Ability to Acquire Drug Resistance Arises Early during the Tumorigenesis Process

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Abstract

Resistance to chemotherapy is one of the principal causes of cancer mortality and is generally considered a late event in tumor progression. Although cellular models of drug resistance have been useful in identifying the molecules responsible for conferring drug resistance, most of these cellular models are derived from cell lines isolated from patients at a late stage in cancer progression. To ask at which stage in the tumorigenesis process does the cell gain the ability to acquire drug resistance, we generated a series of pretumorigenic and tumorigenic cells from human embryonic skin fibroblasts by introducing, sequentially, the catalytic subunit of telomerase, SV40 large T and small T oncoproteins, and an oncogenic form of ras. We show that the ability to acquire multidrug resistance (MDR) can arise before the malignant transformation stage. The minimal set of changes necessary to obtain pretumorigenic drug-resistant cells is expression of telomerase and inactivation of p53 and pRb. Thus, the pathways inactivated during tumorigenesis also confer the ability to acquire drug resistance. Microarray and functional studies of drug-resistant pretumorigenic cells indicate that the drug efflux pump P-glycoprotein is responsible for the MDR phenotype in this pretumorigenic cell model. [Cancer Res 2007;67(3):1130–7]

Introduction

Cytotoxic drugs are used widely for the treatment of cancer. Some tumor types, including pancreatic, renal, colon, and malignant melanoma, often respond poorly to first-line chemotherapy and are classified as intrinsically resistant. The majority of solid tumors, including breast, ovarian, and small cell lung cancers, respond to chemotherapeutic regimes. Nevertheless, in a significant proportion of cases, cancerous cells reappear following initial treatments and no longer respond to further therapy leading to progressive disease. Tumors with this acquired drug resistance fail to respond both to the initial combination of drugs and to new compounds with different mechanisms of action and hence are termed multidrug resistant (MDR; ref. 1).

Several factors contribute to the development of drug resistance. Pharmacologic factors include inadequate access of drug to the tumor cell particularly in solid tumors, inadequate infusion rate or route of delivery, and drug metabolism and excretion. The cellular factors contributing to drug resistance have been intensively studied in model cells selected for resistance to cytotoxic agents in vitro. Mechanisms identified include decreased drug uptake into cells, activation of detoxifying enzymes (e.g., cytochrome P450), activation of DNA repair mechanisms, alterations in drug-induced apoptosis, and increased drug efflux due to overexpression of transporters, such as P-glycoprotein (1).

Although cellular models of drug resistance have led to identification of the molecules responsible for conferring the drug-resistant phenotype, they have proved less successful in identifying the pathways that regulate these events. How drug resistance arises is still unresolved. This is because most cellular models of drug resistance have been developed from transformed cell lines isolated from patients at a late stage in cancer progression and which already exhibit a plethora of karyotypic and physiologic abnormalities.

Recently, several groups have shown it is possible to transform primary human cells into fully tumorigenic cells by altering a few defined pathways ex vivo. This Hahn and Weinberg model of tumorigenesis was first described for human BJ fibroblasts and embryonic kidney epithelial cells by expressing the catalytic subunit of telomerase (to avoid replicative senescence), SV40 large T antigen (SV40 LT; which binds and inactivates the tumor suppressors p53 and pRb controlling the DNA repair and G1 cell cycle checkpoints, respectively) and SV40 small T antigen (SV40 ST; which binds and inactivates PP2A, a serine/threonine phosphatase involved in several signaling pathways) proteins, and oncogenic ras (2). Primary human epithelial cells from the mammary gland, prostate, ovary, trachea, and bronchia have now been transformed by introducing these or similar sets of genes (3).

We have used the Hahn and Weinberg model of tumorigenesis to ask at which stage in the tumorigenic progression does the cell gain the ability to acquire drug resistance? We show that disruption of the tumor suppressors p53 and pRb is sufficient to enable cells to avoid drug-induced senescence and acquire the capacity to develop drug resistance. Global analysis of gene expression and functional studies indicate that acquisition of drug resistance across the tumorigenic pathway is achieved by up-regulation of P-glycoprotein. Thus, in this cell model, the ability to acquire MDR in...
pre-tumorigenic cells is not, as previously supposed, a late event in tumorogenesis resulting from gross genetic instability but is intrinsic to the early steps in the tumorigenic pathway necessary for transformation.

