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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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 p19ARF 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 p19ARF, 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 repression, rather than activation, was a governing factor regulating expression of the candidate genes. 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.

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-RasV12 together with the powerful dominant-negative p53 inhibitor GSE56 (Ossowska 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-RasV12 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 \(5 \times 10^6\) 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 accordingly 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 probes (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 replications 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

| Cell line | Genotype | Phenotype |
|-----------|----------|-----------|
| 56R1      | Wild-type MEFs with GSE56 + H-RAS<sup>12</sup> | Express high levels of RAS<sup>12</sup>, GSE56 and cyclin D1. Unlimited growth at 10% and 1% serum. Growth in methyl cellulose and athymic nu/nu mice. |
| 56R4      | Wild-type MEFs with GSE56 + H-RAS<sup>12</sup> | Express low levels of RAS<sup>12</sup>, 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. |
| 56R2      | 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. |
| 56R7      | Wild-type MEFs with GSE56 | Limited growth at 10% serum and arrest at 1%. No growth in methyl cellulose and in athymic mice. |
| 56SN1     | Wild-type MEFs with EGFP | |
| 56SN5     | Wild-type MEFs with EGFP | |
| EGFP      | Wild-type MEFs with EGFP | |
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 can-
didates that were repeatedly silenced in response to p53
inhibition when primary MEFs were induced to indepen-
dently 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, TGFβ2, and PDGFα.
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, includ-
ing immortalized and partially transformed cells ex-
pressing 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 pro-
cesses occurring at the later stages of epigenetic selec-
tion during transformation.

