adaptive changes in gene expression acquired during the development of p53-knockout mice and a high degree of genomic instability of p53^{-/-} cells that may greatly affect individual gene expression patterns of cell clones. At the same time, expression profiling of Ras-mediated cell transformation can only be carried out in cells that have lost functional p53. To overcome these problems, we have constructed a retroviral vector pL56_IRES_RAS that coexpresses oncogenic H-Ras^{v12} together with the powerful dominant-negative p53 inhibitor GSE56 (Ossovskaya et al. 1996) and allows oncogenic transformation of primary cells, which can be then compared with the parental cell population to determine genetic changes accompanying this process. We next demonstrated that retroviral transduction of MEFs with pL56_IRES_RAS leads to the establishment of morphologically transformed cultures, while expression of GSE56 alone caused effective immortalization of MEFs.

Complete transformation of MEFs is associated with unlimited proliferative capacity, low sensitivity to growth factor withdrawal, reduced contact inhibition leading to formation of multilayered cultures, anchorage independence, and tumorigenicity (Reddy et al. 1982;

Seeburg et al. 1984; Greig et al. 1985; Zhan and Goldfarb 1986). To identify genetic changes associated with the initiation of neoplastic growth that are preserved throughout the entire process of transformation, we generated a set of clonal MEF cultures that includes variants of a complete or incomplete tumorigenic phenotype (Fig. 2). Thus, we generated four phenotypically different transformed clonal cultures (56R1, 56R2, 56R4, 56R7) established from individual transformed colonies infected with retroviral vector encoding GSE56_RAS (Fig. 2A). MEFs infected with GSE56 alone were represented by two cell cultures (56SN1, 56SN5) derived from two independent immortalized clones. These cell lines were compared with a population of MEFs transduced with control vector. Western blot analysis confirmed that all transformed cultures expressed various levels of oncogenic Ras (Fig. 2B), resulting in substantial differences of cyclin D1 induction. Establishment of H-Ras^{v12} transformation was also associated with increased expression of the p53 inhibitor, GSE56, as compared with cell cultures not exposed to oncogenic Ras, presumably because of a less strict requirement for p53 suppression (Serrano et al. 1997). Ras-transformed cultures were all characterized by rapid proliferation and low sensitivity to serum withdrawal (Fig. 2C). As opposed to parental and immortalized cell populations, the majority of Ras-transduced clones were capable of anchorage-independent growth, although this property correlated with relative levels of Ras expression among the four clones used: Clone 56R2

Figure 2. Subcloning and selection of cell cultures with properties of complete neoplastic transformation. (A) Cell morphology at day 14 after MEFs were transduced with indicated constructs and randomly subcloned into cell subcultures for further analysis of their properties. (B) Western blots of cellular lysates that indicate expression levels of GSE56, oncogenic Ras and its downstream target cyclin D1 in described cell cultures. (C) Doubling time and growth factor dependence of MEFs infected with GSE56 (clones 56SN1, 56SN5), GSE56-IRES-Ras (clones 56R1, 56R2, 56R4, 56R7) and with EGFP. (D) Growth of indicated MEF cell subcultures in the absence of anchorage support. (E) Ability of indicated cell subcultures to form tumors in vivo. Cells (5×10^6) were injected into athymic nu/nu mice. Twenty-four days later mice were sacrificed and tumors were weighed.

was not able to grow at all under these condition, while 56R7 produced a lower number of colonies than 56R1 and 56R4 (Fig. 2D). Anchorage independence correlated well with tumorigenicity of MEF cultures as assessed by their ability to form tumors following subcutaneous injection in athymic nu/nu mice: 56R1 and 56R4 were highly tumorigenic, while 56R2 produced much smaller tumors and 56R7 was unable to yield any detectable tumor growth similar to immortalized and control cell populations (Fig. 2E). In summary, a strong correlation was determined between the high levels of expression of oncogenic Ras, its downstream target cyclin D1, and tumorigenicity of transformed MEFs.

Characterization of individual clones of MEFs generated by introducing immortalizing (GSE56) or transforming (GSE56 + Ras) genetic elements allowed us to classify them according to the stage of transformation that they represent (Table 1). We presumed that our genes of interest, those that act to prevent Ras-mediated transformation by causing premature senescence, are likely to be among genes that are either universally down- or up-regulated in all immortalized and transformed cultures (universally p53-dependent) or after introduction of Ras (Ras- and p53-dependent).

Repression of gene expression is a predominant response to p53 inactivation in MEFs

To investigate changes in gene expression resulting from inactivation of p53 and overexpression of oncogenic Ras that might account for acquisition of transformed phenotypes, we analyzed gene expression profiles of all of the above-described MEF populations using NIA 15K Mouse Developmental cDNA microarray, which contains 15,264 genes represented by 52,374 expressed sequence tags derived from pre- and peri-implantation embryos, embryonic day 12.5 female gonadymesonephros, and newborn ovary (Tanaka et al. 2000). Cy3-labeled cDNA probes were prepared from total RNA isolated from each MEF population, then mixed with control cDNA labeled with Cy5 (prepared from total RNA of original MEF) and hybridized with the array. Each RNA

sample was analyzed twice (including synthesis of probes) to produce two independent replicas for comparative analysis of gene expression. Genes demonstrating a reliable twofold or greater difference in expression, as compared with control MEF in all comparative calculations inside and between the replicas, were picked for further analysis. A total of 1327 genes were found to be up- or down-regulated in all genetically modified populations analyzed, which represents ~10% of the entire set of genes included in the array.

Importantly, clusterization of all transformed and immortalized MEF cultures according to the expression patterns of the 1327 selected genes revealed that they group exactly according to their biological properties (transformed and immortalized MEFs formed two separate clusters, while highly tumorigenic MEFs formed a separate subcluster distinct from weakly transformed MEFs) (Fig. 3A). This result allowed us to conclude that indeed we have obtained genetic “fingerprints” of individual stages of MEFs transformation. Since each transformation step was represented by several clonal populations, we could also define and eliminate from further analysis those genes that were specific for individual clones and, therefore, likely to reflect natural clonal variability of MEFs unrelated to the studied phenomenon. More than 70% of genes that passed through this selection criteria were found to be down-regulated in immortalized and transformed MEFs (Fig. 3A), indicating that gene repression was the predominant genetic response in MEFs that lack functional p53, independent of oncogenic Ras expression.

