

# Clonal Selection in Malignant Transformation of Human Fibroblasts Transduced with Defined Cellular Oncogenes

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## Abstract

**Recent evidence has implied that disruption of a limited number of defined cellular pathways is necessary and sufficient for neoplastic conversion of a variety of normal human cell types in tissue culture. We show instead that malignancy in such models results from an iterative process of clonal selection *in vitro* and/or *in vivo*. Normal human fibroblasts underwent malignant transformation after transduction with telomerase, cyclin-dependent kinase 4, dominant-negative p53, and activated Ras or MEK. Furthermore, culture conditions favoring overgrowth resulted in clonal selection, which with added Ras or MEK oncogenes led to the emergence of tumorigenic clones. Such tumors showed variable degrees of malignancy with some even exhibiting metastasis. SV40 small t antigen (ST) has been reported to be necessary and sufficient to convert human fibroblasts with these pathway aberrations to a polyclonal tumor. However, we observed that clonal tumors emerged even with ST addition. Genomic instability was markedly increased by p53 and Rb pathway abrogation. Under the same conditions, fibroblasts with these alterations failed to induce tumors, implying that genomic instability may be necessary but not sufficient for malignant transformation. These findings indicate that the minimum number of events required for malignant transformation of human fibroblasts is greater than has been enumerated by such oncogene addition strategies and support a stochastic cancer progression model initiated by four defined cellular alterations.** [Cancer Res 2008;68(5):1417–26]

## Introduction

Human malignancies are thought to arise by a stepwise series of genetic and/or epigenetic alterations, which select for increasingly malignant clones (1, 2). Advances in identification of signaling pathways commonly altered in cancer cells have led to efforts aimed at defining the minimum number of genetic alterations required for malignant transformation of human diploid cells *in vitro* (3, 4). Early studies indicated that human epidermal keratinocytes expressing Adenoviral and SV40 T antigens in combination with ras induced tumors in immunocompromised

mice (5, 6). More recently, retroviral transduction of normal human fibroblasts or epithelial cells with three elements, SV40 early region (SV40ER), catalytic subunit of telomerase (hTERT), and oncogenic Ras (Ras) was reported to be sufficient for acquisition of the malignant phenotype (7).

Viral oncoproteins, including SV40 large T antigen expressed by SV40ER, and human papillomavirus (HPV) E6 and E7 inactivate p53 and Rb pathways, respectively (8). The inability to functionally substitute SV40ER with HPV E6 and E7 oncoproteins subsequently led to evidence for a critical role of another SV40 protein, small t (ST), expressed by the SV40ER as an alternatively spliced transcript (8–10). The function of ST in malignant transformation was reported to be due to its interaction with and inactivation of phosphatase PP2A, which regulates multiple signaling pathways, including phosphatidyl-inositol 3-kinase–AKT and myc stability (11–13). Further studies showed that a combination of genes solely of cellular origin including hTERT, activated ras, cyclin-dependent kinase 4 (CDK4; R24C mutant) together with Cyclin D1 to inactivate Rb, dominant-negative p53 (Dnp53), and the suppression of B56 $\gamma$  subunit of PP2A induced malignant transformation of human fibroblasts and kidney epithelial cells (8, 14). Thus, evidence from this series of investigations indicated that the minimum cellular requirements for neoplastic conversion of normal human cells include maintenance of telomere length, inactivation of Rb and p53 pathways, perturbation of PP2A, and expression of a constitutively active ras oncogene (4, 15). Other reports using viral and/or cellular elements have implied that the minimum number of alterations required may vary depending on cell type and gene combination (16–21).

Most studies to date have not directly addressed whether the number of genes transduced to create tumorigenic human cells is truly sufficient or whether such cells may only be primed for the selection of additional genetic and/or epigenetic alterations that lead to selection of a malignant clone. It is known that retroviral vectors used to transduce various transforming elements integrate at multiple sites within the cellular genome. Hahn et al. (7) reported that viral integration sites in tumors induced by human cells transduced with SV40ER, hTERT, and Ras were polyclonal for hTERT integration sites. These findings led the authors to conclude that this combination of viral and cellular elements was both necessary and sufficient for malignant conversion of human cells because a polyclonal tumor cell population would imply lack of need for selection of additional events (7). Because tumors are generally thought to evolve clonally, we sought to investigate the role of clonal selection in the acquisition of malignancy in human fibroblast transformation models. Our findings reveal powerful pressures both *in vitro* and *in vivo* that select for malignant clones and provide insights into stochastic tumor progression by primary human cells transduced with defined genetic changes.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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doi:10.1158/0008-5472.CAN-07-3021