Materials and Methods

Vectors. Vectors encoding the catalytic subunit of telomerase (pBABE-hydro-tERT), SV40 LT (pZip-neo-SV40LT), SV40 LT and SV40 ST proteins (pZipSV776-1), or papilloma virus E7 protein (pBABE-puro-E7) have been described elsewhere (4, 5). pBOS-puro-v-Ha-ras was constructed by inserting an EcoRI/XhoI fragment containing the puroycin resistance cassette from PGK-puro (a gift from A. Elefante, Monash University, Victoria, Australia) into the AatII site of pBOS-v-Ha-ras (a gift from J. Downward, Cancer Research UK, London, United Kingdom), which carries the ras oncogene of Harvey murine sarcoma virus. pCMV-p53 (expressing wild-type p53) was from BD Biosciences (San Jose, CA).

Cell culture, transduction, and transfection. Embryonic skin fibroblasts (ESF) were obtained from skin specimens of a 12-week-old normal human embryo by the Medical Research Council (MRC) Tissue Bank (Hammersmith Hospital, London, United Kingdom). Pieces of skin were mechanically and enzymatically disaggregated into single cells by incubation in a combination of 1 mg/mL collagenase (Sigma-Aldrich, St. Louis, MO), 1 mg/mL bovine serum albumin (Sigma-Aldrich), and 1 mg/mL trypsin (Invitrogen, Carlsbad, CA) in PBS at 37°C for 30 min. Isolated ESFs were cultured, expanded, and maintained in DMEM-glutamax supplemented with 10% FCS. Generation of amphotrophic viruses and transduction of primary ESF cultures were essentially as described (4) or using an Amaza nucleofector (Amaza Biosystems, Cologne, Germany) following the manufacturer’s recommendations (program T-16).

Detection of telomerase expression. Telomerase activity was measured with the TRAPEze telomerase detection kit (InterGen, Purchase, NY) as described (6). PCR products were separated on 10% 1:1 acrylamide/bisacrylamide gels and detected by SYBR Green II staining (1:1,000 dilution; manufacturer’s recommendations [program T-16]).

Anchoragen-independent growth assay. Cells (10⁵) were seeded in 0.3% agar in DMEM on 30-mm plates with a bottom layer of solidified 0.6% agar noble in DMEM. Triplicate cultures for each cell type were maintained for 4 weeks at 37°C in an atmosphere of 5% CO₂ and 95% air, with 200 μL fresh medium added after 1 week. Colonies >50 μm in diameter were counted after 4 weeks and photographed under an Axioskop 100 microscope. All experiments were carried out in duplicate.

Tumorigenicity assay. Equal numbers (5 × 10⁶) of TERT-LT, TERT-LT-ST, TERT-LT-RAS, or TERT-LT-ST-RAS cells were harvested by trypsinization, washed twice with 1 × PBS, resuspended in 0.1 mL saline, and injected s.c. into cohorts of 4- to 6-week-old BALB/c athymic nude mice. The mice were kept in a pathogen-free environment and checked every 2 days for 4 months. Mice were killed when tumors reached a maximum size of 1.44 cm³ (maximum length × maximum width). All experiments, done under the appropriate Home Office License, were approved by the Cancer Research UK Animal Ethics Committee.

Karyotype analysis. ESF and TERT cells were analyzed before reaching 1 month in culture, any remaining cells derived from single cell clones or from populations of cell growing in the presence of the drug were expanded in the continuous presence of doxorubicin to generate, after a further 1 to 2 months in culture, resistant lines. All drug-resistant pre-tumorigenic cells were generated independently at least twice. For all subsequent experiments, cells were grown in the absence of drug for a period of 1 week.

Derivation of drug-resistant cell lines. Cells (1 × 10⁶) were seeded at 1,000/cm² and exposed to a single dose of 34 μmol/L doxorubicin. After ~1 month in culture, any remaining cells derived from single cell clones or from populations of cell growing in the presence of the drug were expanded in the continuous presence of doxorubicin to generate, after a further 1 to 2 months in culture, resistant lines. All drug-resistant pre-tumorigenic cells were generated independently at least twice. For all subsequent experiments, cells were grown in the absence of drug for a period of 1 week.

