Transcriptional Programs following Genetic Alterations in p53, INK4A, and H-Ras Genes along Defined Stages of Malignant Transformation

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Abstract

The difficulty to dissect a complex phenotype of established malignant cells to several critical transcriptional programs greatly impends our understanding of the malignant transformation. The genetic elements required to transform some primary human cells to a tumorigenic state were described in several recent studies. We took the advantage of the global genomic profiling approach and tried to go one step further in the dissection of the transformation network. We sought to identify the genetic signatures and key target genes, which underlie the genetic alterations in p53, Ras, INK4A locus, and telomerase, introduced in a stepwise manner into primary human fibroblasts. Here, we show that these are the minimally required genetic alterations for sarcomagenesis in vivo. A genome-wide expression profiling identified distinct genetic signatures corresponding to the genetic alterations listed above. Most importantly, unique transformation hallmarks, such as differentiation block, aberrant mitotic progression, increased angiogenesis, and invasiveness, were identified and coupled with genetic signatures assigned for the genetic alterations in the p53, INK4A locus, and H-Ras, respectively. Furthermore, a transcriptional program that defines the cellular response to p53 inactivation was an excellent predictor of metastasis development and bad prognosis in breast cancer patients. Deciphering these transformation fingerprints, which are affected by the most common oncogenic mutations, provides considerable insight into regulatory circuits controlling malignant transformation and will hopefully open new avenues for rational therapeutic decisions. (Cancer Res 2005; 65(11): 4530-43)

Introduction

Human carcinogenesis can be divided into defined clinicopathological stages. For example, colon cancer progression has been divided into distinct histologic stages directly correlated with genetic alterations in key tumor suppressors and oncogenes (1). Over the last two decades, the molecular nature of genes frequently mutated in human neoplasia was elucidated. Functionally, those genes can be divided to many categories. The most studied genes include signaling molecules (Ras, Src, Akt, tyrosine kinase receptors, etc.), core cell cycle regulators (pRB, p16INK4A, cyclins, etc.), and transcription factors (p53, E2F, NF-kB, etc.). This knowledge leads to the realization that neoplastic transformation involves aberrant signal transduction pathways intimately linked with the deregulated gene expression. Nevertheless, the underlying transcriptional changes, which arise as a consequence of sequential accumulation of genetic alterations and eventually drive the pathologic process, are still elusive.

We addressed this challenge by the microarray technology. Monitoring gene expression changes on a genome-wide scale has proven to be a powerful method to study transcriptional programs involved in carcinogenesis (2). Comparisons between normal tissues and corresponding tumors or between various tumor types revealed significant differences in their mRNA profiles, including hundreds of differentially expressed genes (2). By combining classic supervised statistical methods with unsupervised techniques, such as hierarchical clustering and its advanced variants (3), analysis of microarray data can potentially identify specific biological signatures that reflect profound alterations in cellular pathways and processes. Indeed, molecular signatures that correlate with diagnosis and prognosis were discovered (2, 4–6). Yet, associations of those signatures with specific biological processes and genetic alterations acquired in vivo along transformation are not obvious. The difficulties stem largely from different genetic backgrounds of patients, variable and uncharacterized mutations, and undefined contributions to a resulting expression pattern of several cell types, such as inflammatory, endothelial, and stroma cells in addition to the bona fide tumor cells. Those considerations made it almost impossible to use established malignant cell lines or naturally occurring tumors to dissect the contribution of individual tumor suppressors or oncogenes to the observed changes in gene expression.

Modeling of human carcinogenesis in vitro is an invaluable tool to examine the effects of individual oncogenes, tumor suppressors, and their combinations on the evolution of the transformed phenotype. In this way, recently, the defined combinations of oncogenic events required to convert primary human cells into full-blown tumors were determined. Initially, full transformation was achieved by the combination of viral oncogenes, such as large and small T antigens together with cellular genes, such as mutant Ras and telomerase (7, 8). Later on, the cellular counterparts of viral oncogenes were showed to be sufficient to transform primary human fibroblasts. These include inactivation of p53 and either pRB or p16INK4A tumor suppressors, overexpression of the catalytic
subunit of telomerase (hTERT), inhibition of protein phosphatase 2A, and abnormal activation of Ras downstream pathways (9, 10). Importantly, it was noted that different cell types vary significantly in their susceptibility to the same combination of transforming elements (11, 12).

Thus, to obtain both novel and more reliable results, we set out to study a stepwise process of malignant conversion that makes use of human primary isogenic cells.

Here, we describe a transcriptional program involved in malignant transformation in a unique cellular model. The model consists of WI-38 human diploid fibroblasts in which replicative senescence is overcome by using hTERT, resulting in sustained proliferation of cells. The resulting extraordinary large number of divisions [150 population doublings (PD)] following hTERT introduction eventually gave rise to the INK4A-deficient clones with a significantly higher rate of proliferation, defective contact inhibition checkpoint, and, perhaps most importantly, sensitivity to H-Ras-mediated transformation (13). Recent studies have denoted similar inactivation of p16INK4A and subsequent sensitivity to the H-Ras-mediated transformation in additional strains of human fibroblasts that overcome telomere-independent crisis during immortalization (14–16). Collectively, these studies, including our own, which model malignant transformation in vitro, created a framework of defined oncogenic aberrations that initiate and promote neoplastic process. Based on these analyses, we hypothesized that our in vitro cellular model could recapitulate, with the known limitations of cell transformation in culture, the distinct stages that characterize mesenchymal cell transformation initiation and progression.

We describe here the identification of specific “genetic signatures” associated with each of the genetic changes that lead from normal diploid cells to fully transformed and tumorigenic cells.

Materials and Methods

Cell culture, retroviral constructs, and infection. Primary human embryonic lung fibroblasts (WI-38), amphotropic and ecotropic Phoenix retrovirus-producing cells, and retroviral constructs and infection procedures have been described (13).

Subcutaneous tumorigenicity assay. Immunocompromised athymic nude mice (CD-1 nude; 6–8 weeks old) were irradiated with 4 Gy 24 hours before injection. WI-38/Tfast and its transformed derivatives (1 × 107 cells) were resuspended in 100 μL PBS. Immediately before injection, Matrigel (100 μL, Becton Dickinson, Franklin Lakes, NJ) was added to the cells. Tumor size was monitored every 5 days. Mice were sacrificed when the tumor reached a diameter of 1 to 1.5 cm or after 26 weeks of monitoring. Tumor size was monitored every 5 days. Mice were sacrificed when the tumor reached a diameter of 1 to 1.5 cm or after 26 weeks of monitoring.