## Materials and Methods

**Constructs and retroviral production.** Full-length cDNAs for the individual genes were subcloned into pBABE and pBABE-derived vectors as follows: The telomerase catalytic subunit (hTERT; ref. 22) was subcloned with EcoRI-Sall. CDK4 cDNA (23) was subcloned with BamHI/Sall. Dominant-negative, R248W mutant of p53 (DNP53) was subcloned with BamHI/Ascl. Activated ras (Arg12; Accession number M24154) was subcloned with BamHI. Constitutively active, F53L, S218E, S222E mutant MEKEL was generated by subcloning an HpaI-EcoRI fragment containing the F53L mutation from a clone obtained by expression cloning into MEKE cDNA (Accession number L02526) with S218E, S222E mutations. The entire cDNA was then excised and subcloned with BamHI-Sall. The SV40 ST coding sequence was generated by PCR amplifying ST from a genomic SV40 clone using the forward (5'-GGCGGATCCGCCACCATGGATAAAGTTTT-3') and reverse (5'-AGGCGAATTCTTAGAGCTTAAATCTTG-3') oligonucleotides. The resulting fragment was sequenced and cloned with BamHI/EcoRI.

The calcium phosphate transfection method was used to generate amphotropic virus stocks by transient cotransfection of 293T cells with 5 µg of each retroviral construct and 5 µg of pCL amphi packaging plasmid. Culture fluids were collected 48 h after transfection and filtered through a 0.45-µm filter. All virus stocks were titrated on NIH3T3 cells using the appropriate drug selection. Stocks with titres of >10<sup>5</sup> selectable marker units were used for infections.

**Cell culture and retroviral infection.** HT1080 human fibrosarcoma cells (American Type Culture Collection) and 501T normal human diploid fibroblasts (HDF) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen). For sequential retroviral gene transduction, HDFs were seeded and cultured overnight in growth medium containing 4 µg/mL of polybrene (Sigma) before virus addition. Respective viral stocks were added to cells with fresh medium containing 4 µg/mL polybrene. Medium was aspirated after 16 to 20 h, and cells were allowed to recover in growth medium. The next day, growth medium containing the appropriate selection drug was added. Mass populations of infected cells were selected in growth medium containing 2 µg/mL puromycin, 400 µg/mL neomycin, 50 µg/mL of hygromycin, 2 µg/mL blasticidin, or 50 µg/mL zeocin as appropriate after each sequential infection. In most cases, each gene was transduced using at least two different selection markers in independent experiments. Namely, blasticidin, puromycin, or neomycin for hTERT; neomycin or puromycin for CDK4; hygromycin or puromycin for DNP53; hygromycin or blasticidin for ras; hygromycin for MEKEL; puromycin or zeocin for ST. Mass populations of cells were maintained with all drugs used for selection.

**Limiting dilution cloning.** Clones were isolated from TK4DnR mass culture [HDFs transduced with hTERT (*T*), CDK4 (*K4*), DNP53 (*Dn*), and activated Ras (*R*)] by plating the cells at limiting dilution in 96-well plates. Briefly, cells were serially diluted between 20 and 5 viable cells/mL and plated at 100 µL per well in three 96-well plates, respectively. After incubation at 37°C in 5% CO<sub>2</sub> for 14 days, plates were scored for wells with single colonies, which were expanded.

**Tumorigenicity assay and tumor explantation.** Six-week-old nonobese diabetes/severe combined immunodeficient mice (Taconic Laboratories) were used for tumorigenicity analysis. Cells were trypsinized, centrifuged at 1,000 rpm for 10 min, and washed with PBS. Cell pellets were resuspended in a 100 µL of serum-free medium, mixed with equal volume of Matrigel, and injected s.c. in the flank area. Mice were monitored weekly, and tumor diameter was measured with precision calipers. Mice were sacrificed when tumors reached ~1 cm<sup>3</sup>.