Senescence-associated ß-galactosidase assay. Cells were fixed with 2% glutaraldehyde and 0.2% glutaraldehyde in PBS and incubated in a solution containing 1 mg/mL 5-bromo-4-chloro-3-indolyl-p-nitrophenyl phosphate (pH 6.0), 150 μmol/L NaCl, 2 mmol/L MgCl₂, 5 mmol/L K₂Fe(CN)₆, and 5 mmol/L K₃Fe(CN)₆ overnight at 37°C.

Flow cytometry. Analysis of surface P-glycoprotein expression was by flow cytometry using the phycoerythrin-labeled monoclonal antibody UIC2 (Becton Dickinson, Franklin Lakes, NJ) essentially as described (10). Functional drug efflux was done as described (11) with the following modifications: 0.1 μmol/L BODIPY-Taxol (Invitrogen) was added in complete medium to culture dishes containing exponentially growing cells (5 × 10⁶) and incubated for 30 min at 37°C. Where indicated, inhibitors XR9576 (300 μmol/L), cyclosporin A (1 μmol/L), or verapamil (1 μmol/L) were added 1 h before addition of the fluorescent substrate.

Microarray hybridization, processing, and data analysis. All microarray protocols are fully described at the Microarray Centre Web site. Briefly, double-stranded cDNA was generated from 10 μg total RNA isolated from three successive and equivalent passages for each cell type and used as a template to generate biotin-labeled cRNA. Drug-resistant cells were grown in the absence of doxorubicin for 1 week before the isolation of RNA. Affymetrix GeneChip Human Genome U133 series A and B (Affymetrix, Santa Clara, CA) were used. Array data were normalized to the median overall brightness with dChip, and the expression of each gene in all samples was calculated (12). Unsupervised cluster analysis was done with Cluster 3.0 and visualized with TreeView. Paired comparisons

http://rana.lbl.gov/EisenSoftware.htm

http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm

http://microarray.csc.mrc.ac.uk/

http://www.dchip.org/

http://microarray.csc.mrc.ac.uk/mcresearchSoftware/clustergenesearch.html

http://rana.lbl.gov/EisenSoftware.htm

http://www.dchip.org/
between triplicate arrays of doxorubicin-sensitive and doxorubicin-resistant cell types were done to identify genes reliably differentially expressed between the two groups per each cell type. Filtering criteria included a P value for the hypothesis that the two groups in comparison had the same mean using a t test to be >0.05 and differential expression had to be observed in the three replicates for cell type analyzed. Two further criteria were used to vary the output number: low stringent (fold change, at least 1.3; fluorescence, at least 50) and high stringent (fold change, at least 2.0; fluorescence, at least 200). Using the high stringent settings, the output list of differentially expressed probes is skewed toward genes with high expression. The three set of probes differentially regulated in each comparison were sorted by applying several Boolean algorithms with dChip to look for specific expression signatures. Expression data have been deposited in Array Express under accession number E-MIMR-11.

**RNA and reverse transcription-PCR.** Total RNA was reverse transcribed as previously described (13). Detection of oncogenic and cellular ras mRNAs was done by reverse transcription-PCR (RT-PCR) using the same antisense primer (OLEY40, 5′-ACACTTGCACTCATGCAAGCC-3′) and sense primers OLEY38 for v-Ha-ras (5′-GTGGTGGTGGGCGCTAGA-3′) or OLEY39 for c-ras (5′-GTGGTGGTGGGCGCCGTC-3′). Glyceraldehyde-3-phosphate dehydrogenase primers have been described (13). Quantitative real-time RT-PCR with the gene-specific primers as described in Supplementary Table S1 was done as described (8). Primers were purchased from either MWG Biotech AG (Ebersberg, Germany) or Sigma-Genosys (St. Louis, MO).

### Results

**Generation of a series of pretumorigenic and tumorigenic human ESF by sequential expression of hTERT, SV40 LT and ST proteins, and oncogenic ras.** We used primary human ESFs to recapitulate the Hahn and Weinberg model of tumorigenesis, generating a defined series of pretumorigenic and tumorigenic cells that recapitulate the key genetic alterations that cells undergo to become malignant (2, 14): TERT (expressing the catalytic subunit of human telomerase, hTERT), TERT-LT (expressing hTERT and SV40 LT), TERT-LT-ST (expressing hTERT, LT, and the SV40 ST), TERT-LT-RAS (expressing hTERT, LT, and oncogenic ras, v-Ha-ras), and TERT-LT-ST-RAS (expressing hTERT, LT, ST, and oncogenic ras) cells (Supplementary Fig. S1).