Microarray hybridization and processing. Double-stranded cDNA was generated from 15 μg total RNA using the SuperScript Choice System from Invitrogen (Carlsbad, CA), with an oligo(dT)18 primer containing a T7 promoter site at the 3’ end (Genset, La Jolla, CA). cDNAs were purified via phenol/chloroform extraction followed by ethanol precipitation. Purified cDNA was used as template for in vitro transcription, using the Enzo BioArray High-Yield RNA Transcript Labeling kit (Enzo Diagnostics, New York, NY). Labeled in vitro transcripts were purified over RNeasy mini columns according to the manufacturer’s instructions. The labeled cRNA was fragmented at 94°C for 35 minutes in fragmentation buffer [40 mmol/L Tris acetate (pH 8.1), 100 mmol/L potassium acetate, 30 mmol/L magnesium acetate], and a hybridization mixture was generated by addition of 0.1 mg/mL herring sperm DNA, 0.5 mg/mL acetylated bovine serum albumin (Invitrogen), 1 mol/L NaCl, 10 mmol/L Tris-acetate, and 0.0001% Tween 20. A mixture of four control bacterial and phage cRNA panels (1.5 pmol/L BioI, 5 pmol/L BioC, 25 pmol/L BioD, and 100 pmol/L Cre) was included as an internal control for hybridization efficiency. Aliquots of each sample (12 μg cRNA in 200 μL hybridization mix) were hybridized to a GeneChip Human Genome Focus Array (Affymetrix, Santa Clara, CA). After hybridization, each array was washed according to the manufacturer’s protocol and stained with streptavidin-phycocyanin conjugate (Molecular Probes, Eugene, OR). The hybridization signal was amplified using biotinylated anti-streptavidin...
antibodies (Vector Laboratories, Burlingame, CA) followed by restaining with streptavidin-phycocerythin. Arrays were scanned by the GeneArray scanner G2500A (Hewlett Packard, Palo Alto, CA), and scanned images were visually inspected for hybridization imperfections. Arrays were analyzed using Affymetrix Microarray Suite software version 5.0 by scaling to an average intensity of 250.

Data analysis. Gene expression analysis was done in duplicate on 12 data points. Each transcript represented on the array was designated by Microarray Suite software version 5.0 as either absent, present, or marginal, and only those genes that had a present call ($P < 0.05$) in both repeats of at least one data point were retained. The log-transformed expression values of each gene were mean centered (by subtracting the average) and normalized to generate the final expression matrix, of 5,581 rows (genes) and 24 columns (samples), which served as the input for the super-paramagnetic clustering analysis (SPC; ref. 18). Stable, statistically significant gene clusters (3) were identified using this algorithm. Fold change: to meet the condition of a “2-fold increase” in condition A versus condition B, the lower of the two repeats of A must have at least twice the value of the higher one of B.

Results

Conversion of the INK4A locus–deficient WI-38/hTERT fibroblasts into tumor cells requires inactivation of p53 and H-RasV12 expression. To define the possible requirements for tumorigenesis in vivo, we first determined which of the oncogenic changes in WI-38 cells results in cells capable of forming tumors in mice. We showed previously that hTERT-induced immortalization of WI-38 human diploid fibroblasts results in the spontaneous emergence of rapidly proliferating variants (WI-38/Tfast). Those clones do not express INK4A locus genes (p16INK4A and p14ARF) and have elevated levels of the c-myc oncogene. In addition, these cells could be further transformed with a constitutively active H-RasV12 gene, which confers them with anchorage-independent growth (WI-38/Tfast/R cells). Inactivation of wild-type p53, using the dominant-negative polypeptide GSE56 (19), and H-Ras expression was induced by infection at the indicated time points (Fig. 2; Table 1). Samples were taken in duplicates at 12 points and hybridized with the GeneChip Human Genome Focus Array; the relative mRNA abundance of ~8,500 human genes was monitored. After standard preprocessing steps (see Materials and Methods), >200,000 expression values were collected from 24 microarrays. Of these, 5,581 probe sets passed a filter (see Materials and Methods) and were analyzed by an unsupervised clustering algorithm, SPC (18), to identify genes with a correlated pattern of expression. Our working hypothesis was that genes forming a cluster represent unique genetic signatures, which are able to distinguish between the stages of transformation. In addition, the unique expression profiles over the samples may contain information about transcriptional programs initiated by tumor suppressor inactivation (p16INK4A and p53) versus oncogene activation (H-Ras). By using this approach, we were able to identify 10 predominant, stable, and significant gene clusters. We focus below on the most informative of them.

A defect in myogenic differentiation program characterizes early stages of transformation. By using SPC analysis, we identified a set of 397 genes that showed orchestrated down-regulation at the early stages of transformation (Fig. 3A; Figure 1. Conversion of WI-38/Tfast fibroblasts into tumor cells requires inactivation of p53 and H-RasV12 expression. A and B, nude mice injected with either 1 x 10⁶ WI-38 cells expressing H-RasV12 (A) or H-RasV12 and the p53-inactivating peptide, GSE56 (B). S.c. tumor mass of ~1 cm diameter is clearly visible in (B). C, H&E staining of WI-38/Tfast/R/G tumor sample (cross-section magnification, ×100) revealing an unencapsulated but well-demarcated mass composed of densely packed spindle cells. This histologic pattern is most consistent with fibrosarcoma. D, summary of in vivo tumorigenicity assay. Representative of one experiment of three, which gave similar results.
Importantly, this set of genes allowed us to discriminate between samples bearing an intact INK4A locus (WI-38 and Tslow), in which expression of these genes was relatively high, versus the INK4A locus–deficient samples and their derivatives (Tfast, Tfast/G, Tfast/R, and Tfast/R/G), all of which showed reduced expression of the same set of genes. By analyzing functional annotations of the down-regulated genes in this group, we found that a significant fraction of them (62 of 397 genes) is involved in various aspects of mesenchymal cell development and differentiation and, more specifically, in the differentiation of the smooth muscle lineage. These include extracellular signaling molecules (BMP1, BMP2, and NOTCH3) and their modulators (IGFBP3, IGFBP4, LTBP1, LTBP2, etc.), mesenchymal lineage-specific transcription factors (TWSG1, PITX1, HHHEX, MSX2, SOX11, PMX1, and OSR2), and smooth muscle differentiation markers (ACTA2, ACTG2, FHL1, CALD1, MYL6, etc.; refs. 20–22). Coordinated reduction in the expression of numerous differentiation markers, as evidenced by the DNA microarrays results, led us to consider the possibility that WI-38 cells at this stage of transformation develop a defect in the smooth muscle terminal differentiation program.