Tumors were explanted in culture by mincing and digestion for 3 h with agitation at 37°C in HBSS containing 0.5mg/mL collagenase (Sigma) and 1mg/mL dispase (Sigma). Dissociated cells were centrifuged at 1,200 rpm for 5 min, and the pellet was resuspended and plated in DMEM containing 10% FBS. After the cells had attached, the medium with appropriate selection drug was added. A pure population of tumor cells devoid of contaminating mouse cells was generally obtained within 1 week.

**Anchorage-independent growth assay.** Growth of cells in soft agarose was determined by seeding 1 × 10<sup>5</sup> or 1 × 10<sup>4</sup> cells per 60-mm dish in 0.5%

sea plaque agarose (Cambrex) in DMEM supplemented with 10% FBS on a semisolid bottom layer of growth medium containing 1.0% agarose. Cells were fed once weekly with 1 mL of growth medium containing 0.5% agarose. Representative fields were photographed at 3 weeks.

**Protein analysis.** Cells were washed once with PBS and lysed on ice in lysis buffer [50 mmol/L HEPES (pH 7.6), 250 mmol/L NaCl, 0.1% NP40, 5mmol/L EDTA supplemented with 1× proteinase inhibitor cocktail (Roche), and 2 mmol/L sodium orthovanadate]. Lysates were cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Protein concentration was determined by the bicinchoninic acid protein assay kit (Pierce). Fifty micrograms of protein was subjected to SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. Immunoblot analysis was performed under standard conditions for enhanced chemiluminescence. The following proteins were detected with the corresponding antibodies: CDK4 (sc-260); p21 (sc-397) from SantaCruz; ras (05-516) from Upstate; p53 (1801) from hybridoma center Mount Sinai School of Medicine, NY; and β-actin (A-5441) from Sigma.

**Southern analysis.** Genomic DNA was extracted by the Proteinase K/SDS-phenol-chloroform extraction method. Ten micrograms of DNA were then digested with either HindIII or EcoRI and fractionated in a 0.8% agarose gel, transferred to Hybond N membrane (Amersham), and hybridized with appropriate <sup>32</sup>P-labeled probes. The membranes were hybridized overnight at 60°C in ExpressHyb hybridization solution (BD Biosciences). Membranes were washed twice in 2× SSC; 0.05% SDS at room temperature for 15 mins each; and twice in 0.1× SSC, 0.1% SDS at 50°C for 15 mins each, followed by autoradiography. The EcoRV-Sall fragment of hTERT cDNA, HindIII-ClaI hygromycin fragment from the pBABE hygromycin vector, and BamHI-EcoRI fragment from the SV40 ST expression construct were used as templates to synthesize <sup>32</sup>P-labeled probes using the Rediprime II kit (Amersham) to detect retroviral hTERT, Ras, MAP/ERK kinase (MEK), and SV40 ST fragments, respectively. The hTERT probe also detected endogenous genomic hTERT fragments. The Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) probe was PCR amplified from the pBABE vector to detect LTRs present in each proviral integrant. HindIII and EcoRI, which were used for genomic DNA digestion, each cut only once within the proviral DNA and did not cleave any of the probes for the individual genes added, allowing detection of a unique junction fragment involving viral and host DNA for each integration site. Thus, polyclonality was reflected as a smear and clonal selection by the presence of predominant band(s). Multiple bands of comparable intensities suggested multiple retroviral integration events in the same cell (24).

**Spectral karyotyping.** SKY analysis was performed on methanol-acetic acid-fixed cells obtained from wild-type and gene-transduced HDFs. HDFs with one or more gene combination were matched for passage unless otherwise indicated. Briefly, cultures were harvested after 6 to 10 h of treatment with Colcemid (0.005 µg/mL; Sigma). After hypotonic treatment with 0.075 mol/L KCl for 20 mins at 37°C, cells were fixed in methanol-acetic acid (3:1) and dropped onto slides. Hybridization and detection with the Human SKY Paint kit (Applied Spectral Imaging Ltd.) was performed according to the manufacturer's protocol. SKY images were acquired with an SD300 Spectracube (Applied Spectral Imaging) mounted on a Nikon Eclipse E800 microscope, using a custom designed optical filter (SKY-1; Chroma Technology) and analyzed with the SKY View 1.2 software (ASI). Breakpoints on the SKY-painted chromosomes were determined by comparison with corresponding inverted 4',6-diamidino-2-phenylindole images and the banded karyotype. In each case, between 10 and 40 metaphases were analyzed. All chromosome abnormalities were analyzed according to ISCN (1995).