After a finite number of cell divisions, and as a consequence of telomere shortening, primary cells cease to divide and enter a state of replicative senescence. Ectopic expression of hTERT has been used in some cell systems to overcome telomere shortening and thus artificially immortalize cells (2). However, in the ESFs described here, as well as in MRC-5 fetal fibroblasts, adult human mammary fibroblasts, and endothelial cells, expression of hTERT (Supplementary Fig. S1A) failed to overcome replicative senescence (data not shown; refs. 4, 15). Both ESF and TERT cells ceased to proliferate after an equivalent time in culture (60 days or ~30 population doublings; data not shown). However, hTERT expression was a prerequisite for the development of tumorigenesis because we were unable to generate ESF-derived cells transfected only with the SV40 LT and SV40 ST in the absence of hTERT coexpression (data not shown). Thus, in this cell model, hTERT alone is insufficient to overcome replicative senescence, but its expression is necessary for the survival and proliferation of SV40 LT and ST–expressing cells.

SV40 LT oncoprotein binds p53, causing its inactivation and accumulation. Consequently, cells expressing LT (Supplementary Fig. S1B) have a nonfunctional DNA damage checkpoint (16). Assessment of p53 inactivation was determined following exposure to doxorubicin. In the absence of doxorubicin, p53 was not detected in ESF and TERT cells, whereas following exposure to the drug both p53 and its downstream target p21WAF accumulated in a time-dependent manner, indicating that both these cell types had an intact DNA damage checkpoint (Supplementary Fig. S1E, left).

### Table 1. Sensitivity of experimentally transformed ESFs and their drug-resistant derivatives

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Doxorubicin (μmol/L)</th>
<th>Paclitaxel (μmol/L)</th>
<th>Cisplatin (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESF</td>
<td>112 ± 8</td>
<td>42 ± 2</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>TERT</td>
<td>187 ± 62</td>
<td>45 ± 2</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>TERT-LT</td>
<td>161 ± 15</td>
<td>60 ± 5</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>R-TERT-LT</td>
<td>304 ± 67</td>
<td>307 ± 42</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>TERT-LT-RAS</td>
<td>70 ± 7</td>
<td>12 ± 8</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>R-TERT-LT-RAS</td>
<td>320 ± 12</td>
<td>136 ± 7</td>
<td>11 ± 7</td>
</tr>
<tr>
<td>TERT-LT-ST</td>
<td>19 ± 3</td>
<td>7 ± 1</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>R-TERT-LT-ST</td>
<td>333 ± 83</td>
<td>157 ± 32</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>TERT-LT-ST-RAS</td>
<td>6 ± 2</td>
<td>9 ± 3</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>R-TERT-LT-ST-RAS</td>
<td>57 ± 7</td>
<td>152 ± 7</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

**NOTE:** Data represent the mean ± SD of at least three separate experiments.

*Concentration of drug at which proliferation after 4 d was reduced by 50%, estimated from the corresponding dose-response curves.
inactivate p53 but insufficient to sequester enough pRb molecules, thus retaining a functional G1 checkpoint. Therefore, in this cellular model, TERT-LT cells have a p53<sup>-</sup> pRb<sup>+</sup> phenotype comparable with papillomavirus E6–expressing TERT fibroblasts (14). In contrast, the other LT-expressing cells generated in this study (TERT-LT-ST, TERT-LT-RAS, and TERT-LT-ST-RAS) had higher levels of LT, which inactivated both the DNA damage and the G<sub>1</sub> cell cycle checkpoints (the p53<sup>-</sup> pRb<sup>-</sup> phenotype; Supplementary Fig. S1F).