To validate this hypothesis, we treated, in parallel, WI-38/hTERTslow (a representative of the normal cells) and WI-38/hTERTfast (INK4A locus–inactivated cells with a suspected defect in muscle differentiation) with TGF-β. This cytokine is a principal inducer of myogenic differentiation (23). We measured the changes in the expression of typical myofibroblast and smooth muscle cell markers, such as smooth muscle specific α isoform of actin (ACTA2 or α-SMA), caldesmon (CALD), and calponin 1 (CNN1). WI-38/hTERTfast and their progeny expressed lower basal levels of all three markers compared with the parental WI-38 fibroblasts and WI-38/TERTslow as was predicted by the microarrays analysis and validated by QRT-PCR. More significantly, on TGF-β treatment, dramatic induction of these markers was evident in WI-38/hTERTslow cells, whereas only marginal changes were observed in WI-38/hTERTfast cells under the same treatment regimen (Fig. 3B). These results were further explored by α-SMA immunofluorescence.

Table 1. Description of the samples selected for the microarray experiment

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample name</th>
<th>PDLs</th>
<th>Exogenously introduced genes</th>
<th>Karyotype</th>
<th>INK4A status</th>
<th>Tumor formation</th>
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<td>1</td>
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<td>puro</td>
<td>46, XX</td>
<td>Intact</td>
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<td>GSE56-puro</td>
<td>NA</td>
<td>Intact</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
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<td>puro</td>
<td>NA</td>
<td>Intact</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>WI-38/G, senescent</td>
<td>42</td>
<td>GSE56-puro</td>
<td>NA</td>
<td>Intact</td>
<td>NA</td>
</tr>
<tr>
<td>5</td>
<td>WI-38/T\text{flow}, G</td>
<td>40</td>
<td>hTERT-puro</td>
<td>46, XX</td>
<td>Intact</td>
<td>NA</td>
</tr>
<tr>
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<td>Intact</td>
<td>NA</td>
</tr>
<tr>
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<td>hTERT-puro</td>
<td>NA</td>
<td>Intact</td>
<td>NA</td>
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<tr>
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<td>hTERT-puro</td>
<td>46, X</td>
<td>Promoter region</td>
<td>hypermethylation</td>
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<tr>
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<td>hTERT-puro</td>
<td>der(X)t(X;17)</td>
<td>Promoter region</td>
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<td>hypermethylation</td>
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<tr>
<td>11</td>
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<td>12</td>
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<td>hTERT-puro</td>
<td>der(X)t(X;17)</td>
<td>Promoter region</td>
<td>hypermethylation</td>
</tr>
</tbody>
</table>

Abbreviation: NA, not assessed.
Figure 3. Identification of a genetic signature associated with the earliest detectable transformation stage. A, cluster of down-regulated genes correlating with INK4A locus expression. Set of 397 of 5,581 genes (that passed the filtering criteria using all 24 samples) identified by clustering all samples according to the similarity of their gene expression profiles. The cluster was identified by unsupervised analysis using the SPC clustering procedure. Rows, genes; columns, samples. The sample names are denoted at the top of the cluster and detailed in Table 1. Logarithmic color scale representing centered and normalized values (bottom left). All the samples with a silenced INK4A locus (Tfast and derivatives) showed reduced expression. Selected genes from the cluster, which are discussed in the text, are indicated at the right of the cluster. Smooth muscle differentiation markers are shown in red. A complete list of genes is available in Supplementary Fig. S1.

B, validation of smooth muscle differentiation in WI-38/hTERTslow and WI-38/hTERTfast cells, representing INK4A locus intact and silenced stages, respectively. QRT-PCR of smooth muscle differentiation markers: α-SMA (ACTA2), calponin 1 (CNN1), and caldesmon (CALD). Subconfluent WI-38/hTERTslow (65 PDLs, Tslow) and WI-38/hTERTfast (560 PDLs, Tfast) were made quiescent by serum deprivation for 24 hours. Then, the medium was replaced with medium containing 1 ng/mL TGF-β1 for 36 hours. Gene expression was normalized to the GAPDH expression in the same sample.

C, effect of TGF-β1 on the induction of α-SMA fibers in Tslow and Tfast cells exposed to serum-free medium or TGF-β1 for 36 hours and analyzed by immunofluorescence. Nuclei were stained with 4’,6-diamidino-2-phenylindole to show cell number in both control and TGF-β1-treated cells. Cells were prepared and treated as described in Materials and Methods in (B) above. Representative of several fields photographed at >1,000 magnification.

D, Western blot analysis showing the effect of TGF-β1 on the induction of α-SMA protein in the Tslow and Tfast cells. Blotting was done after exposure of cells to serum-free medium or 1 ng/mL TGF-β1 for 36 hours using antibodies specific to α-SMA and β-tubulin (β-tub; control).
analysis. Whereas a small fraction (~10%) of WI-38/hTERTslow cells showed low levels of constitutive α-SMA staining, exposure to TGF-β induced marked cytoskeleton reorganization and development of a prominent network of brightly stained actin filaments in almost 100% of the cells, suggestive of myofibroblast differentiation. In stark contrast, WI-38/hTERTfast cells showed no staining of actin fibers in the control sample, and only a few isolated cells developed slight staining of actin on TGF-β treatment (Fig. 3C). Western blotting analysis gave very similar results (Fig. 3D). These results provide compelling evidence that the myogenic differentiation program is impaired in WI-38/hTERTfast cells.

In addition to the smooth muscle differentiation markers, several cell cycle regulators showed a marked reduction of their expression during the transition from the Tslow to the Tfast phenotype. Among these are several Rb regulators, such as CDKN2A (p16INK4a), in agreement with our previous findings; ref. 13), CDKN1C (p57KIP2), negative regulators of cell proliferation (CREG and QSCN6), and genes with known tumor suppressor activity (LDOC1 and FAT). To confirm this trend, we measured the expression of two representative genes, p57KIP2 and DLOC1, by QRT-PCR. In agreement with the microarray results, both genes showed a dramatic reduction in their expression (Supplementary Fig. S2).

An additional functional group that showed decreased expression in the Tfast cells is composed of genes promoting apoptosis, such as TNFRSF1B, TNFRS21, DAPK3, HTATIP2, and Bnip3L, suggesting acquisition of increased resistance to some apoptotic stimuli starting from the premalignant stage.

**Early stages of transformation: high biosynthetic activity and embryonic marker reexpression negatively correlate with INK4A locus status.** In addition to the group of genes downregulated during hTERT-mediated immortalization and INK4A inactivation, we identified a large cluster of genes that show the opposite pattern of expression; that is, their expression was relatively low in primary and INK4A locus-intact hTERT-immortalized samples and elevated in cells with inactivated INK4A, including both premalignant and malignant samples (Fig. 4A; Supplementary Fig. S3).