The second group of potential Ras cooperators is rep-
resented by the genes whose down-regulation was found
unique only to transformed phenotypes after compara-
tive analysis of p53-mediated immortalization and trans-
formation (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 ca-
pable of cooperating with Ras during the course of trans-
formation or directly and indirectly result from Ras sig-
aling 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 trans-
formation 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, Gadd45γ, 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 elf4G3. The results of hybridizations
with genes selected from both groups were in complete
accordance with microarray data and, more impor-
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 undergoing 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, \( GADD45 \), \( HSP25 \), \( TGF \), 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 aide of PickSi software were synthesized and cloned into retroviral pLHXPS vector under the control of an H1 promoter, thus allowing RNA polymerase III-driven expression of 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 isof orm | MYRL2         |
| H3162A04-3    | Insulin growth factor-II                        | IGF-II        |
| H305B02-3     | Tropomyosin α*                                  | TPM1*         |
| H315D11-3     | β-Tropomyosin isof orm 2                        | TPM2(2)       |
| H305F05-3     | Lysozyme M                                     | LYZ           |
| H300C07-3     | β-Tropomyosin isof orm 1                        | TPM2(1)       |
| H305F09-3     | B-cell translocation gene 2*                    | BTG2*         |
| H305E04-3     | Calponin 1                                      | CN1           |
| H305C02-3     | Growth arrest and DNA damage-inducible γ 45*   | GADD45γ*      |
| H3141F12-3    | Four and a half LIM domains 1                   | FHL1          |
| H301F03-3     | Melanoma X-actin                                | ACTX          |
| H300C10-3     | Serine protease inhibitor 6                     | SPI6          |
| H308D09-3     | Small heat-shock protein 25*                    | HSP25*        |
| H305G07-3     | Transforming growth factor β 2*                 | TGFβ2*        |
| H310H11-3     | Cysteine-rich protein 2                         | CRIP2         |
| H302B07-3     | Cyclin G*                                       | CCNG1*        |
| H307F12-3     | Dipeptidyl peptidase IV                        | DPP4          |
| H307H02-3     | Gap junction membrane channel protein β 3      | GJB3          |
| H312A09-3     | Glutathione peroxidase 3                        | GPX3          |
| H306F06-3     | Nuclear myosin 1 β                             | NM1           |
| H316C02-3     | Platelet derived growth factor α                | PDGFα         |
| H301A03-3     | Protein-L-isoaspartate-D-aspartate O-methyltransferase 1 | PCMT1        |
| H304F12-3     | Serine protease inhibitor 4                     | SPI4          |
| H313G02-3     | 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.
Table 3. Genes repressed in a p53 + HRasv12-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                | COL1α2 |
| 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 |
| H303E03-3      | FRKC apoptosis WT1 regulator*      | PAWR*  |
| H3059B02-3     | Regulator of G-protein signaling 12 | RGS12  |
| H303H07-3      | Similar to kinase-like 4           | KNSL4  |
| H3152G04-3     | α-2 collagen type VI subunit       | COL4α-2|
| H309G04-3      | Red-1 gene                         | MXN    |
| H312B09-3      | Amyloid β precursor protease       | APP    |
| H301F05-3      | P-cadherin 3                       | CDH3   |
| H302G09-3      | Pro-α1 II collagen chain gene      | COL2α-1|
| H305F12-3      | Spi2 proteinase inhibitor          | SPI2/E1B|
| H313C06-3      | Transglutaminase                   | TGA2E  |
| H315G06-3      | α-1 type IV collagen               | COL4α-1|
| H314F04-3      | Annexin A6                         | ANXA6  |
| H309B01-3      | Bone morphogenetic protein         | BMP-1  |
| H309H01-3      | Calponin 2                         | CNN2   |
| H301E11-3      | C-terminal Lim domain protein 1    | PDLM1  |
| H309F04-3      | Chondroitin 4-O-sulfotransferase   | C4ST-2 |
| H305D05-3      | Colony-stimulating factor 1 macrophage | CSF1  |
| H300E03-3      | Cysteine-rich protein              | CSRP   |
| H305A08-3      | Cytochrome P450                     | CYP1A1 |
| H312E04-3      | CXC chemokine ligand 12            | CXCL12 |
| H312A07-3      | elf4E-like protein*                | 4E-LP* |
| H312B11-3      | fibulin 2                          | FBLN2  |
| H304A09-3      | Fk506-binding protein 6            | FKBPs  |
| H311H03-3      | Fvb/N collagen pro-α1 type I chain | COL1α-1|
| H315E07-3      | Growth-arrest-specific 6           | GAS6   |
| H306G03-3      | Guanine nucleotide-binding protein α 14 | GNA14 |
| H312F05-3      | Insulin-like growth factor-binding protein 5 | IGFBP5 |
| H301H04-3      | Integrin β-5                      | ITGβ5  |
| H312E01-3      | Leman coiled-coil protein variant 1 | LCCP  |
| H315E06-3      | Lymphocyte-specific 1              | LSP1   |
| H304F11-3      | Lysosomal thiol reductase          | IFI30  |
| H313G09-3      | Luman basic leucine zipper protein | LZIP   |
| H305D07-3      | Matrix metalloproteinase 23        | MMP23  |
| H314E06-3      | S-Gicerin/Muc18                    | MCAM   |
| H312G06-3      | Neurophilin                        | NRP    |
| H303G07-3      | Nuclear receptor corepressor 2     | NCOR2  |
| H313H12-3      | Osteoblast-specific factor 2 fasciclin I-like | OSF2 |
| H312H01-3      | Paxillin-like protein gene         | HIC5   |
| H302H10-3      | Peptidylprolyl isomerase C         | PPIC   |
| H314C02-3      | Platelet-derived growth factor receptor β | PDGFβ |
| H315E09-3      | Procollagen type V α 2             | COL5α2 |
| H301E11-3      | Retinoblastoma-binding protein 7   | RBBP7  |
| H300H12-3      | Ribosomal protein S6 kinase 90kd polypeptide 2 | RPS6KA2 |
| H300E01-3      | Selenoprotein R                    | SEPR   |
| H301C08-3      | Sh3 domain protein 5               | SH3D5  |
| H315E04-3      | Shroom                             | SHRM   |
| H319A03-3      | Synapsin I                         | SYN1   |
| H310C02-3      | Tissue inhibitor of metalloproteinase 3 | TIMP3 |
| H311E07-3      | Transmembrane 4 superfamily member 6 | TM4SF6 |
| H315E09-3      | Unc-51-like kinase 2 C. elegans    | ULK2   |
| H300A09-3      | Ras homolog gene family, member B  | RhoB   |
| H304H07-3      | Hras-revertant gene 107*           | HREV107*|
| H310H02-3      | Myotonic dystrophy kinase-related Cdc42-binding kinase | MRCK |
| H312C10-3      | Steroid-sensitive gene-1           | SSG-1  |