## Results

**Malignant potential of HDFs transduced with cellular genes.** HDFs were sequentially infected with retroviruses containing hTERT to immortalize the cells, CDK4 to inactivate the Rb pathway, and dominant-negative p53 to inactivate the p53 pathway. We then introduced either control vector or oncogenic

Ras. Table 1 shows that TK4DnV cells formed no detectable tumors upon subcutaneous inoculation. Under the same conditions, HDFs transduced with retroviruses containing hTERT, CDK4, dominant-negative p53, and oncogenic Ras induced late-appearing tumors, regardless of the order of gene introduction (Table 1, *experiments 1–2*). When cultures achieved confluence and were passaged weekly at 1:4 [ $\sim 2$  population doublings (PD) per transfer] for an additional  $\sim 50$ PD, the vector-containing cells remained non-tumorigenic, whereas the oncogenic Ras-transduced cultures showed strikingly increased tumor forming ability (Table 1). Progressive tumors developed within 1 to 7 weeks compared with 16 to 27 weeks for the parental cells in two independent experiments (Table 1, *experiments 1–2*).

To quantitate the increased tumor forming ability of TK4DnR cells with continued passage in culture, we titrated the number of cells required. As shown in Fig. 1A, as few as  $10^3$  TK4DnR (+50PD) cells induced progressive tumors by 6 to 7 weeks compared with 16 to 17 weeks with  $5 \times 10^6$  TK4DnR cells. These results indicated that *in vitro* passage of these cells led to a >5,000-fold increase in their malignant potency. Additionally, when late-appearing tumors induced by either TK4DnR or K4TDnR were explanted into culture and reinoculated *in vivo*, they formed tumors much more rapidly (1–3 weeks; Table 1). Of note, *in vitro* or *in vivo* passage of TK4DnR cells resulted in their ability to form tumors with time course similar to HT1080 tumor cells, a line established from a human fibrosarcoma (Table 1; ref. 25). These findings strongly suggested that selection pressures both *in vitro*

and *in vivo* resulted in the emergence of more tumorigenic variants, and that selection pressures *in vitro* could be as powerful as those *in vivo* for emergence of highly tumorigenic TK4DnR cells.

Figure 1B shows further that although TK4DnV (+50PD) remained relatively contact inhibited in monolayer culture and failed to form colonies in semisolid agarose, TK4DnR cells grew more densely and formed small- to medium-sized colonies under the same conditions. TK4DnR (+50PD) and TK4DnR (T1) cells closely resembled each other and exhibited similarly increased morphologic alterations and anchorage-independent growth ability compared with the parental TK4DnR cells (Fig. 1B). Comparison of the expression levels of CDK4, Ras, and the functional inactivation of p53 in these cell lines revealed no differences. These results excluded the possibility that differences in protein levels of the transduced genes might account for the selection of highly malignant variants *in vitro* or *in vivo* (Fig. 1C).