Among the 250 genes that comprise this cluster, 138 genes were found to be involved in various aspects of cell metabolism and to a large extent (93 of 138) to have a direct role in protein synthesis (Fig. 4A). For example, 47 genes encode ribosomal proteins, which belong to the 40S (17 genes) and 60S (30 genes) ribosomal subunits. In addition to the ribosomal proteins, multiple genes that participate in different steps of protein biosynthesis showed increased expression. This group consists of genes associated with amino acid metabolism, amino acid activation (NARS, TARS, VARS2, VARS1, KSRRP, CARS, and QARS), translational initiation (EIF3S7, EIF3S5, EIF33, EIF2B1, etc.), translational elongation (EEF1B2 and TUFM), protein modification and protein folding (CCT7, CCT4, PPIA, HSPBP1, and PFDN2), and regulation of translation (ETF1, EIF4EBP1, etc.). A third group of genes up-regulated in these cells and associated with translation are nucleolar proteins (U5-200KD, SNRPD2, PABPC4, SF3A3, etc.) that were shown to regulate ribosome assembly and nucleocytoplasmic transport of mature ribosomal subunits (24).

Global up-regulation of genes associated with ribosomal biogenesis and translation could explain the increased proliferation rate of the WI-38/Tfast cells versus WI-38 and WI-38/Tslow. Indeed, the rate of protein synthesis was found to be a limiting factor in proliferation and growth of cells in several experimental models (24).

Another group of genes that was dramatically induced in all WI-38/Tfast samples was enriched for members of the cancer/testis-associated gene family. This group included the subfamilies MAGE (10 transcripts), GAGE (6 transcripts), SPANXC, and SSX1. Expression of SSX1 and MAGE was validated by QRT-PCR (Fig. 4B and C). Indeed, their expression was almost undetectable in primary cells and in the WI-38/Tslow cells but was induced 1,000-fold in the WI-38/Tfast cells and became even higher in the tumor sample derived from the WI-38/Tfast/R/G cells. The expression of these genes was described in many cancers, including sarcomas (25). This similarity suggests that this in vitro model reflects some of the physiologic changes that occur during neoplastic initiation in the presumed cell of origin of sarcoma.

Expression of several genes involved in apoptosis was also up-regulated in the WI-38/Tfast cells. Among them are both antiapoptotic (AATF and MCL1) and proapoptotic (TP53, TNFSF7, IL24, PDCD2, and SMAC) genes. The increase in the expression of these proapoptotic genes represents the possible activation of a cellular anticancer response at this stage.

As evident from this cluster, the protein biosynthetic pathway and embryonic antigen expression constitute major transcriptional programs, which are abnormally activated during the transition from normal to premalignant cells.

**Identification of p53 target genes along the process of malignant transformation: the “proliferation signature” emerges following p53 inactivation in WI-38/Tfast cells.** p53 inactivation serves as a hallmark of the malignant transformation process. Its role in the onset of cell death, cell cycle regulation, and genome stability has been extensively studied (26). However, the effect of p53 inactivation on the pattern of gene expression at different stages of the transformation process was not addressed previously. In contrast to previous studies, which aimed to identify p53 target genes by p53 overexpression or activation by different stresses in the context of cancer cells (27–29), we inactivated endogenous wild-type p53 protein in the normal cells as well as at the different stages of the transformation process and then searched for down-regulated target genes. By doing a pairwise comparison between isogenic samples isolated at each stage of the transformation process (WI-38, young; WI-38, senescent; WI-38/hTERTslow; WI-38/hTERTfast; and WI-38/hTERTfast/R) and differing only in their p53 status, we identified 210 transcripts that showed at least 1.6 reduction in at least two of six pairs (Table 2; data not shown). As shown in Table 2, well-known p53 direct transcriptional targets, such as cyclin G1, p21WAF1, PA26, DDR2, DlxR, WIG1, and TNFRS6 (CD95 or Fas antigen), showed reduced expression in all pairs (score 6). When we classified p53-responsive genes into functional groups, we found that p53 in the nonstressed cells regulates genes involved in a plethora of physiologic processes. The most prominent group consists of genes participating in apoptosis, such as TNFRS6 (CD95), TNFRSF10B (KILLER/DR5), TNFSF7, TNFRSF10D, APLP1, and NLK. Additional functional categories involve genes participating in cell cycle control (cyclin G1, p21WAF1, and WIG1), signal transduction (GRPS1, PDE5A, and RGS20), extracellular matrix (ECM) and cytoskeleton organization (COLL11A1, TAGLN, and ACTA2), and angiogenesis (THBS3). These results suggest that under regular cell culture conditions p53 constitutively transactivates many genes participating in a variety of physiologic processes.

The identification of many known p53 transactivation targets by this approach further supports the value of our experimental model in addressing the importance of p53 inactivation in the
Figure 4. Identification of a genetic signature of up-regulated genes associated with accelerated growth of premalignant and malignant cells. A, cluster of up-regulated genes that correlate with INK4A locus expression. Set of 250 of 5,581 genes (that passed the filtering criteria using all 24 samples) clustering all samples according to the similarity in their gene expression profiles. This cluster was identified and organized as described in Fig. 3A. All the samples with a silenced INK4A locus (T<sup>fast</sup> and its derivatives) showed high expression of this cluster. Selected genes from the cluster, which are discussed in the text, are indicated at the right of the cluster. A complete list of genes is available in Supplementary Fig. S3. B and C, QRT-PCR of MAGEA1 (B) and SSX1 (C) expression in young (p, young), senescent (p, sen.), T<sup>slow</sup>, T<sup>fast</sup>, and Tumor1 cells. Tumor1 cells were recovered from a tumor formed by T<sup>fast</sup>/R/G cells following injection into nude mice. Gene expression was normalized to the GAPDH expression in the same sample. Columns, averages of duplicate QRT-PCR measurements.
transformation process. To further understand the downstream effects of p53, we next identified genes that are transrepressed by p53. We found that inactivation of p53 by the dominant-negative peptide GSE56, at every stage of transformation, resulted in a concerted up-regulation of 168 transcripts (Fig. 5A; Supplementary Fig. S4). The highest expression was observed when p53 function was ablated in WI-38/T\textsuperscript{fast} cells (T\textsuperscript{fast}/G and T\textsuperscript{fast}/R/G samples). The same group of genes had the lowest expression in senescent WI-38 cells (p, senescent) and was only slightly up-regulated by p53 inactivation (G, senescent). Importantly, their expression patterns are correlated with the proliferation rate of each sample (data not shown). A detailed examination of this gene cluster revealed that it includes mainly genes associated with various aspects of cell proliferation, such as DNA replication, cell cycle progression and its control, DNA repair, and metabolic demands of cell growth. Due to this functional similarity, we termed this group of genes “inactivated p53-associated proliferation signature.” Genes that function in the G2-M phase of the cell cycle represented the largest functional category. More specifically, cyclin-dependent kinase CDC2 and its regulators such as cyclin B2, cyclin A2, CKS1B, CKS2, CDC25A, and CDKN3, whose function is critical for entrance to mitosis, showed marked up-regulation.