As has been shown previously (2), only ESF-derived cells expressing hTERT, LT, ST, and an oncogenic form of ras (Supplementary Fig. S1C and D) formed colonies in soft agar and produced tumors in nude mice (Supplementary Table S2) and therefore exhibited a fully transformed phenotype. Two additional differences between the tumorigenic TERT-LT-ST-RAS cells and all other pretumorigenic cells were an accelerated proliferation rate (Supplementary Table S3) and a doubling of chromosome number (Supplementary Tables S4 and S5). Other cells transformed

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![Image](image_url)
using this or a similar set of genes have been reported to have decreased doubling times and to be either diploid or tetraploid (19, 20).

Due to their rapid growth rate and altered cell cycle checkpoints, tumorigenic ESFs (TERT-LT-ST-RAS cells) were extremely sensitive to doxorubicin (IC₅₀ 6 nmol/L versus 112 nmol/L for ESF), moderately sensitive to paclitaxel (IC₅₀ 9 nmol/L versus 42 nmol/L for ESF), and only slightly sensitive to cisplatin (IC₅₀ 10 µmol/L versus 18 µmol/L for ESF). Cells earlier in the tumorigenesis process were only marginally more sensitive to doxorubicin and paclitaxel than parental ESFs, but the sensitivities to these drugs increased with the number of genetic alterations introduced (Table 1).

**Cell fate following exposure to doxorubicin.** We used this series of progressively transformed ESFs to determine whether drug resistance could be acquired in cells before malignant transformation, and if so, at which stage. We assessed the responses of each of the ESF derivatives generated above 28 days after exposure to a single therapeutically relevant dose of the DNA-damaging agent doxorubicin. Several variables were used to monitor cell fate after exposure to doxorubicin. BrdUrd was used to mark the cells before drug treatment such that the proportion of the original starting population surviving after doxorubicin exposure could be determined, trypan blue exclusion was used to calculate the total number of viable cells, and a clonogenic assay was used to assess and quantitate the number of doxorubicin-resistant clones.

The response to doxorubicin varied considerably with stage in the tumorigenic pathway. ESF and TERT cells, which had functional DNA damage and G₁ cell cycle checkpoints (p53⁺ pRb⁺), responded either by entering drug-induced senescence or by cell death. The proportion of surviving cells were dose dependent (Fig. 1A), and these cells displayed characteristics typical of drug-induced senescence: increase in cell size, flat vacuolated morphology, activation of senescence-associated β-galactosidase (SA-β-gal; Fig. 1D), and arrest in G₁ as indicated by hypophosphorylation of pRb (Fig. 1E, left; ref. 21).

The proportion of TERT-LT cells that survived 28 days exposure to doxorubicin was comparable with that for TERT cells (Fig. 1A), although their response differed. Whereas TERT cells entered drug-induced senescence, the surviving TERT-LT cells proliferated in the presence of doxorubicin (Fig. 1C). These surviving cells were elongated, did not arise from or produce defined clones (Fig. 1B), and formed a network of closely intertwined cells that did not express SA-β-gal (Fig. 1D). When subcultured, TERT-LT cells were able to continue proliferating in the presence of doxorubicin to generate resistant TERT-LT (R-TERT-LT) cells. Inactivation of the G₁ cell cycle checkpoint occurred during growth of R-TERT-LT cells in the presence of doxorubicin (Fig. 1E, right). These cells proliferated at a slow rate in the presence of doxorubicin (Supplementary Table S3) and were moderately resistant to doxorubicin and paclitaxel (Table 1).

In contrast, tumorigenic TERT-LT-ST and TERT-LT-RAS cells, as well as tumorigenic TERT-LT-ST-RAS cells, all of which have inactivated DNA damage and G₁ checkpoints (Supplementary Fig. S1E and F), were more sensitive to doxorubicin than were ESF or TERT cells (Table 1). Exposure of these cells to 34 to 136 nmol/L doxorubicin resulted in significant cell death (Fig. 1A). However, after 28 days in culture, drug-resistant clones appeared in all three cell types in a dose-dependent manner (Fig. 1B). The cells forming the clones had a similar morphology to the drug-resistant cells (Fig. 1D). The three types of drug-resistant variants obtained (R-TERT-LT-ST, R-TERT-LT-RAS, and R-TERT-LT-ST-RAS) showed a MDR phenotype because they were cross-resistant to at least one structurally and functionally different drug: paclitaxel (Table 1). The proliferation rates of the resistant derivatives in the presence of 34 nmol/L doxorubicin were lower than their respective drug-naïve cells (Supplementary Table S3) but comparable when doxorubicin was omitted from the growth medium (data not shown). Thus, tumorigenic cells acquire the ability to develop drug resistance at a stage before becoming fully transformed.