Establishment of the list of tumor suppressor candidates

After determining that phenotypically similar cell lines show clear hierarchical clustering, we have intersected their microarray profiles of down-regulated transcripts to obtain clone-independent sets of genes repressed in each phenotype (Fig. 3B). Our microarray analysis of immortalized and transformed phenotypes has revealed that inhibition of p53 results in the suppression of the genes

Table 1. Summary of the properties of genetically modified MEFs used for microarray profiling

Cell line	Genotype	Phenotype
56R1 56R4	Wild-type MEFs with GSE56 + H-RAS ^{v12}	Express high levels of RAS ^{v12} , GSE56 and cyclin D1. Unlimited growth at 10% and 1% serum. Growth in methyl cellulose and athymic nu/nu mice.
56R2 56R7		Express low levels of RAS ^{v12} , high levels of GSE56, no cyclin D1 induction. Unlimited growth at 10% and 1% serum. Weak or no growth in methyl cellulose and/or athymic mice.
56SN1 56SN5	Wild-type MEFs with GSE56	Express low to moderate levels of GSE56. Unlimited growth at 10% serum and arrest at 1%. No growth in methyl cellulose and/or athymic mice.
EGFP	Wild-type MEFs with EGFP	Limited growth at 10% serum and arrest at 1%. No growth in methyl cellulose and in athymic mice.

Figure 3. Microarray profiling of transformation mediated by suppression of p53 and overexpression of H-Ras^{v12}. (A) Hierarchical clustering of expression measurements from 1327 genes in MEFs with down-regulated p53 (56SN1, 56SN5) and in MEFs with down-regulated p53 that overexpress H-RAS^{v12} (56R1, 56R2, 56R4, 56R7) and that show a consistent pattern of up- or down-regulation compared with primary MEFs. Each row represents a genetically modified MEF cell line and each column represents expression of a single gene across MEF specimens. Red indicates increased gene expression and blue indicates decreased gene expression relative to the median expression level in a primary MEF culture. The right panel shows the proportion of up- and down-regulated genes that exhibit the same pattern of regulation in hierarchically close MEF species. (B) Venn diagram of the number of genes whose suppression was common and unique between p53 dependent immortalization and transformation of MEFs. Down-regulated genes that exhibit the same pattern of regulation in hierarchically close transformed MEFs were intersected with down-regulated genes from immortalized MEFs to identify the targets whose inhibition was mediated only by repression of p53, as opposed to the genes that required additional activity of oncogenic Ras in order to be repressed.

implicated in a wide variety of cellular processes. To distinguish which of those genes function as mediators of a p53-dependent tumor suppressive mechanism, we intersected clone-independent gene expression profiles of immortalized and transformed cells with inactivated p53 (Fig. 3B). Genes that were common for both profiles (Table 2) include potentially new tumor suppressor candidates that were repeatedly silenced in response to p53 inhibition when primary MEFs were induced to independently undergo immortalization or transformation. Not surprisingly, the list of genes presented in Gene Table 2 contains a number of known p53-responsive targets, such as *Cyclin G1*, *BTG2*, *IGF-II*, *TGF β 2*, and *PDGF α* . This set could contain genes, down-regulation of which determines the nature of cooperation of p53 suppression and oncogenic Ras in transformation of primary cells. In particular, 21 genes had been identified that exhibited clone-independent mode of regulation and were found to be down-regulated at all stages of transformation, including immortalized and partially transformed cells expressing GSE56 (Gene Table 2, in bold). In addition, 16 genes had been found that are not specific for partially transformed cells and might provide clues to the processes occurring at the later stages of epigenetic selection during transformation.

The second group of potential Ras cooperators is represented by the genes whose down-regulation was found

unique only to transformed phenotypes after comparative analysis of p53-mediated immortalization and transformation (Table 3). These genes are repressed as a result of oncogenic Ras expression in the absence of p53 and could include p53-independent targets that are either capable of cooperating with Ras during the course of transformation or directly and indirectly result from Ras signaling activity. Altogether, this list includes 62 genes that are repressed in all cell variants carrying oncogenic Ras (Table 3, in bold) and 29 genes that require high levels of Ras expression associated with complete transformation in order to be repressed. In addition, there are 27 unknown or uncharacterized EST sequences. The list of candidates contains several known genes directly related to Ras induced transformation such as *H-Rev107* and *Par-4* (Hajnal et al. 1994; Barradas et al. 1999).

We next sought to confirm the results of microarray analysis and picked three genes from each group of down-regulated genes to compare their expression in RNA samples isolated from transformed, immortalized, and primary cells. From the group of p53-dependent genes, we selected *Cyclin G1*, *Gadd45 γ* , and *Hsp25*; from the group of genes that responded to oncogenic Ras activity in addition to p53 inhibition, we selected *H-Rev107*, *Par-4*, and *eIF4G3*. The results of hybridizations with genes selected from both groups were in complete accordance with microarray data and, more impor-

Table 2. *Genes repressed in a p53-dependent manner*

NIA 15K ID	Name	Symbol
H3136H11-3	Fibronectin leucine-rich transmembrane protein 2	FLRT2
H3005D09-3	Follistatin-like 3	FSTL3
H3147B03-3	Myosin regulatory light chain 2 smooth muscle isoform	MYRL2
H3126A04-3	Insulin growth factor-II	IGF-II
H3035B02-3	Tropomyosin α^*	TPM1*
H3135D11-3	β -Tropomyosin isoform 2	TPM2(2)
H3054F05-3	Lysozyme M	LYZ
H3002C07-3	β -Tropomyosin isoform 1	TPM2(1)
H3059F09-3	B-cell translocation gene 2*	BTG2*
H3053E04-3	Calponin 1	CNN1
H3054C02-3	Growth arrest and DNA damage-inducible γ 45*	GADD45 γ^*
H3141F12-3	Four and a half LIM domains 1	FHL1
H3041F03-3	Melanoma X-actin	ACTX
H3009C10-3	Serine protease inhibitor 6	SPI6
H3082D09-3	Small heat-shock protein 25*	HSP25*
H3055G07-3	Transforming growth factor β 2*	TGF β 2*
H3109H11-3	Cysteine-rich protein 2	CRIP2
H3024B07-3	Cyclin G*	CCNG1*
H3037F12-3	Dipeptidyl peptidase IV	DPP4
H3007H02-3	Gap junction membrane channel protein β 3	GJB3
H3125A09-3	Glutathione peroxidase 3	GPX3
H3016F06-3	Nuclear myosin I β	NM1
H3146C02-3	Platelet derived growth factor α	PDGF α
H3011A03-3	Protein-L-isoaspartate D-aspartate O-methyltransferase 1	PCMT1
H3042F12-3	Serine protease inhibitor 4	SPI4
H3137G02-3	Glucocorticoid-inducible protein 5	GIS5

Table listing genes repressed in all of the cell lines expressing GSE56 in bold face type plus the genes whose down-regulation was unique only to highly tumorigenic cell lines. Genes marked with an asterisk were selected for further analysis.

tantly, support the overall pattern of gene repression obtained in silico for the rest of the genes delineated in immortalized and transformed cells (Fig. 4). The “p53-only-dependent” group of genes show little dependency for hyperactive Ras in order to decrease their expression to the level that permits primary cells to undergo transformation (Fig. 4A,B). By contrast, genes whose inhibition requires activity of oncogenic Ras in the absence of p53 demonstrate a different pattern of suppression. Abolishment of functional p53 by GSE56 has very minimal effect on the expression of these genes as seen from Northern blot analysis of *H-Rev107* and *Par-4* (Fig. 4C,D) and might serve only as an initiation step toward their silencing (Fig. 4C, cf. EGFP and GSE56), a process that in this case drastically depends on the activity of mutant Ras.