In addition, genes with distinctive function in mitosis, including mitotic spindle organization (TTK and kinesins), mitotic spindle checkpoints (BUB1, BUB1B, MAD2L1, and BIRC5), and chromosome segregation (CDC20, CENPF, ESPL1, UBE2C, PLK1, and STK12), were also up-regulated. This cluster also includes genes that are responsible for DNA packaging (HAT1, CHCI, SUV39H1, and TOP2A) and chromosome organization (H1FX).

In addition to the enhanced expression of the CDC2 kinase and its positive regulators, two inhibitors of CDC2, which are direct p53 transcriptional targets, CDKNIA and GADD45, showed marked down-regulation (data not shown).

p53 inactivation as well as deregulated expression of PLK1, STK12, and CDC2 were shown to promote aneuploidy (30, 31). Furthermore, tetraploid cells were readily detectable in the T\textsuperscript{fast}/R/G as well as in the resulting tumors (ref. 13; data not shown). To analyze if this p53-dependent and transformation progression-dependent up-regulation in the expression of multiple mitotic genes correlates with the development of polyploidy, we exposed cells to nocodazole, a microtubule-depolymerizing drug. Then, the DNA content of the cells was quantitated by FACS analysis. As shown in Fig. 5B, normal parental WI-38 (WI-38, puro) fibroblasts were unable to progress through the cell cycle in the presence of nocodazole, whereas their p53-deficient counterpart (WI-38, GSE56) replicated their DNA, resulting in 9% of polyploid cells. In agreement with the increased expression of proliferation signature genes in the WI-38/hTERT\textsuperscript{fast} cells, upon exposure to nocodazole, they produced 12.5% of polyploid cells. Inactivation of p53 in these cells (WI-38/hTERT\textsuperscript{fast}/G) resulted in the maximal expression of the proliferation signature, further promoting the fraction of the cells with more than 4N DNA content (20%). Less than 2% of polyploid cells were consistently seen in the untreated samples.

Fifty-one of the 168 transcripts form our inactivated p53-associated proliferation cluster (31% of the genes) belong also to the proliferation cluster found by Roesty et al. (32) in cervical cancer samples. They reported that the expression levels of the genes of their cluster were predictive of outcome in cervical cancer. To evaluate the clinical significance of our inactivated p53-associated proliferation signature genes, we tested its ability to predict the clinical outcome of cancer patients. To this end, we studied their expression levels in the large breast cancer gene expression data set of (33). We found that 71 genes from our proliferation cluster (42%) showed alteration in the 98 primary breast cancers included in the breast cancer study. More significantly, we were able to segregate all patients according to the expression of this signature into two groups (Fig. 5C). Those tumors that were characterized by a high level of expression had a significantly higher risk of death due to development of distant metastases within 5 years ($P = 4.9 \times 10^{-5}$). These results show that this inactivated p53-associated proliferation signature contributes to tetraploidy generation and has a strong predictive value regarding aggressive tumor behavior.

"Tumor-forming" genetic signature. Inactivation of wild-type p53 and expression of constitutively active H-Ras conferred WI-38/hTERT\textsuperscript{fast} cells with tumorigenic potential (Fig. 1). Therefore, identification of unique transcriptional changes in the WI-38/T\textsuperscript{fast}/G/R cells is of great significance for understanding the transforming efficiency of both genetic hits. To this end, we carried out pairwise comparisons of WI-38/T\textsuperscript{fast}/R versus their counterparts with dominant-negative p53 (GSE56), H-Ras, or a combination of both. Next, we identified a group of genes that showed the highest extent of Ras/GSE56 synergism; that is, their expression in the T\textsuperscript{fast}/R samples compared with the T\textsuperscript{fast}/R control had a higher fold change than the additive effects of H-Ras and GSE56 alone (Table 3). This tumor-forming genetic signature was composed mainly of secreted molecules that belong to the CXC chemokine family (CXCL1, CXCL2, IL8, CXCL6, and CXCL10), cytokines (IL1B, IL6, and CSF2), and modifiers of ECM (TFPI2, MMP3, PRSS2, CIQTNF1, PRSS3, and ADAMTS8).

The CXC chemokines were shown to play a role in processes essential for tumor growth, such as autocrine stimulation, angiogenesis, invasion and metastasis (34). High levels of expression and secretion of these chemokines was detected in melanoma, colon, pancreatic and breast tumors. In some cases, a direct correlation between metastatic potential and CXCL8 (IL8) secretion was described (35).

Because we observed the strongest synergism between H-Ras and p53 inactivation on the expression of CXCL1, we used QRT-PCR to study its expression in independent samples derived from WI-38 at different stages of transformation (Fig. 6). We found that expression of CXCL1 was extremely low in young WI-38 fibroblasts as well as in WI-38/T\textsuperscript{fast} and WI-38/T\textsuperscript{fast}/Neo cells. Inactivation of p53 or expression of constitutively active Ras up-regulated CXCL1 by 10- and 36-fold, respectively, compared with the Neo control. However, the combination of p53 inactivation and constitutive Ras expression resulted in >10,000-fold induction. Importantly, expression of CXCL1 was even further up-regulated in cells retrieved from a tumor sample originating from WI-38/T\textsuperscript{fast}/G/R, suggesting that this chemokine confers a selective advantage in tumor formation. We detected a similar pattern of expression of the IL1B gene (Fig. 6). The regulation of these secreted factors by H-Ras and p53 was not reported previously, to the best of our knowledge.

ECM turnover, which is governed mainly by matrix metalloproteinases (MMP), is essential for the ability of malignant cells to promote neovascularization, invasion, and metastasis (36). The activities of MMPs are under tight control of the physiologic tissue inhibitors of MMPs (TIMP). We observed profound changes in the expression pattern of several MMPs and TIMPs following
Table 2. Genes whose expression was down-regulated upon endogenous p53 inactivation