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BTG2 mediates anti-Ras function of p53

Table 3. (continued)

| NIA 15K ID  | Name                           | Symbol |
|------------|--------------------------------|--------|
| H305G12-3  | 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  |
| H3101D09-3 | 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 | Cdh9 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                | COL3α1 |
| 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 + HRasV12 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-RasV12 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-RASV12, 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
Expression of the gene in shBTG2 + Ras-transformed cells. Since functional inactivation of BTG2 did not affect p53-mediated induction of p21 by 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 (Voorhees and Agami 2003). Apparently, 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 passing 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-RasV12 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-
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 pRb 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-κB 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 de-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 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-

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**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-Rasv12 + Neo' 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-Rasv12 + Neo' 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.
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 [Khwaia 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 tumor cell lines (Melamed et al. 2002) and reduced BTG2 expression is highly conserved in several normal tissues, including kidney proximal tubules, lung alveolar bronchial epithelia, and prostate acinar cells (Melamed et al. 2002), which BTG2 is highly expressed in several normal tissues, including kidney proximal tubules, lung alveolar bronchial epithelia, and prostate acinar cells (Melamed et al. 2002).

The BTG2 gene, containing 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 32P-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], 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 [Finsley 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 [Kamiyo 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 candidate 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 p19ARF.

Recently, a BTG2-knockout mouse was generated that appeared to be viable but exhibited abnormalities in vertebral 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 be 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 finds 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-RasV12 mutant into pLXSN (Clontech). To generate pL56_Ras plasmid pLXSN vested 24, 36, and 48 h post-transfection, pooled, and filtered. Supernatants containing infectious viral particles were harvested (Amersham Pharmacia Biotech) according to manufacturer's instructions. p53 and p19ARF mRNA quantitation was performed using semiquantitative RT–PCR technology (TaqMan Real-Time PCR) with probe-primers according to the manufacturer's protocol. BTG2 mRNA quantitation was performed using a fluorescence-based real-time RT–PCR [5'-CCCGAGTATCTGGAAGACAG-3'; p53 antisense: 5'-ATAGTTCGCGGTTCAT-3'; p19ARF antisense: 5'-TCAACCACAAGATGGACCC; shBTG2(2): GGTCTTGGC TATCGCTGTA-3'; and mp53: 5'-GTACATGTGTAATAGCT CC-3'. p16 was reported in Voorhoeve and Agami (2003); hps53 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, F325], p53 [Pab 246], GSE65 [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 [TaqlMan Real-Time PCR] with probe-primers for mouse and human BTG2 [ABI Assay-on-Demand FAM-MGB probes], according to manufacturer's protocol. p53 and p19ARF mRNA quantitation was performed using semiquantitative RT–PCR. The following primers were used: p53 sense: 5'-CCCGAGTATCTGGAAGACAG-3'; p53 antisense: 5'-ATACTGCCTGAGGAGCAGAC-3'; p19ARF sense: 5'-GTACATGTGTAATAGCT CC-3'; p19ARF antisense: 5'-CTCTCACATGTGTAATAGCT CC-3'.

For Northern blot analysis of GADD45b, Hsp25, Cyclin G1, H-rev107, Par-4, c-fosG3, 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-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 K3Fe(CN)6, 3.3 mM K4Fe(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).
p53 reporter assay

For measuring p53 transcriptional activity cells were infected with pUST_WafConA_mCMV LacZ 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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