The identification of known p53-responsive and oncogenic Ras-regulated genes in our experimental setup and the confirmation of their down-regulation pattern by Northern analysis provided strong supportive evidence that our list of selected candidate tumor suppressors indeed contains biologically relevant genes.

Screening of gene-specific shRNAs in Ras cooperation assay reveals BTG2 as a repressor of Ras-mediated transformation

Identification of gene repression as a predominant pattern of global transcriptional changes in MEFs undergo-

ing transformation, as well as candidate gene targets themselves, have provided a basis for a screening design aimed at the isolation of new tumor suppressor genes. This approach was based on the combination of RNA interference (RNAi)-mediated gene silencing technology (Mello and Conte 2004) together with a Ras cooperation screening assay to identify the genes whose suppression would promote transformation of primary cells.

Our hypothesis was that genes acting as mediators of p53 tumor-suppressive function were likely to be among those that were universally down-regulated in all transformed and immortalized cultures that express GSE56. From genes delineated in Table 2, we selected six candidates (encoding TPM1, BTG2, GADD45 γ , HSP25, TGF β 2, and CCNG1) with most relevant biological function for testing whether their down-regulation would promote Ras-mediated transformation of primary cells. Nonetheless, we could not completely exclude those genes that, in addition to p53 inactivation, required H-Ras^{v12} signaling to be repressed and mediate induction of the neoplastic phenotype. Thus, we included a subset of transcripts from Table 3 (*PAR-4*, *DOK-1*, *H-Rev107*, *eIF4G3*) to the previously selected group of genes. Short inverted repeats homologous to the identical regions of mouse and human genes selected from Tables 2 and 3 and picked with the aid of PickSi software were synthesized and cloned into retroviral pLHXP vector under the control of an H1 promoter, thus allowing RNA polymerase III-driven expression of

Boiko et al.

Table 3. *Genes repressed in a p53 + HRas^{v12}-dependent manner*

NIA 15K ID	Name	Symbol
H3011B01-3	Mitogen inducible gene	MIG-2
H3114H08-3	37 Kda leucine-rich repeat LRR protein	p37NB
H3125D01-3	Collagen type I α 2	COL1 α 2
H3085H02-3	Protein disulfide isomerase-related	PDIR
H3116A08-3	General transcription factor 3C polypeptide 2	GTF3C2
H3115C09-3	Lim domain-containing translocation partner in lipoma	LPP
H3136G08-3	Caldesmon 3' UTR	CALD1
H3152F10-3	Pmepa1 protein	PMEPA1
H3038E03-3	PRKC apoptosis WT1 regulator*	PAWR*
H3059B02-3	Regulator of G-protein signaling 12	RGS12
H3038D07-3	Similar to kinesin-like 4	KNSL4
H3152G04-3	α -2 collagen type VI subunit	COL4 α -2
H3098G04-3	Red-1 gene	MXN
H3124B09-3	Amyloid β precursor protease	APP
H3018F05-3	P-cadherin 3	CDH3
H3026G09-3	Pro- α 1 II collagen chain gene	COL2 α -1
H3052F12-3	Spi2 proteinase inhibitor	SPI2/EB1
H3137C06-3	Transglutaminase	TGASE
H3159G06-3	α -1 type IV collagen	COL4 α -1
H3140F04-3	Annexin A6	ANXA6
H3009B01-3	Bone morphogenic protein	BMP-1
H3039H01-3	Calponin 2	CNN2
H3015E11-3	C-terminal Lim domain protein 1	PDLIM1
H3090F04-3	Chondroitin 4-O-sulfotransferase	C4ST-2
H3057D05-3	Colony-stimulating factor 1 macrophage	CSF1
H3005E03-3	Cysteine-rich protein	CSRP
H3053A08-3	Cytochrome P450	CYP1A1
H3124E04-3	CXC chemokine ligand 12	CXCL12
H3124A07-3	eIF4E-like protein*	4E-LP*
H3120B11-3	Fibulin 2	FBLN2
H3046A09-3	Fk506-binding protein 6	FKBP6
H3119H03-3	Fvb/N collagen pro- α -1 type I chain	COL1 α -1
H3154E07-3	Growth-arrest-specific 6	GAS6
H3066G03-3	Guanine nucleotide-binding protein α 14	GNA14
H3126F05-3	Insulin-like growth factor-binding protein 5	IGFBP5
H3031H04-3	Integrin β -5	ITG β 5
H3122E01-3	Leman coiled-coil protein variant 1	LCCP
H3150E06-3	Lymphocyte-specific 1	LSP1
H3040F11-3	Lysosomal thiol reductase	IFI30
H3136G09-3	Luman basic leucine zipper protein	LZIP
H3058D07-3	Matrix metalloproteinase 23	MMP23
H3146E06-3	S-Gicerin/Muc18	MCAM
H3122G06-3	Neuropilin	NRP
H3038G07-3	Nuclear receptor corepressor 2	NCOR2
H3134H12-3	Osteoblast-specific factor 2 fasciclin I-like	OSF2
H3122H01-3	Paxillin-like protein gene	HIC5
H3028H10-3	Peptidylprolyl isomerase C	PPIC
H3140C02-3	Platelet-derived growth factor receptor β	PDGFR β
H3156E09-3	Procollagen type V α 2	COL5 α 2
H3017E11-3	Retinoblastoma-binding protein 7	RBBP7
H3001H12-3	Ribosomal protein S6 kinase 90kd polypeptide 2	RPS6KA2
H3002E01-3	Selenoprotein R	SEPR
H3031C08-3	Sh3 domain protein 5	SH3D5
H3115E04-3	Shroom	SHRM
H3119A03-3	Synapsin I	SYN1
H3109C02-3	Tissue inhibitor of metalloproteinase 3	TIMP3
H3116G07-3	Transmembrane 4 superfamily member 6	TM4SF6
H3159E09-3	Unc-51-like kinase 2 <i>C. elegans</i>	ULK2
H3008A09-3	Ras homolog gene family, member B	RhoB
H3040H07-3	Hras-revertant gene 107*	HREV107*
H3101H02-3	Myotonic dystrophy kinase-related Cdc42-binding kinase	MRCK
H3129C10-3	Steroid-sensitive gene-1	SSG-1