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Down-regulation upon p53 inactivation at the selected stage of the transformation*</th>
<th>Sum score †</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNG1</td>
<td>Cyclin G1</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>Cyclin-dependent kinase inhibitor 1A (p21, Cip1)</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>PA26</td>
<td>p53-regulated PA26 nuclear protein</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>DDB2</td>
<td>Damage-specific DNA binding protein 2, 48 kDa</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>FDXR</td>
<td>Ferrodoxin reductase</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>WIG1</td>
<td>p53 target zinc finger protein</td>
<td>1 1 1 1 1 1</td>
<td>6</td>
</tr>
<tr>
<td>TNFRSF6</td>
<td>TNF receptor superfamily, member 6 (CD95, Fas antigen)</td>
<td>1 1 0 1 1 1</td>
<td>5</td>
</tr>
<tr>
<td>C12orf5</td>
<td>Chromosome 12 open reading frame 5</td>
<td>1 1 1 1 1 0</td>
<td>5</td>
</tr>
<tr>
<td>ANXA4</td>
<td>Annexin A4</td>
<td>1 1 0 1 0 1</td>
<td>4</td>
</tr>
<tr>
<td>TNFRSF10B</td>
<td>TNF receptor superfamily, member 10B (KILLER/DR5)</td>
<td>1 1 1 1 1 0</td>
<td>4</td>
</tr>
<tr>
<td>APLP1</td>
<td>Amyloid β (A4) precursor-like protein</td>
<td>0 1 1 1 1 0</td>
<td>4</td>
</tr>
<tr>
<td>GAMT</td>
<td>Guanidinoacetate N-methyltransferase</td>
<td>1 0 1 1 0 1?</td>
<td>3</td>
</tr>
<tr>
<td>COL11A1</td>
<td>Collagen, type XI, α1</td>
<td>1 0 1 1 0 1</td>
<td>3</td>
</tr>
<tr>
<td>GPR51</td>
<td>G protein-coupled receptor 51</td>
<td>1 1 1 1 1 1</td>
<td>4</td>
</tr>
<tr>
<td>PDE5A</td>
<td>Phosphodiesterase 5A, cyclic guanosine 3',5'-monophosphate specific</td>
<td>1 0 1 1 1 1</td>
<td>4</td>
</tr>
<tr>
<td>PSTPIP2</td>
<td>Proline-serine-threonine phosphatase interacting protein 2</td>
<td>0 1 1 1 1 4</td>
<td>4</td>
</tr>
<tr>
<td>TAGLN</td>
<td>Transgelin</td>
<td>0 0 1 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>ACTA2</td>
<td>Actin, α2, smooth muscle, aorta</td>
<td>1 0 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>MFGE8</td>
<td>Milk fat globule-epidermal growth factor 8 protein</td>
<td>1 0 1 1 0 0</td>
<td>3</td>
</tr>
<tr>
<td>OSEF-2</td>
<td>Osteoblast-specific factor 2 (fasciclin I-like)</td>
<td>1 0 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Serine (or cysteine) proteinase inhibitor, clade E (nexitin, plasminogen activator inhibitor type 1), member 1</td>
<td>0 0 1 1 1 1</td>
<td>3</td>
</tr>
<tr>
<td>NET-6</td>
<td>Transmembrane 4 superfamily member tetraspan NET-6</td>
<td>0 0 1 1 1 0</td>
<td>3</td>
</tr>
<tr>
<td>CUL4B</td>
<td>Cullin 4B</td>
<td>0 0 1 1 0 1</td>
<td>3</td>
</tr>
<tr>
<td>RNASE6P1</td>
<td>RNase 6 precursor</td>
<td>0 0 1 1 1 0</td>
<td>3</td>
</tr>
<tr>
<td>NIK</td>
<td>Nemo-like kinase</td>
<td>0 0 1 1 0 1</td>
<td>3</td>
</tr>
<tr>
<td>PG56</td>
<td>Pregnancy-specific β1-glycoprotein 5</td>
<td>1 0 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>UGGCG</td>
<td>UDP-glucose ceramide glucosyltransferase</td>
<td>0 0 0 1 1 1</td>
<td>3</td>
</tr>
<tr>
<td>THBS3</td>
<td>Thrombospondin 3</td>
<td>1 0 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>TM7SF2</td>
<td>Transmembrane 7 superfamily member 2</td>
<td>1 0 0 1 1 0</td>
<td>3</td>
</tr>
<tr>
<td>COL4A5</td>
<td>Collagen, type IV, α5 (Alport syndrome)</td>
<td>0 1 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>COP22</td>
<td>Coatomer protein complex, subunit 2</td>
<td>1 1 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>CTP24</td>
<td>Cytochrome P450, subfamily XXIV (vitamin D 24-hydroxylase)</td>
<td>0 1 0 1 1 3</td>
<td>3</td>
</tr>
<tr>
<td>APG-1</td>
<td>Heat shock protein (hsp110 family)</td>
<td>0 1 1 1 0 0</td>
<td>3</td>
</tr>
<tr>
<td>RGS20</td>
<td>Regulator of G-protein signaling 20</td>
<td>1 1 0 0 0 0</td>
<td>3</td>
</tr>
<tr>
<td>TNFSF7</td>
<td>TNF (ligand) superfamily, member 7</td>
<td>1 0 0 0 1 1</td>
<td>3</td>
</tr>
<tr>
<td>KCNN2</td>
<td>Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2</td>
<td>1 0 1 1 0 0</td>
<td>3</td>
</tr>
<tr>
<td>PRSC</td>
<td>Corin</td>
<td>1 1 0 1 0 1</td>
<td>3</td>
</tr>
<tr>
<td>TNFRSF10D</td>
<td>TNF receptor superfamily, member 10D, decoy with truncated death domain</td>
<td>1 0 1 1 0 1</td>
<td>3</td>
</tr>
</tbody>
</table>

NOTE: Pairwise comparison between normalized values of each gene expression was done between the above samples infected with either control virus or GSE56-containing one (G). Gene that showed at least 1.6-fold reduction in the GSE56-containing sample in the corresponding pairwise comparison received score 1; otherwise, the score was 0.

*Comparisons were listed in the following order: (1) WI-38/p, young versus WI-38/G, young; (2) WI-38/p, senescent versus WI-38/G, senescent; (3) WI-38/Tlow (40 PDLs) versus WI-38/Tlow/G (140 PDLs); (4) WI-38/Tlow (215 PDLs) versus WI-38/Tlow/G (140 PDLs); (5) WI-38/Tfast versus WI-38/Tfast/G; and (6) WI-38/Tfast/R versus WI-38/Tfast/R/G.

† Only genes that showed significant changes in at least three of six comparisons (sum score 3) were enlisted.
introduction of H-Ras and GSE56 (Table 3). The expression of MMP3 was further validated by QRT-PCR analysis (Fig. 6). Once again, we observed a synergistic effect of H-Ras expression and p53 inactivation on MMP3 induction, and the high expression persisted during in vivo growth of the corresponding tumor. Of note, matrix MMP1 and MMP3 were reported to be targets of Ras transformation (37).

Thus, it seems that strong induction of proangiogenic and autocrine chemokines in concert with potent ECM modifiers is characteristic of the tumor-forming genetic signature. Those genes could form the basis of the in vivo tumorigenic potential conferred on cells by expression of H-Ras and by p53 ablation.