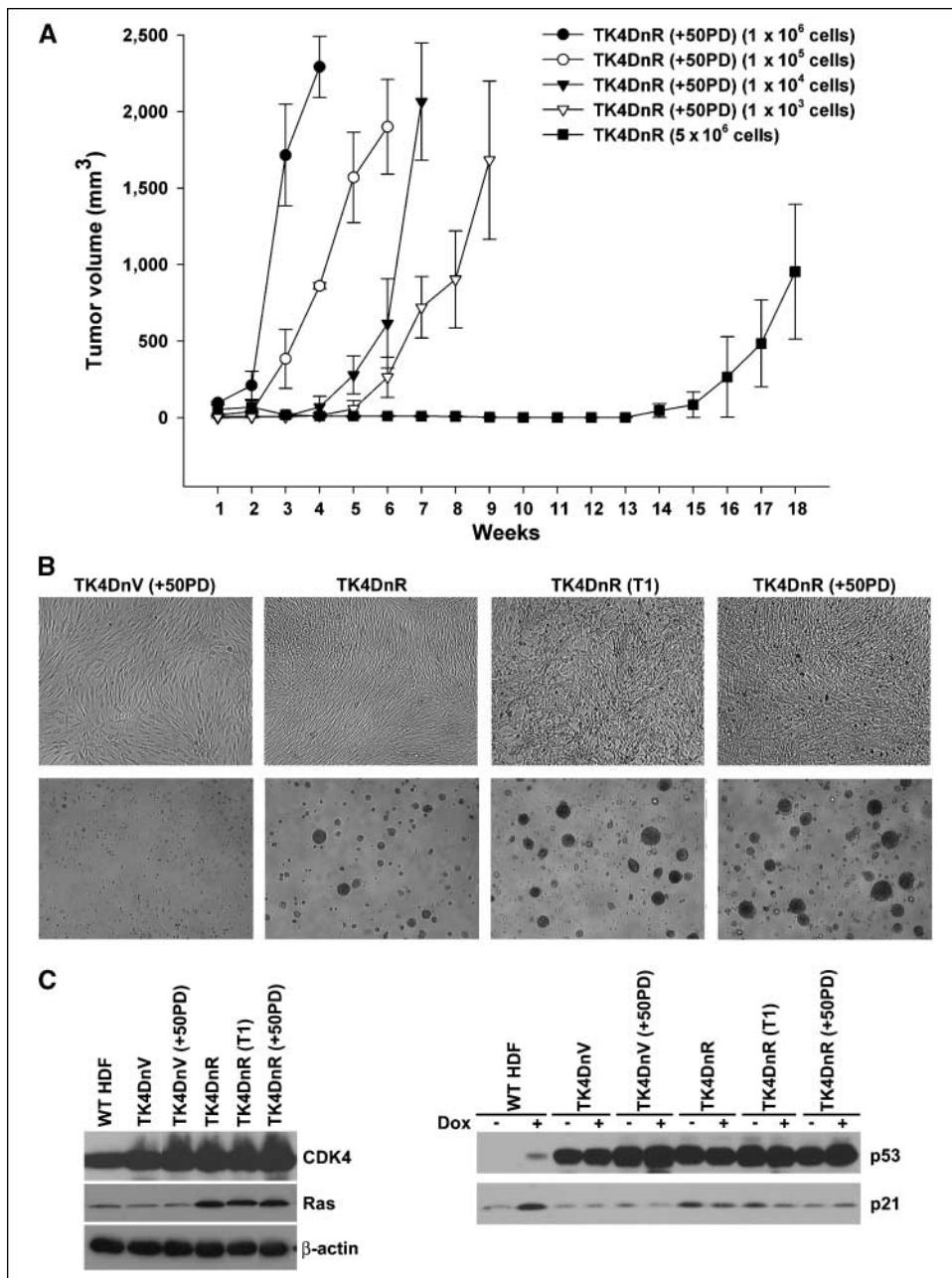
**Analysis of retroviral integration sites establishes clonal selection *in vitro*.** To establish directly that additional events required for the acquisition of highly malignant properties were selected *in vitro* as well as *in vivo*, we performed Southern blot analysis and compared proviral integration profiles in TK4DnV, TK4DnR cell lines passaged *in vitro* or *in vivo*. Retroviral vectors integrate at many sites within the cellular genome (26, 27), and proviral integration sites provide markers for individual transduced cells. Thus, the demonstration of distinct viral integration site(s) in a mass culture of retrovirus-infected cells can be considered strong evidence for selection of predominant clone(s).

**Table 1.** Comparison of tumor formation after *in vitro* or *in vivo* passage of oncogene transduced HDFs

Cells	Designation	Tumors/sites inoculated	Latency (wk)
HDFs-Expt 1			
	TK4DnV	0/4	>45
	TK4DnV (+50PD)	0/4	>45
	TK4DnR	3/4	16, 17, 17
	TK4DnR (+50PD)	8/8	1, 1, 1, 2 2, 2, 2, 2
	TK4DnM	0/4	>20
	TK4DnM (+50PD)	4/4	1, 1, 11, 11
	Explanted tumor cells		
	TK4DnR (T1)	4/4	1, 1, 2, 2
	TK4DnR (T2)	4/4	1, 2, 3, 3
HDFs-Expt 2			
	K4TDnV	0/4	>45
	K4TDnV (+50PD)	0/8	>45
	K4TDnR	2/4	16, 27
	K4TDnR (+50PD)	5/8	4, 4, 6, 6, 7
	Explanted tumor cells		
	K4TDnR (T1)	4/4	1, 1, 2, 2
Human fibrosarcoma	HT1080	5/5	1, 1, 2, 2, 2