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Table 3. (continued)

NIA 15K ID	Name	Symbol
H3055G12-3	Thrombin receptor	CF2R
H3123A05-3	Staufen RNA-binding protein homolog 1	STAU1
H3153A05-3	Socs box-containing Wd protein	SWIP-2
H3113F12-3	Short incubation prion protein	PRNPA
H3061D06-3	Fat 1 cadherin	MFAT1
H3151E12-3	Downstream of tyrosine kinase 1*	DOK1*
H3115F11-3	Nidogen 2	NID2
H3020G05-3	Nebulin-related anchoring protein	NRAP
H3010D09-3	Vinculin	VCL
H3104D07-3	Mammary-derived growth inhibitor	MDGI
H3120B07-3	Gelsolin	GSN
H3121C02-3	Follistatin-like protein	FSTL
H3110F10-3	Epoxide hydrolase 1	EPHX1
H3056G12-3	Enigma homolog 2	ENH2
H3152D01-3	Cyclin D2	CCND2
H3116C03-3	Subcomponent C polypeptide	C1QC
H3121G09-3	Chondroitin sulfate proteoglycan 2	CSPG2
H3097B09-3	Cd97 antigen	CD97
H3079D07-3	Coiled-coil protein 1	CC1
H3081D02-3	Bcl-2-related ovarian killer	BOKL
H3125C05-3	Prolyl 4-hydroxylase	P4HB
H3114H12-3	Collagen 3 α -1	COL3 α 1
H3134D09-3	Semaphorin III family homolog	SEMA3
H3019H02-3	Vanilloid receptor 1	VR1
H3075F12-3	Tumor endothelial marker 8	TEM8
H3053A07-3	Spinocerebellar ataxia 1	SCA1
H3116A11-3	Px19-like protein	PX19
H3017H10-3	Lim and Sh3 protein 1	LASP1
H3116E09-3	Core histone macroh2a2.2	MACROH2A2

Table listing genes repressed in all of the cell lines expressing GSE56 + HRas^{v12} in bold plus the genes whose downregulation was unique only to highly tumorigenic cell lines. Genes marked with an asterisk were selected for further analysis.

shRNAs against the genes of choice. As a control, mouse p53 cDNA was also included in the list of target genes to monitor the efficiency of inhibitory oligo sequence selection as well as their ability to mediate Ras transformation in functional assays that followed the synthesis and cloning of shRNAs.

Primary MEF cultures were infected first with shRNA-expressing retroviruses targeting gene candidates, either individually or as a mixture. Seventy-two hours later, each cell population was infected with retroviral constructs expressing H-Ras^{v12} in combination with neomycin resistance gene followed by G418 selection to eliminate cells that were not transduced with oncogenic Ras. After 14 d of selection, cells infected with control shRNA (targeting GFP) failed to grow, forming a culture of sparse growth-arrested cells displaying characteristics of a senescent phenotype, while cells transduced with a mixture of shRNAs as well as with individual shRNAs against p53 and one of the candidate genes, *BTG2*, produced numerous colonies of proliferating cells with grossly transformed morphology (Fig. 5A). The inhibitory effect of shRNAs on the expression of target genes was confirmed by measuring the level of their corresponding mRNAs using semiquantitative RT-PCR or real-time RT-PCR assays (shown for *BTG2* and *p53* in Fig. 5B,C).

BTG2 was previously isolated as an early growth-response gene (Bradbury et al. 1991) whose expression can be modulated by p53 when induced by DNA-damaging agents (Rouault et al. 1996). Here, for the first time, we show that down-regulation of *BTG2*, similarly to p53, makes cells susceptible to oncogenic transformation.

To further investigate the tumorigenic properties of the cells transformed with *BTG2* shRNA and activated Ras, we have analyzed their anchorage dependence and tumorigenicity. When plated in semisolid media containing methylcellulose, these cells, in sharp contrast to those transduced with shRNA against GFP and H-RAS^{v12}, were able to grow and formed numerous colonies (Fig. 5D). For in vivo tumorigenicity assays, 10⁷ cells of each cell type were injected subcutaneously in both flanks of four female nu/nu or SCID/Beige mice. After 14 d, cells transformed with *BTG2* shRNA and mutant Ras formed tumors at all sites of injection, indicating that they have acquired complete neoplastic phenotype (Fig. 5E).

We next sought to determine whether p53 modulates expression of *BTG2* during immortalization and transformation of the primary cells. Real-time PCR analysis of MEFs expressing GSE56 with or without oncogenic Ras revealed that functional inactivation of p53 is always accompanied by the significant reduction in *BTG2*

Figure 4. Comparison of behavior of the genes that become repressed after p53 knockdown in the presence or absence of oncogenic Ras among three types of cell cultures. (A) Relative expression of the genes that were commonly down-regulated in both immortalized and transformed cells. Values of expression are calculated relative to average gene expression level (dotted line) determined for all genes represented in the array. (B) Northern blot analysis of indicated genes that were down-regulated in MEFs in response to p53 suppression and independent of oncogenic Ras overexpression. (C) Relative expression of the genes that were down-regulated exclusively in transformed cells. Values of expression are calculated relative to average gene expression level (dotted line) determined for all genes represented in the array. (D) Northern blot analysis of indicated genes whose down-regulation was only specific for transformed MEFs where p53 inhibition was accompanied by overexpression of H-Ras^{v12}.

expression (Fig. 5F). Similar to GSE56, cells expressing shRNA targeting p53 had reduced the level of *BTG2* mRNA (Fig. 5B). Cells transduced with shRNA against *BTG2* retain p53 mRNA levels unchanged (Fig. 5C); moreover, p53 in *BTG2*-knockdown cells was functional as judged by the activation of p53 reporter construct, pWafConA_mCMVLacZ, in response to p53-inducing stimuli (Fig. 5G). We then analyzed expression of p21, one of the main p53-dependent target genes that mediate growth arrest function of p53 upon DNA damage (el-Deiry et al. 1993). shRNA-mediated knockdown of *BTG2* did not affect p53-mediated induction of p21 by oncogenic Ras (Fig. 5H). Since functional inactivation of p53 in MEF can be reached by the loss of *ARF* expression (Inoue et al. 2000; Kamijo et al. 1997), we analyzed the expression of this gene in sh*BTG2* + Ras-transformed MEF. *ARF* expression was detectable in all MEF cell populations, including those expressing shRNA against *BTG2*, and its expression became elevated after transduction of oncogenic Ras (Fig. 5J), thus showing the expected pattern of regulation (Palmero et al. 1998; Lin and Lowe 2001).

Our data, therefore, demonstrates that suppression of p53 target gene, *BTG2*, can indeed sensitize cells to the

acquisition of tumorigenic phenotype in the presence of functional p53, thus providing a missing link between p53 activation and growth suppression.