Discussion

Deregulated transcriptional programs resulting from the activation of oncogenes and inactivation of tumor suppressors underlie many important aspects of cancer. In this study, primary human fibroblasts were the cell of origin, gradually transformed to a tumorigenic state. Then, complex and multistep biological phenomenon of transformation was dissected to a distinct number of transcriptional programs that exhibit well-defined orderly temporal organization. This was achieved by the identification of stable clusters of gene expression along defined stages of the transformation process. The identification of these specific genetic signatures reflects the acquisition of specific physiologic features.

Figure 5.

Identification of the inactivated p53-associated proliferation signature. A, a cluster of 168 genes, which showed similar patterns of expression and was associated with p53 inactivation and increased cell proliferation rate. A complete list of genes is available in Supplementary Fig. S4. Note that expression is highest in the Tfast/GSE56 (Tfast/G) and lowest in the WI-38/puro senescent cells. This cluster was identified and organized as described in Fig. 3A. Selected genes from the cluster, which are discussed in the text, are indicated. B, effect of p53 inactivation on the different stages of transformation on DNA rereplication in response to nocodazole. Asynchronous cultures of WI-38/puro, WI-38/GSE56, WI-38/hTERTfast, and WI-38/hTERTfast/GSE56 were seeded into medium with or without 0.05 μg/mL nocodazole. After 72 hours, the cells were fixed and stained with PI for flow cytometry (FACS) analysis. Cells with DNA content greater than 4N were considered as polyploid and were quantitated by gating cells to the right of the 4N peak on histogram plots. C, prognostic value of the inactivated p53-associated proliferation signature in breast cancer patients described in the study of van’t Veer at al. (33). Gene symbols of the inactivated p53-associated proliferation signature (168 genes) were intersected with the gene symbols of the breast cancer profiling experiments. From our panel of 168 genes (Fig. 5A), 71 were also found in the study of van’t Veer at al. Then, a new expression matrix of 71 x 96 was created, such that each column represented a patient sample and each row represented a gene. Each column was labeled according to the patient prognosis: blue, good prognosis (no distant metastasis were developed within 5 years); green, poor prognosis (distant metastasis developed within 5 years). The samples were then sorted in an unsupervised manner according to their expression using the SPIN algorithm (58). The P for the separation was calculated using the Wilkinson rank-sum test. Tumors with high inactivated p53-associated proliferation signature had worse prognosis than tumors with low inactivated p53-associated proliferation signature ($P = 4.9 \times 10^{-5}$).
increasingly transformed cell populations suggests its active
differentiate. The fact that this genetic signature persisted in the
the disruption of the cell cycle regulation and inability to properly
that this genetic signature provides a mechanistic link between
types of soft tissue sarcomas (40–45). Our results strongly suggest
tissues similar to WI-38 fibroblasts, such as synovial and other
inactivation of p16INK4A and p57KIP2 were observed previously in
several molecules associated with myogenic differentiation was
transformation fingerprints. Nevertheless, recently, two studies
combinations of transforming genes might result in additional
differentiation-proficient and differentiation-deficient cells are still
proliferation is one of the hallmarks common to many tumor
transformation of different origins.

Table 3. Tumor-forming genetic signature

<table>
<thead>
<tr>
<th>Functional category, gene symbol and name</th>
<th>Fold induction relative to ( \text{T}^{\text{fast}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokines and cytokines</td>
<td>GSE56</td>
</tr>
<tr>
<td>CXCL1, chemokine (C-X-C motif) ligand 1*</td>
<td>1.24</td>
</tr>
<tr>
<td>CXCL3, chemokine (C-X-C motif) ligand 3</td>
<td>0.65</td>
</tr>
<tr>
<td>CXCL2, chemokine (C-X-C motif) ligand 2</td>
<td>2.64</td>
</tr>
<tr>
<td>IL1B, IL-1β*</td>
<td>1.17</td>
</tr>
<tr>
<td>IL8, IL-8</td>
<td>2.90</td>
</tr>
<tr>
<td>CSF2, colony-stimulating factor 2 (granulocyte-macrophage)</td>
<td>0.32</td>
</tr>
<tr>
<td>IL6, interleukin-6 (IFN, β2)</td>
<td>2.11</td>
</tr>
<tr>
<td>CXCL6, chemokine (C-X-C motif) ligand 6</td>
<td>0.87</td>
</tr>
<tr>
<td>ECM modulators</td>
<td></td>
</tr>
<tr>
<td>MMP1, matrix metalloproteinase 3 (stromelysin 1)*</td>
<td>1.16</td>
</tr>
<tr>
<td>PRSS3, protease, serine, 3 (mesotrypsin)</td>
<td>1.33</td>
</tr>
<tr>
<td>TIMP1, tissue inhibitor of metalloproteinase 1</td>
<td>1.60</td>
</tr>
<tr>
<td>PRSS2, protease, serine, 2 (trypsin 2)</td>
<td>2.42</td>
</tr>
<tr>
<td>ADAMTS8, a disintegrin-like and metalloprotease (reprolysin type)</td>
<td>0.96</td>
</tr>
<tr>
<td>with thrombospondin type 1 motif, 8</td>
<td></td>
</tr>
<tr>
<td>Cell death</td>
<td></td>
</tr>
<tr>
<td>BCL2A1, BCL2-related protein A1</td>
<td>1.43</td>
</tr>
<tr>
<td>BIRC3, baculoviral IAP repeat-containing 3</td>
<td>0.67</td>
</tr>
<tr>
<td>CST1, cystatin SN</td>
<td>1.22</td>
</tr>
</tbody>
</table>

NOTE: Selected genes whose expression was affected in a cooperative manner by p53 inactivation and H-Ras overexpression. The fold change is calculated by pairwise comparison between normalized values of each gene expression in the corresponding samples.

*Genes for which the pattern of expression was validated by QRT-PCR (Fig. 5).

essential for initiation and progression of mesenchymal cell
transformation (Fig. 7). It is important to stress that transformation
of other lineages, such as epithelial or myeloid, by similar
combination of genetic elements might lead to the discovery of
different genetic signatures. Equally possible is that other
combinations of transforming genes might result in additional
transformation fingerprints. Nevertheless, recently, two studies
(38, 39) found expression modules shared between many cancer
types, suggesting common tumor progression mechanisms and
essential transcriptional features that evolve along neoplastic
transformation of different origins.

Disruption of the fine balance between differentiation and
proliferation is one of the hallmarks common to many tumor
types. However, molecular markers that distinguish between
differentiation-proficient and differentiation-deficient cells are still
elusive in many cases. We found here a particular genetic
signature that describes the defect in the smooth muscle
differentiation program. Furthermore, a correlated expression
pattern between the Rb regulators p16INK4A and p57KIP2 and
several molecules associated with myogenic differentiation was
found. Importantly, alterations in the expression of mesenchymal
cell developmental and differentiation markers as well as
inactivation of p16INK4A and p57KIP2* were observed previously in
tissues similar to WI-38 fibroblasts, such as synovial and other
types of soft tissue sarcomas (40–45). Our results strongly suggest
that this genetic signature provides a mechanistic link between
the disruption of the cell cycle regulation and inability to properly
differentiate. The fact that this genetic signature persisted in the
increasingly transformed cell populations suggests its active
contribution to the more aggressive phenotype as well. Taking into
consideration that the cell of origin for fibrosarcoma is still obscure
(46), acquisition of a differentiation defect relevant to fibroblasts, as
we found, suggests that human lung embryonic fibroblasts (such as
WI-38) could be a cell of origin for this tumor type.