**p53 and pRb control cell fate following doxorubicin exposure.** The importance of both the DNA damage and G₁ checkpoints in controlling the fate of the cells following exposure to doxorubicin was further shown by separately expressing wild-type p53 and human papillomavirus E7 protein, which binds and inactivates pRb (22), in TERT-LT cells, generating TERT-LT-P53 and TERT-LT-E7 cells, respectively. TERT-LT-P53 cells responded to doxorubicin like ESF or TERT cells; cell death at high doses and drug-induced senescence at low doses (Fig. 1D; data not shown). In contrast, TERT-LT-E7 cells responded to doxorubicin exposure like TERT-LT-ST, TERT-LT-RAS, and TERT-LT-ST-RAS cells, generating doxorubicin-resistant clones after 28 days in culture (Fig. 1B and D). This confirms that inactivation of both the p53 and pRb checkpoints is required before cells gain the ability to acquire MDR.
Global analysis of gene expression in pretumorigenic drug-resistant cells. Approximately 1,000 genes were differentially expressed in at least one of the drug-resistant pretumorigenic cell types (Supplementary Fig. S2A). We specifically compared paired drug-naive and drug-resistant variants to identify differentially expressed genes. We validated these changes in expression for 10 selected genes by quantitative RT-PCR (Supplementary Fig. S2B) and for 2 by Western blot analysis (Supplementary Fig. S2C and D). In addition, ABCC1 was assayed by monitoring the function of its product, P-glycoprotein (Fig. 2C). A high degree of correlation was found between data obtained by microarray analysis and quantitative RT-PCR, Western blot, and functional assays, showing that the microarray data accurately represent the differential gene expression signature associated with the development of drug resistance.

ABCC1 is up-regulated in all drug-resistant pretumorigenic cells. Microarray analysis showed a common signature of 21 differentially expressed genes in all drug-resistant pretumorigenic cells (Table 2). These genes encoded some extracellular matrix and cytoskeleton components, proteins involved in adhesion, in the secretory process, transcription factors, as well as membrane carriers and transporters. Only one of these, ABCC1, encoding the membrane-associated ATP-dependent efflux pump P-glycoprotein, has previously been linked to MDR both in vitro and in vivo (23). Surface P-glycoprotein was also up-regulated in all drug-resistant cells as expected (Fig. 2B), with the exception of R-TERT-LT cells.

In R-TERT-LT cells, although ABCC1 mRNA was up-regulated, there was no corresponding increase in P-glycoprotein determined with the antibody UIC2 (Fig. 2A and B). R-TERT-LT cells isolated on multiple independent occasions consistently exhibited this phenotype. R-TERT-LT cells were able to efflux BODIPY–Taxol, a robust P-glycoprotein surrogate (11), albeit not as efficiently as R-TERT-LT-RAS and R-TERT-LT-ST cells (Fig. 2C), suggesting that there is some P-glycoprotein at the plasma membrane but below minimum antibody detection levels. The efflux activity of R-TERT-LT cells is insufficient to account for the moderate drug resistance of these cells (1.9-fold) because verapamil (24) and the P-glycoprotein inhibitor XR9756 (9) did not significantly reduce the doxorubicin IC₅₀ values (Table 3). The gene expression signature of R-TERT-LT cells also showed down-regulation of caspase-2, an initiator caspase necessary for the onset of apoptosis triggered by DNA damage, and up-regulation of BCL2-A1, which reduces the release of proapoptotic cytochrome c and blocks caspase activation (25, 26). Thus, it is most likely that, at this very early pretumorigenic stage, moderate