Suppression of BTG2 overcomes Ras-induced growth arrest of human fibroblasts

Since our experiments with mouse cells demonstrated that inhibition of *BTG2* can mimic suppression of p53 in its ability to rescue Ras-mediated senescence, our next step was to test whether *BTG2* suppression could play a similar role in human cells.

Similar to mouse cells, activation of oncogenic Ras in human fibroblasts induces terminal growth arrest phenotypically similar to cellular senescence (Serrano et al. 1997; Lin et al. 1998). However, in human cells, induction of senescence in response to oncogenes depends on the integrity of p53 and p16/pRb pathways. In some reports, p16/pRb function was shown to be a major factor preventing senescence-associated growth arrest in human fibroblasts transduced by oncogenic Ras (Serrano et al. 1997; Wei et al. 2001), while others suggest that p53 is of equal importance (Voorhoeve and Agami 2003). Apparent controversy between these reports most likely is due to the fact that the levels of p16 vary significantly among substrains of human fibroblasts and their passaging history in cell culture.

We transduced early-passage human diploid fibroblasts IMR90 having low basal expression levels of p16 (data not shown) with lentiviral vectors expressing inhibitory shRNAs against human *BTG2*, *p53* (Brummelkamp et al. 2002), or *GFP*, followed by infection with another lentiviral vector coexpressing *H-Ras^{v12}* and neomycin resistance genes. After 2 wk of G418 selection, it became evident that introduction of shRNA against *BTG2* or *p53* rescued IMR90 cells from growth arrest caused by transduction of oncogenic Ras (Fig. 6A). While ~95% of IMR90 cells transduced by control shRNA against *GFP* and *Ras* stained positive for a senescence marker (acidic β -galactosidase) and stopped growing, only 30% of cells expressing shRNAs against *BTG2* and 5% of cells expressing shRNA against *p53* showed positive β -galactosidase staining and these cultures were able to proliferate, regardless of the presence of oncogenic Ras, until they reached confluence (Fig. 6B). Further passaging of these cells indicated that they indeed escaped (rather than delayed) Ras-mediated growth arrest. The efficacy of inhibitory effect of previously isolated mouse shRNA for human gene homolog of *BTG2* was confirmed using real-time RT-PCR (Fig. 6C). Thus, similar to mouse cells, inhibition of *BTG2* allows human fibroblasts to overcome the growth suppressive effect of Ras oncoprotein.

Knockdown of BTG2 results in up-regulation of cyclins D1 and E1 and phosphorylation of Rb and contributes to transformation of human fibroblasts

Loss of protective response against oncogenic stress is an essential step toward neoplastic transformation. There-

Figure 5. shRNA-mediated knockdown of BTG2 or p53 allows Ras-mediated transformation of primary MEFs. (A) Primary MEFs were infected with a mixture as well as with individual shRNAs against candidate tumor suppressor genes; all cultures were then superinfected with LRasSN virus-containing supernatants and then seeded at 5×10^5 cells per 100-mm plate. Infected populations were grown in the presence of 0.4 mg/mL of G418. Morphologically transformed colonies appearing 2 wk later were photographed, stained, and counted. (B) Real-time PCR analysis of BTG2 mRNA levels in cells expressing shRNAs for BTG2, p53, and GFP. (C) Semi-quantitative RT-PCR analysis of p53 mRNA levels in cells expressing shRNA for p53, BTG2, and GFP. (D) MEFs transformed by a combination of oncogenic Ras and shRNAs for the indicated genes were resuspended in normal culture medium containing 1.4% methyl cellulose and seeded at 10^4 cells per well, and in duplicates into six-well plates coated with 1.2% low-melting-point agarose. Cultures were grown under standard tissue culture conditions, cells were fed twice weekly, and the number of macroscopically visible foci was scored after 3 wk. (E) The ability to form tumors in vivo was assessed by injecting 4×10^6 transformed cells under the skin of both flanks of athymic nu/nu mice; growth of tumors was analyzed 18 d later. (F) Levels of BTG2 mRNA in MEFs expressing GSE56 + HRas, GSE56, or EGFP used for microarray analysis determined by real-time PCR. (G) p53 retains its transactivation ability in the cell with down-regulated BTG2 (results of detection of the activity of p53-responsive lacZ after induction with the indicated DNA-damaging drugs). (H) Western blot analysis of p21 expression in MEFs transduced with constructs expressing shRNAs against the indicated genes with or without oncogenic H-RAS^{V12}. β -Actin is used as loading control. (I) Semi-quantitative RT-PCR analysis of *ARF* mRNA levels in MEFs expressing shRNA against p53, BTG2 and GFP, with or without oncogenic H-RAS^{V12}.

fore, we next examined whether suppression of *BTG2* can promote and be part of a multistep transformation process in human cells. With identification of a minimal set of genetic elements sufficient for neoplastic transformation of human primary cells (Hahn et al. 1999, 2002; Voorhoeve and Agami 2003), it has become possible to address the individual role of candidate tumor suppressor genes in human oncogenesis. We transduced IMR90 cells with lentiviruses expressing catalytic subunit of human telomerase (H-TERT), small t-antigen of SV40 (*st*), and oncogenic Ras (IMR90-TtR). Expression of all these proteins is essential, but not sufficient, for full transformation of human fibroblasts, which also requires inactivation of both p53 and pRb pathways (Hahn et al. 2002; Voorhoeve and Agami 2003; Rangarajan et al. 2004).

Since *BTG2* was functioning as a downstream member of the p53 pathway in mouse cells, we expected that

repression of this gene might substitute inhibition of p53 and, in cooperation with pRb repression, lead to full transformation of human cells. To test this assumption, we delivered shRNA against *BTG2*, with or without shRNA against *p16* (Voorhoeve and Agami 2003), to IMR90-TtR cells. shRNA against *p53* and *GFP* were used as positive and negative controls, respectively. Surprisingly, suppression of *BTG2* did not induce anchorage-independent growth of IMR90-TtR cells even if introduced in combination with shRNA against *p16* (data not shown). At the same time, shRNA against *BTG2* dramatically induced anchorage independent growth of IMR90-TtR cells if combined with p53 inhibitory element GSE56 (Fig. 7A). This effect of shRNA against *BTG2* was similar to that of shRNA against *p16*, suggesting that *BTG2* is involved in regulation of Rb pathway and therefore suppression of *BTG2* can cooperate with suppression of p53 in transformation of human fibro-

blasts. Recent reports indicate that BTG2 can be induced by p53-independent mechanisms including NF- κ B activation and ARF signaling (Kuo et al. 2003; Kawakubo et al. 2004) that might explain why additional down-regulation of BTG2 is required, even when p53 is repressed, in order to reach transformed phenotype.