Our gene expression array analysis allowed us to identify a list of
genes down-regulated upon p53 inactivation in the context of
nonstressed cells, among them are both novel and known p53
targets genes participating in a variety of physiologic processes.
Those genes may be of primary importance in the understanding
the role of p53 in the normal cell cycle. Keeping in mind that germ
line mutations in the p53 gene (as in Li-Fraumeni syndrome)
predispose to development of sarcoma (47), we suggest that at least
some of the genes we identified as endogenous p53 targets may be
particularly exciting. It could provide a testable hypothesis that
p53 inactivation confers cells with the increased resistance to death
stimuli mediated by TNF-α and its family members.

Acquisition of genomic instability is an additional hallmark of
malignant transformation. We identified a unique genetic signature
associated with p53 inactivation and development of increased
polyploidy. This signature consists mainly of genes regulating cell
cycle progression and mitosis. Importantly, maximum expression
of these genes required the INK4A-deficient background, suggest-
ing a novel level of cooperation between p53 and p16INK4A tumor
suppressors. Phenotypically, our finding supports existence of
this cooperation; that is, duplication of DNA in the presence of
mitotic inhibitor attained maximal levels when both tumor suppressors were inactivated. Several reports showed that alterations in mitosis genes found in our cluster (31, 48) facilitate the acquisition of additional mutations, thereby further promoting cancer progression. Taking into account that tetraploid metaphases were readily identified in Tfast/G and Tfast/R/G cells and in the tumor samples retrieved from mice, we suggest that inactivation of p53 in the INK4A-deficient cells primarily leads to the acquisition of chromosomal instability, a key event in human tumorigenesis (49–51).

The ability to offer accurate survival prognosis of cancer patients is one of the critical issues in cancer medicine. According to our results, the inactive p53-associated proliferation signature is a strong predictor of a poor outcome in several common solid tumors, including breast (Fig. 5C) and prostate carcinomas (data not shown). Similar proliferation signatures were identified in several studies as a partial characteristic of highly proliferative and more aggressive tumors (52, 53). In addition, a similar genetic signature was found in the tetraploid progenitors of esophageal cancer, which contained mutant p53 (54). Based on these facts, an apparent link between a high rate of proliferation and tumor aggressiveness could be predicted. Finally, these observations suggest that this particular signature, which we have identified by defined genetic manipulations in vitro, represents an authentic physiologic genetic program common to many cancer types. This transformation fingerprint is mainly regulated by the p53 and p16INK4A/pRb tumor suppressors and affects both genome destabilization and the malignant potential of cells.

Tumorigenicity in our strain of fibroblasts is strictly dependent on mutant H-Ras expression and p53 inactivation, suggesting that both oncogenic events are required for this malignant phenotype (55, 56). Indeed, we identified a specific group of genes, which was induced in a highly synergistic manner only when both H-Ras

Figure 6. QRT-PCR validation of the tumor-forming genetic signature. Expression of CXCL1 (top), MMP3 (middle), and IL1B (bottom) as representative genes from the tumor-forming genetic signature (see also Table 3) was tested at different stages of transformation. Sample stages are detailed in Table 1. Tumor1 cells were obtained from a tumor formed by Tfast/G cells on injection into nude mice. Gene expression was normalized to GAPDH expression in the same sample. Columns, averages of duplicate QRT-PCR measurements.

Figure 7. Outline of a stepwise malignant transformation process based on the transcriptional programs identified in this study. Microarray profiling revealed specific genetic signatures associated with the particular stages in our in vitro transformation model. (Selected genes are shown in the boxes colored according to their expression level: yellow-red, high expression; blue, low expression.) These alterations in gene expression reflect the biological features spontaneously acquired by cells [der(X);t(X;17) and INK4A locus silencing] or induced by engineered mutations (GSE56 and H-Ras) along the transformation process. We suggest that the genetic signatures identified in our study provide a conceptual framework for similar transcriptional alterations associated with the transition from normal tissue to hyperplasia, dysplasia, and then to cancer.
and p53 inactivation occurred (Table 3). This tumor-forming genetic signature involves important genes required for angiogenesis, autostimulate, and metastasis. The expression of genes that enable neoplastic cells to modulate their stromal environment represents a critical stage in the tumorigenic process. There are several possible explanations for the transcriptional synergy we observed between H-Ras and p53 in inducing the tumor-forming genetic signature: it is possible that both activated H-Ras and p53 deficiency are required for maximal activation of a single key transcription factor, such as nuclear factor-κB (NF-κB). An alternative possibility could be comodulation of distinct transcriptional factors (e.g., activator protein-1, NF-κB, and CBP/p300) that are involved in the transcriptional regulation of those genes (34). It is also possible that similar transcriptional programs provide the basis for the long-known cooperative effect between pairs of oncogenes in transformation (56, 57). Furthermore, modulation of the signal transduction pathway elements downstream to p53 and Ras provide a promising avenue for future therapy.

Although many of the genes identified in our study were already known to be associated with cancer, the novelty of our findings lies in the fact that we identified specific classes of genes that underlie the acquisition of specific transformation hallmarks. It seems that fully transformed cells contain a limited number of deregulated transcriptional programs. We hypothesize that the information obtained from our in vitro model of malignant transformation accurately reflects, to the extent one could modulate in vivo, the changes that occur during tumor initiation and progression in vivo. Such common features should be carefully evaluated in naturally occurring malignancies. More significantly, our data provide new knowledge essential for better understanding of the transcriptional programs that underlie the transformed state.

Acknowledgments


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References


17. Blatt B, Wiseeman S, Domany E. Proliferation gene expression signature involves important genes required for angiogenesis of distinct transcriptional factors (e.g., activator protein-1, known to be associated with cancer, the novelty of our findings lies in the fact that we identified specific classes of genes that underlie the acquisition of specific transformation hallmarks. It seems that fully transformed cells contain a limited number of deregulated transcriptional programs. We hypothesize that the information obtained from our in vitro model of malignant transformation accurately reflects, to the extent one could modulate in vivo, the changes that occur during tumor initiation and progression in vivo. Such common features should be carefully evaluated in naturally occurring malignancies. More significantly, our data provide new knowledge essential for better understanding of the transcriptional programs that underlie the transformed state.

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References