NOTE: Tumor frequency and latency upon subcutaneous inoculation of oncogene transduced HDFs in immunocompromised mice. HDFs were transduced with hTERT; CDK4; Dnp53; and either control vector (*V*), activated Ras (*R*), or constitutively active MEK (*M*). Mass cultures expressing the respective genes are designated as per the sequence of addition of each gene. After sequential gene introduction and marker selection, individual cultures, which were transferred weekly ( $\sim 2$  PDs per transfer) for an additional  $\sim 50$  PDs are designated with a (+50PD) suffix. Cells explanted from tumors that formed with long latency were also analyzed for tumor formation. Cells ( $5 \times 10^6$ ) were inoculated per site in experiment 1, and  $2 \times 10^6$  cells were inoculated per site in experiment 2. Tumor growth was monitored weekly with precision calipers, and tumors were scored as positive at 4 mm diameter. Animals were sacrificed when tumors reached a diameter of  $\geq 1.0$  cm.

Abbreviation: Expt, experiment.



**Figure 1.** Enhanced tumorigenic and transformed properties of *in vitro* or *in vivo* passaged TK4DnR cells. **A**, comparison of tumor growth kinetics of *in vitro* passaged TK4DnR (+50PD) and parental TK4DnR cells after subcutaneous inoculation in immunocompromised mice at indicated cell numbers. Points, mean tumor volume; bars, SE versus time (wk). **B**, comparison of saturation density (top) and agarose colony formation (bottom) by cultures as indicated. Assays were performed in duplicate, and representative fields from plates seeded with the  $10^4$  cells are shown. **C**, comparison of CDK4 and Ras protein levels (left), and Dnp53 and p21 protein levels in the absence or presence of doxorubicin (0.1 mg/mL; right) in cultures as indicated.

Southern blotting with the hTERT probe after HindIII digestion of DNA from control HDFs revealed a major 10-kb hTERT hybridizing fragment representing the endogenous hTERT gene (Fig. 2A, left). A distinct doublet migrating at around 6.8 kb was seen in TK4DnR, TK4DnR (T1), TK4DnR (+50PD), as well as TK4DnV (+50PD) cellular DNAs (Fig. 2A, left, lanes 2, 3, 4, and 6). Moreover, the signal intensity of the doublet in each of these lanes was similar to that of the endogenous gene fragment, indicating that the retroviral integrant must reflect a predominant clone. The signal intensity of this doublet was less strong in TK4DnV cellular DNA compared with the endogenous hTERT fragment (Fig. 2A, left, lane 5), indicating that only a subpopulation of TK4DnV cells contained this hTERT integration site, which became further enriched with passage in TK4DnV (+50PD; Fig. 2A, left, lane 6). Because the same integrant was observed in all cultures, a selection

event marked by this integrant must have occurred before retroviral transduction of activated ras. We confirmed these results using an independent restriction enzyme EcoRI (Fig. 2A, right). Southern blot analysis of EcoRI digested cellular DNAs with the hTERT probe showed selection for the same sized fragments (~6.8 kb and 10 kb) in TK4DnR cells and its derivatives (Fig. 2A, right, lanes 1–3). TK4DnM cells were generated by transducing the same TK4Dn cells with constitutively activated MEK, a downstream component of the ras signaling pathway. Figure 2A (right) shows that the hTERT fragments (~6.8 kb and 10 kb) selected in TK4DnR cells and its derivatives were also observed in TK4DnM (+50PD) cells (Fig. 2A, right, lanes 5–6). Of note, the selection of the hTERT integrant alone was not sufficient to explain the increased malignancy of TK4DnR (+50PD) because the same hTERT integrant was already selected in poorly tumorigenic TK4DnR parental cells.

Moreover, TK4DnV (+50PD) cells did not exhibit any tumor growth in spite of independently selecting for this integrant with *in vitro* passage.

We next performed integration site analysis for Ras, the last gene added in TK4DnR cells. The selectable hygromycin marker, present in the Ras retroviral vector, was used as probe. In parental TK4DnR cells, two faintly hybridizing bands were observed (Fig. 2B, left, lane 2), one of which was selected in both *in vitro* derived TK4DnR (+50PD) and *in vivo* derived TK4DnR (T1) cells (Fig. 2B, left, lanes 3–4). The *in vitro* and *in vivo* selection of this same ras integrant was confirmed by analysis with a different restriction enzyme, EcoRI (Fig. 2B, right, lanes 1–3). Based on the relative intensities of the observed ras integration sites in the three cultures, we estimated that around 5% to 20% of parental TK4DnR cells exhibited this proviral integration site even within the period required for Ras gene transduction and analysis. However, as the tumor-forming ability of *in vitro* derived TK4DnR (+50PD) cells was increased by >5,000-fold compared with the parental TK4DnR cells (Fig. 1A), the enrichment for this integrant alone could not account for its increased tumorigenic potency. Instead, the results suggested selection of at least one additional genetic alteration within the subpopulation of parental TK4DnR cells possessing this integrant.