To gain insights in the mechanisms of cooperation between down-regulation of BTG2 and p53 in transformation of IMR90-TtR fibroblasts, we analyzed expression of various cell cycle regulatory proteins known to be down-regulated by oncogenic Ras and involved in the establishment of neoplastic phenotype. Western blot analysis data indicate that expression of shRNA against BTG2 in IMR90-TtR cells with GSE56 results in the up-regulation of cyclins D1 and E1 (Fig. 7B) as compared with the nontransformed IMR90-TtR-GSE56 cells. Thus, for the

Figure 6. Inhibition of BTG2 rescues human diploid embryonic lung fibroblasts, IMR90, from oncogene-mediated growth arrest. (A) IMR-90 cells infected with shRNAs against BTG2, p53, and GFP were superinfected with lentivirus-expressing H-Ras^{v12} + Neo^r cassette and selected in G418-containing media for 14 d, after which surviving resistant cells were fixed and stained with Methylene Blue. (B) Resistant IMR-90 cells expressing H-Ras^{v12} + Neo^r and shRNAs for the indicated genes were stained for senescence-associated endogenous β-galactosidase activity and photographed. Results were calculated as the percentage of visible cells that had positive staining (at least five different fields were counted for each plate). (C) BTG2 expression is down-regulated in IMR-90 cells that express shRNA for BTG2 or p53 as measured by real-time PCR.

Figure 7. BTG2 cooperates with p53 to prevent transformation of human IMR90 cells. (A) Primary IMR90 cells were sequentially infected to express a combination of oncogenic elements that are required but not sufficient for tumorigenic transformation (TERT/st/GSE56/Ras), and then super infected with shRNA lentiviral constructs against the indicated genes. Cells were grown in methyl cellulose and the number of macroscopically visible foci was scored after 3 wk. (B) Western blot analysis of genetically transduced IMR90 cells for the expression of the indicated proteins. (C) A model in which BTG2 integrates p53 and other signaling networks to control cell division. Cell proliferation and, ultimately, formation of malignant tumors largely depends on pRb phosphorylation status, which controls the activity of the E2F family of transcription factors necessary for induction of the genes that regulate DNA synthesis. Rb phosphorylation status is, in turn, mediated by CDKs bound to activating cyclins. Oncogenic and other stress signals that might lead to the formation of a malignant phenotype activate BTG2 through p53-dependent and p53-independent mechanisms leading to reduced expression of D- and E-type cyclins, hypophosphorylation of Rb, and the inhibition of cell growth.

first time up-regulation of G1-type cyclins has been directly linked to BTG2 down-regulation in human cells. Previous reports on the down-regulation of cyclin D1 mRNA level in 3T3 cells (Guardavaccaro et al. 2000) or cyclin E1 in 293T cells (Lim et al. 1998) which ectopically overexpressed BTG2, complement our data. Cyclins D1 and E1 are key regulators of CDK4, CDK6, and CDK2, which are activated upon binding to corresponding cyclins (Koff et al. 1991; Matsushime et al. 1992; Xiong et al. 1992; Meyerson and Harlow 1994) after their induction by growth-activating signals (Won et al. 1992; Ohtani et al. 1995). We further analyzed whether up-regulation of cyclins D1 and E1 by shRNA against BTG2 affected the phosphorylation status of Rb, the main tar-

BTG2 mediates anti-Ras function of p53

get of CDK4/Cyclin D1 and CDK2/Cyclin E1 complexes, which controls G1/S entry checkpoint and is one of the most critical factors affecting cellular decision between proliferation and growth arrest or apoptosis (Sherr 1993; Weinberg 1995). While the protein level of Rb was not largely affected by the BTG2 suppression, phosphorylation of Ser807 but not Ser795 was strongly induced in the cells expressing shRNA against *BTG2* (Fig. 7B).

Part of the biological effects of Ras are mediated via Akt pathway (Khwaja et al. 1997) which can result in modulation of cyclin D1 expression both at protein and mRNA levels (Diehl et al. 1998; Burgering and Medema 2003). Hence, we tested whether the effect of *BTG2* suppression on the expression cyclin D1 could be due to the differences of Akt activation. As shown in Figure 7B, oncogenic Ras induces phosphorylation of Akt at Ser473, however, none of the above described genetic modifications, including expression of shRNAs against *BTG2* or *p16* had any effect on the protein level of activated Akt.

Based on these results, we propose a model (Fig. 7C) in which BTG2 is a critical point of convergence of signaling pathways, including p53, that regulates phosphorylation of Rb through the expression of D- and E-type cyclins, which is necessary to inactivate Rb-mediated block of proliferation and turn on E2F-dependent promoters required for cell cycle progression and survival (Dyson 1998; Harbour et al. 1999; Ezhevsky et al. 2001).

Down-regulation of *BTG2* in human cancers

The *BTG2* gene localizes to chromosomal locus 1q32 (Rouault et al. 1996), which is frequently lost in a subset of breast cancers (Chen et al. 1989). Immunohistological studies have shown that BTG2 is highly expressed in several normal tissues, including kidney proximal tubules, lung alveolar bronchial epithelia, and prostate acinar cells (Melamed et al. 2002) and reduced BTG2 expression was recently reported in various cancer cell and malignant tissue samples (Ficazzola et al. 2001; Kawakubo et al. 2004; Struckmann et al. 2004). After establishing the link between BTG2 suppression and transformation in both mouse and human cell lines, we wished to determine whether deregulation of this gene has any clinical relevance. To test how common the changes in BTG2 expression in human tumors are, we used the BD Clontech Cancer Profiling Array I, which contains multiple pairs of cDNA samples generated from mRNAs of matched tumor and normal tissue samples of the same patient. Overall, this array includes 13 tissues, most of which are represented by 10 or more patients. Nylon membranes containing cDNA samples normalized to the housekeeping genes, β -actin and ubiquitin, were hybridized with the 32 P-labeled *BTG2* cDNA probe. Relative intensities of the signals, reflecting relative abundance of *BTG2* mRNA in normal versus tumor tissues, were quantitated and the results were plotted using logarithmic scale. After analysis of obtained results we found that expression of BTG2 was down-regulated in a large proportion of renal and breast cell carcinomas and to lesser extent in patients with stomach cancer (Fig. 8),

Figure 8. BTG2 expression analysis in human cancers using BD Clontech Cancer Profiling Array I, consisting of genetically matched cDNA pairs derived from tumor and adjacent normal tissue samples.

thus arguing for the importance of *BTG2* down-regulation in naturally occurring human cancer.