<table>
<thead>
<tr>
<th>Differential expression</th>
<th>Gene group</th>
<th>Gene name</th>
<th>R-TERT-LT</th>
<th>R-TERT-LT-RAS</th>
<th>R-TERT-LT-ST</th>
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<tr>
<td>Down-regulated in</td>
<td>Proteases</td>
<td>MMP2, matrix metalloproteinase 2</td>
<td>−3.1</td>
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<td>−2.1</td>
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<td>resistant cells</td>
<td></td>
<td>CSP62, versican</td>
<td>−2.1</td>
<td>−5.9</td>
<td>−1.8</td>
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<td>Extracellular matrix</td>
<td></td>
<td>AGC1, aggrecan 1</td>
<td>−3.5</td>
<td>−4.2</td>
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<td></td>
<td></td>
<td>COL8A1, collagen, type VIII α1</td>
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<td>−3.1</td>
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<td></td>
<td></td>
<td>LTRB2, latent transforming growth factor-β binding protein 2</td>
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<td>−8.7</td>
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<tr>
<td></td>
<td>Cytoskeleton</td>
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<td></td>
<td></td>
<td>CALD1, caldesmon 1</td>
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<td></td>
<td></td>
<td>TNS1, tensin</td>
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<td></td>
<td>Adhesion</td>
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<td></td>
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<td>HNT, neurotomin</td>
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<td>−1.7</td>
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<td></td>
<td>Cell cycle</td>
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<td>−2.0</td>
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<tr>
<td></td>
<td>Unknown function</td>
<td>SART2, squamous cell carcinoma antigen</td>
<td>−2.3</td>
<td>−1.7</td>
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Up-regulated in resistant cells

<table>
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<tr>
<th>Differential expression</th>
<th>Gene group</th>
<th>Gene name</th>
<th>R-TERT-LT</th>
<th>R-TERT-LT-RAS</th>
<th>R-TERT-LT-ST</th>
</tr>
</thead>
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<tr>
<td>Transcription factors</td>
<td>SALL1, sal-1-like 1</td>
<td>4.1</td>
<td>3.3</td>
<td>7.6</td>
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<tr>
<td>Enzymes</td>
<td>HSD17B2, hydroxysteroid (17-β) dehydrogenase 2</td>
<td>2.6</td>
<td>5.3</td>
<td>9.0</td>
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<tr>
<td>Channels, carriers,</td>
<td>MANIC1, mannosidase</td>
<td>3.2</td>
<td>3.2</td>
<td>5.5</td>
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<tr>
<td>transporters</td>
<td>ABCB1, P-glycoprotein</td>
<td>1.8</td>
<td>4.4</td>
<td>12.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLC16A14, solute carrier family 16 member 14</td>
<td>1.8</td>
<td>2.1</td>
<td>2.2</td>
<td></td>
</tr>
<tr>
<td>Secretory process</td>
<td>TBC1DS, TBC1 domain family member 8</td>
<td>4.6</td>
<td>1.6</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>PHLD1A1, pleckstrin homology-like</td>
<td>2.2</td>
<td>1.6</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>domain, family A, member 1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Unknown function</td>
<td>IMAGE clone 2322114</td>
<td>1.8</td>
<td>2.0</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

*Values represent fold difference in expression obtained by microarray analysis between drug-naive and drug-resistant pretumorigenic cells.
levels of drug resistance are achieved both through apoptosis evasion and drug efflux.

Drug-resistant R-TERT-LT-ST and R-TERT-LT-RAS cells failed to accumulate doxorubicin intracellularly when cultured in the presence of 34 nmol/L doxorubicin (data not shown), and BODIPY-Taxol efflux was cyclosporin A inhibitable (Fig. 2), indicating the involvement of a drug efflux pump. Furthermore, verapamil and XR9576 reverted the IC₅₀ value of R-TERT-LT-RAS indicating the involvement of a drug efflux pump. Furthermore, levels of drug resistance are achieved both through apoptosis evasion and drug efflux.

Drug-resistant R-TERT-LT-ST and R-TERT-LT-RAS cells failed to accumulate doxorubicin intracellularly when cultured in the presence of 34 nmol/L doxorubicin (data not shown), and BODIPY-Taxol efflux was cyclosporin A inhibitable (Fig. 2C), indicating the involvement of a drug efflux pump. Furthermore, verapamil and XR9576 reverted the IC₅₀ value of R-TERT-LT-RAS cells to that of drug-naive TERT-LT-RAS cells (Table 3). Thus, resistance to doxorubicin in pretumorigenic R-TERT-LT-RAS and R-TERT-LT-ST cells is primarily due to drug efflux mediated by P-glycoprotein.