Discussion

p53 is one of the most frequently mutated genes found in human cancers (Levine 1997). Inactivation of p53 makes cells susceptible to transformation by activated oncogenes in vitro and makes both mice and humans (Li-Fraumeni syndrome patients) highly cancer prone (Finlay et al. 1989; Donehower et al. 1992; Malkin 1994). Consistently, wild-type p53 can inhibit tumor cell growth in vitro and in vivo and makes primary cells resistant to oncogenic transformation (Eliyahu et al. 1989; Baker et al. 1990). The mechanisms of p53 activation by dominant oncogenes, including oncogenic Ras, are relatively well understood and involve transcriptional activation of tumor suppressor ARF detracting Mdm2 from p53 (Kamijo et al. 1997; Jacobs et al. 1999; Inoue et al. 2000). In contrast, factors acting downstream of p53 and capable of inhibiting cell growth in response to oncogenic signaling remain largely unknown. Although there is a strong belief that p53 exerts its tumor suppressor function through transcriptional regulation, it remained unclear which, among the wealth of p53-responsive genes identified, are responsible for Ras-induced growth arrest.

In this work we developed an approach to identification of such genes. It involved gene expression profiling of different stages of transformation of mouse fibroblasts by the combination of p53-suppressing element and oncogenic Ras followed by the establishment of the list of candidate suppressors of Ras-mediated transformation and their functional validation via screening of focused shRNA in Ras transformation assays. Although microarray-based gene expression profiling of p53-dependent transcription has been applied previously to several cell

systems (Polyak et al. 1997; Kannan et al. 2000; Zhao et al. 2000), we for the first time compared p53-dependent changes in gene expression between normal nontransformed MEFs and MEFs that underwent a single-step transformation. In fact, the most common models used in the studies of Ras-induced transformation involve immortalized cells (such as NIH 3T3), which are suitable for transformation by a single oncogene. Hence, such cells have presumably already acquired genetic alterations, including reduced expression of tumor suppressor genes, which prevent primary MEF from transformation. Indeed, loss of p53 function seems to be a prerequisite for transformation, but the genes through which it exerts its growth arrest activity in response to Ras remained unknown, although they are likely to be found among the p53-responsive ones. To avoid analysis of established immortalized cell cultures that have passed through numerous generations in vitro and could acquire genetic alterations irrelevant to predisposition to transformation, we have designed a retroviral construct coexpressing GSE56, and H-Ras^{v12}, which was capable of full single-step transformation of primary cells. The use of GSE56-only retrovirus allowed us to distinguish between p53- and Ras-dependent effects and to pick those genes that were stably deregulated by p53 suppression alone and in combination with Ras. Another technically important solution that allowed us to dramatically reduce the number of candidates for testing was parallel analysis of several independently picked clonal cell populations, which made it possible to get rid of those differentially expressed genes that had no relation to transformation and simply reflected clonal variability of cells.

An observation made at this stage of the work was a strong prevalence of down-regulated over up-regulated genes in the list of candidates. Since p53 is both transcriptional activator and transcriptional repressor, this result argues that loss of p53-dependent transcriptional activation is likely to have stronger impact on the tumorigenic phenotype rather than the release of p53-mediated repression. It also allowed us to focus primarily on identification of candidate tumor suppressors rather than oncogenes. We therefore generated a set of shRNA-expressing constructs against a selected subset of candidates and screened them in Ras transformation assay with the desire to find those that would allow Ras-induced transformation without repression of p53. As we show here, BTG2 appeared to possess all these properties.

BTG2 is an early growth response gene (Bradbury et al. 1991) whose promoter contains p53-binding sites and is regulated by p53 (Rouault et al. 1996). The importance of BTG2 expression in the ability of p53 to resist oncogenic transformation was further demonstrated in the experiments showing that tumorigenic phenotype acquired by cells as a result of BTG2 inactivation and Ras signaling did not require inactivation of p53 itself or its upstream regulator p19^{ARF}.

Recently, a BTG2-knockout mouse was generated that appeared to be viable but exhibited abnormalities in ver-

tebrae formation during early development (Park et al. 2004). Thus far, BTG2-null mice have not been reported to develop any spontaneous tumors in the early stages of their lives. The absence of sporadic tumor phenotype could be explained by the fact that BTG2 belongs to the class of closely related antiproliferative genes known as the *tob* family, which includes *TOB*, *TOB2*, *ANA*, *PC3B* and *BTG1* (Matsuda et al. 2001) and which could have a compensatory tumor suppressive effect in BTG2-null mice. Similarly to Rb-deficient mice, where it was demonstrated that only multiple knockouts of Rb family members made mice prone to spontaneous tumorigenesis (Classon and Harlow 2002), long-term studies as well as interbreeding experiments between knockout members of the *tob* family are required to uncover their tumor suppressive properties in vivo.

There has been evidence suggesting that BTG2 might be growth suppressive when overexpressed in some tumor cell lines (Montagnoli et al. 1996; Guardavaccaro et al. 2000); however, until now there were no data showing that reduced expression of BTG2 can sensitize cells to the acquisition of tumorigenic phenotype. Here we first demonstrate that shRNA-mediated knockdown of BTG2 in primary human lung fibroblasts IMR-90 abolishes oncogene-mediated growth arrest and allows their proliferation in the presence of hyperactive Ras. Unlike mouse cells, human cells are not completely transformed by the combination of BTG2 knockdown and Ras activation. This is well in line with prior reports indicating that inactivation of p53 is not sufficient to mediate Ras-induced transformation and requires the involvement of oncogenes of DNA containing viruses such as E1A of adenovirus, large and small T-antigens of SV40, and papilloma virus E6/E7—all known to target both p53 and pRb pathways (Hahn et al. 1999; Morales et al. 1999; Elenbaas et al. 2001). Interestingly, we found that in human cells shRNA-mediated down-regulation of BTG2 results in hyperphosphorylation of Rb at Ser807, presumably via up-regulation of cyclins D1 and E1 and, in combination with dominant-negative p53 mutant (GSE56), small T antigen of SV-40, and catalytic subunit of H-TERT, was capable of inducing full transformation of primary human fibroblasts IMR-90. This effect of shRNA against BTG2 was similar to that of shRNA against *p16*, suggesting that p53-responsive BTG2 is involved in regulation of Rb pathway, and therefore suppression of BTG2 can cooperate with suppression of p53 in transformation of human fibroblasts. This finding indicates another regulatory link between p53 and Rb pathways that is mediated by BTG2. However, the need to have both anti-BTG2 shRNA and GSE56 for transformation of human cells might simply reflect that neither alone can reach strong enough level of inhibition of BTG2.

Recent reports on reduced expression of BTG2 in different sets of human malignancies (Ficazzola et al. 2001; Kawakubo et al. 2004; Struckmann et al. 2004) have been extended by our analysis of a panel of matching tumor and normal tissue samples showing that a large proportion of breast and kidney carcinomas have significantly

less *BTG2* mRNA expression than adjacent normal tissues, thus further confirming relevance of *BTG2* deregulation to naturally occurring cancer. Obviously, reduced levels of p53-responsive gene *BTG2* in some cancers can be due to p53 inactivation by mutations. This hypothesis, however, cannot explain frequent down-regulation of *BTG2* in renal cell carcinoma, the type of cancer that is known to rarely acquire mutations in *p53* gene (see the International Agency for Research on Cancer TP53 Mutation Database, <http://www.IARC.fr/p53/index.html>).