**Discussion**

Failure of chemotherapy in cancer patients is clinically associated with progressive disease and is responsible for over half a million deaths annually in the United States (27). Many of the efforts to overcome clinical drug resistance have focused on modulating the well-characterized effectors, such as P-glycoprotein, or activation of apoptosis, yet the clinical application of this strategy has thus far failed to deliver significant therapeutic benefits (28). An alternative strategy to circumvent MDR would be to target the master regulators controlling the upstream targets for the acquisition of drug resistance.

The development of cellular models for tumorigenesis (2, 29) has, for the first time, allowed us to ask whether the capacity to develop drug resistance arises before or after tumorigenic transformation and what is the minimum number of altered pathways required to permit this event. In our defined series of progressively transformed ESFs, the ability to acquire doxorubicin resistance requires both inactive DNA damage and G₁ cell cycle checkpoints. Thus, the minimum number of genetic transformations necessary for a primary cell to become drug resistant, in addition to hTERT expression, is inactivation of the pathways controlled by p53 and pRb (Fig. 3). The role of p53 as a central mediator of the DNA damage and other cellular stress responses is well established (30, 31). However, the influence of p53 status on chemosensitivity and the development of drug resistance have previously been elusive (32). Thymocytes derived from p53-null mice are resistant to the induction of apoptosis in response to DNA-damaging agents, including etoposide and ionizing radiation (33, 34), whereas normal human fibroblasts depleted of functional p53 by LT or human papillomavirus E6, and primary embryo fibroblasts from p53-null mice, are more sensitive to paclitaxel than the nonaltered cells (35). In addition, pRb can specifically enhance the ability of p53 to induce apoptosis by facilitating the action of p53 as a transcriptional suppressor. Thus, p53 and pRb are pivotal in deciding cell fate after drug treatment: senescence, apoptosis, or drug resistance (21).

Microarray analysis showed up-regulation of ABCB1 encoding the membrane-associated ATP-dependent efflux pump P-glycoprotein in all drug-resistant pretumorigenic cells. In soft tissue sarcoma, which most closely resembles ex vivo–transformed fibroblasts, P-glycoprotein expression is a predictor for drug resistance correlating with high tumor size and stage and with poor prognosis and response to chemotherapy (36–38). The increased expression of ABCB1 detected in the doxorubicin-resistant pretumorigenic ESFs (Fig. 2A) is probably mediated by the transcription factor SALL1, which was also up-regulated in all ESF drug-resistant derivatives. SALL1 enhances the canonical Wnt signaling pathway (39, 40), enabling the nuclear import of β-catenin and subsequent gene induction via binding to LEF/TCF elements. A complex of β-catenin and TCF4 has been shown to bind the ABCB1 promoter and activate ABCB1 expression in colorectal carcinogenesis (41).

**Table 3. Selective reversal of doxorubicin resistance by P-glycoprotein modulators in pretumorigenic cells**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>IC₅₀* (nmol/L doxorubicin)</th>
<th>P-glycoprotein modulator</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 µmol/L verapamil</td>
</tr>
<tr>
<td>TERT-LT</td>
<td>161 ± 15</td>
<td>160 ± 11</td>
</tr>
<tr>
<td>R-TERT-LT</td>
<td>304 ± 67</td>
<td>237 ± 67</td>
</tr>
<tr>
<td>TERT-LT-RAS</td>
<td>70 ± 7</td>
<td>78 ± 11</td>
</tr>
<tr>
<td>R-TERT-LT-RAS</td>
<td>320 ± 12</td>
<td>90 ± 8</td>
</tr>
</tbody>
</table>

NOTE: Data represent the mean ± SD of at least three separate experiments.

*Concentration of drug at which proliferation after 4 d was reduced by 50%, estimated from the corresponding dose-response curves.

P < 0.0001.
In conclusion, we have shown that the ability to acquire drug resistance arises as an intrinsic part of the tumorigenic process. Disruption of the p53 DNA damage checkpoint enables cells to avoid drug-induced senescence, and subsequent inactivation of the pB2-controlled G1 cell cycle checkpoint allows the generation of drug-resistant pretumorigenic cells. Drug efflux by P-glycoprotein is the main mechanism mediating resistance to doxorubicin in this pretumorigenic cell model.

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References
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