By defining *BTG2* as the major player in anti-Ras activity of p53 we have validated the approach we used (consisting of microarray profiling and functional screening in Ras transformation assays) to identify new components of p53 pathway acting downstream of p53. On the other hand, the differences in scales of the effects of *BTG2* and *p53* knockdowns both in vitro and in transgenic animals suggest that *BTG2* is not the only mediator of anti-Ras activity of p53. We therefore believe that the extension of this work toward characterization of the rest of the candidate genes listed in Tables 2 and 3 is important to find the remaining tumor suppressors of this type.

Materials and methods

Cells

Primary MEFs were prepared as described previously (Zou et al. 2002). IMR-90 cells are human diploid embryonic lung fibroblasts that were obtained from American Type Culture Collection.

Recombinant retro- and lentiviral vectors

The plasmid-encoding dominant-negative inhibitor of p53, GSE56, was described in Ossovskaya et al. (1996). pLRasSN was generated by inserting full-length cDNA of H-Ras^{V12} mutant into pLXSN (Clontech). To generate pL56_Ras plasmid pLXSN vector was used to replace SV40-Neo^r region with a full-length H-RAS^{V12} cDNA, the 5' end of which was previously ligated to the internal ribosomal entry site (IRES) sequence; GSE56 was then inserted into polycloning site under BamHI and EcoRI sites. To generate lentiviral pLV56_Ras plasmid, the fragment containing GSE56_IRES_Ras was recloned into pLVCMVH4 vector under control of CMV promoter. Lentiviral p53 reporter plasmid, pUST_WafConA_mCMVLacZ, was described previously (Razorenova et al. 2005). To generate H-TERT and *st* lentiviral constructs, their respective cDNAs (kind gifts of R.A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA) were cloned into lentiviral vector pLVCMVH4.

Retroviral/lentiviral transduction

For the preparation of retroviral stocks Phoenix-ampho packaging cells (Grignani et al. 1998) were used, while 293T cells were used for preparation of lentiviral stocks. Cells were transfected with the vectors, using Lipofectamin-Plus reagent (Invitrogen Inc.); in the case of lentivirus, two helper plasmids, pCMVΔR8.2 and pVSV-G, were cotransfected along with experimental vector. Supernatants containing infectious viral particles were harvested 24, 36, and 48 h post-transfection, pooled, and filtered.

Infections of exponentially growing cells were performed every 5 h for 2 d with various dilutions of virus-containing supernatant supplemented with 4 μg/mL polybrene (Sigma).

Selection and cloning of shRNA oligonucleotides

PickSI Software was used to select appropriate 19-nucleotide (nt) fragment from the target gene cDNA sequence (homologs in mouse and human) and then to generate 19-nt inverted repeat of the selected sequence. The two fragments were then joined together by the 9-nt spacer TTCAAGAGA and a poly(T) stretch was added at the 3' end of the resultant 47-nt oligo as a stop codon for RNA polymerase III. The BamHI and HindIII cohesive ends were added at the 5' and 3' ends, respectively. Resulting 64-nt short interfering RNA (siRNA) oligos were synthesized at Integrated DNA Technologies, annealed according to manufacturer's protocol, and cloned into modified pBabe-puromycin vector. This vector contains the RNA polymerase III-dependent H1 RNA promoter cloned into NheI site of the U3 region of the 3' long terminal repeat (LTR), which was used to drive the expression of the siRNAs. siRNA oligos were cloned into BglII and HindIII sites immediately downstream of H1 RNA promoter. The successful 19-nt target sequences used were shBTG2(1): 5'-TCAACCACAAGATGGACCC; shBTG2(2): GGTTCTGGC TATCGCTGTA-3'; and mp53: 5'-GTACATGTGTAATAGCT CC-3'. p16 was reported in Voorhoeve and Agami (2003); hp53 was described in Brummelkamp et al. (2002).

Western immunoblotting

Protein lysates preparation, electrophoresis, and blotting followed standard protocol. The following primary antibodies were used: H-Ras (F235), p16 (C-20), p21 (F5, F235), p53 (Pab 246), GSE56 (G59-12), cdk4 (C-22) (Santa Cruz), pRb (14001-A, Pharmingen), Phospho-Rb (Ser795), Phospho-Rb (Ser807/811) (Cell Signaling), cyclin D1(Sc-450), and cyclin E1 (M20, C19).

RNA expression analysis

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. *BTG2* mRNA quantitation was performed using a fluorescence-based real-time RT-PCR technology (TaqMan Real-Time PCR) with probe-primers sets for mouse and human *BTG2* (ABI Assay-on-Demand FAM-MGB probes), according to manufacturer's protocol. p53 and p19^{ARF} mRNA quantitation was performed using semiquantitative RT-PCR. The following primers were used: p53 sense: 5'-CCCGAGTATCTGGAAGACAG-3'; p53 antisense: 5'-ATAG GTCGGCGGTTTCAT-3'; p19^{ARF} sense: 5'-GTCGCAGGTTCT TGGTCACTGTGA-3'; p19^{ARF} antisense: 5'-CTTCTCAAGAT CCTCTCTAGCCTCAACA-3'.

For Northern blot analysis of GADD45γ, Hsp25, Cyclin G1, H-rev107, Par-4, eiF4G3, and GAPDH, 10 μg of total RNA was electrophoresed and transferred to the Hybond-NX membrane (Amersham Pharmacia Biotech) according to manufacturer's instructions. RNA blots were hybridized with specific ³²P radio-labeled probes for indicated genes in ExpressHyb solution (BD Biosciences Clontech) according to manufacturer's instructions.

Senescence-associated β-galactosidase assay

Cells were fixed with 0.5% glutaraldehyde (Sigma) and incubated in staining solution [1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆, 3.3 mM K₃Fe(CN)₆, 0.02% NP-40, 0.2% X-Gal (Sigma) at pH 5.5 at 37°C]. Plates were photographed and results were calculated as the percentage of visible cells that had positive staining (at least five different fields were counted for each plate).

Boiko et al.

p53 reporter assay

For measuring p53 transcriptional activity cells were infected with pUST_WafConA_mCMVLacZ plasmid, seeded on 96-well plates, and treated with p53-inducing agents: Doxorubicin (250 ng/mL), 5'-FU (50 µg/mL), MMS (50 µg/mL). β -Galactosidase activity was measured as previously described (Razorenova et al. 2005).

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Boiko et al.

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