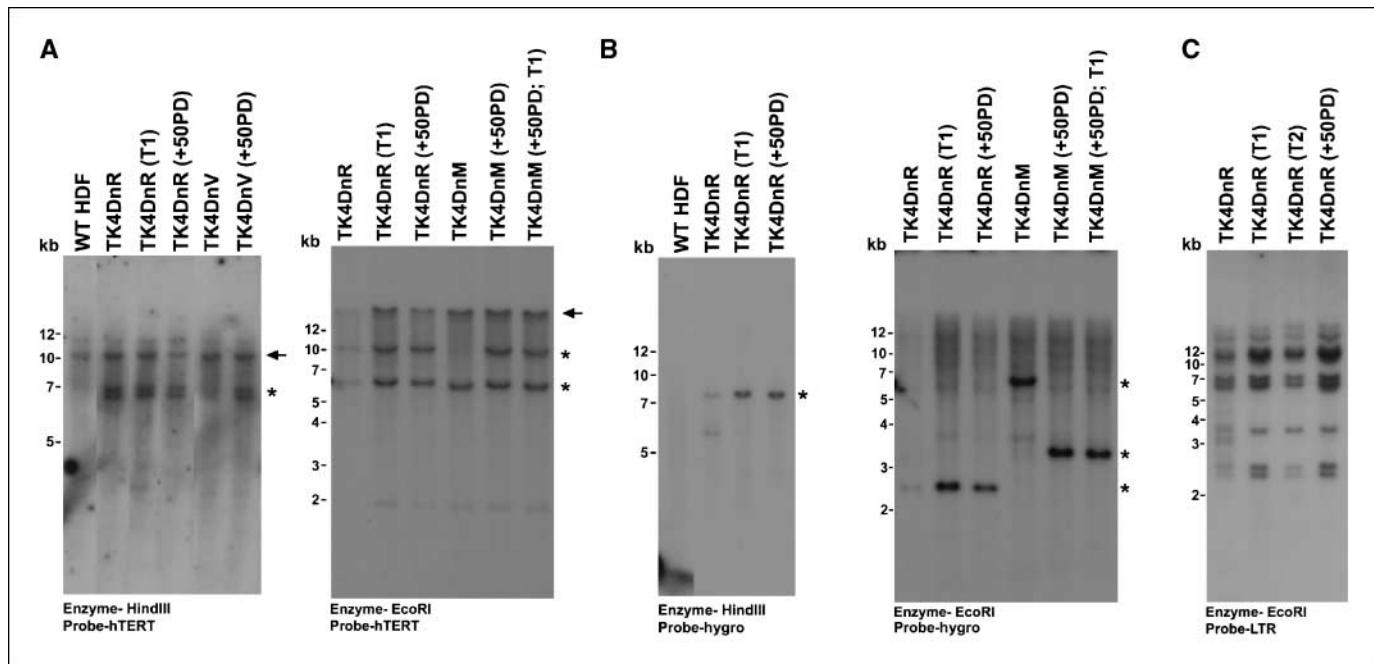
Independent evidence for *in vitro* selection was observed in TK4DnM (+50PD) cells. Figure 2B (right) shows early selection of a predominant MEK integrant (~6.0 kb) in TK4DnM cells (right, lane 4). Of note, this integrant was overtaken by another MEK integrant (~3.5 kb) with continued *in vitro* passage (Fig. 2B, right, lane 5). This selected integrant was also stably retained after *in vivo* passage (Fig. 2B, right, lane 6). In summary, at least two sequential *in vitro* clonal selection events seemed to be involved in the evolution of highly malignant TK4DnR (+50PD) and TK4DnM

(+50PD) cells. The first resulted in a common hTERT integration site, and the second was shown by selection of individual Ras and MEK integrants in each independently propagated line, associated with markedly increased tumorigenic potency (Fig. 2; Table 1).

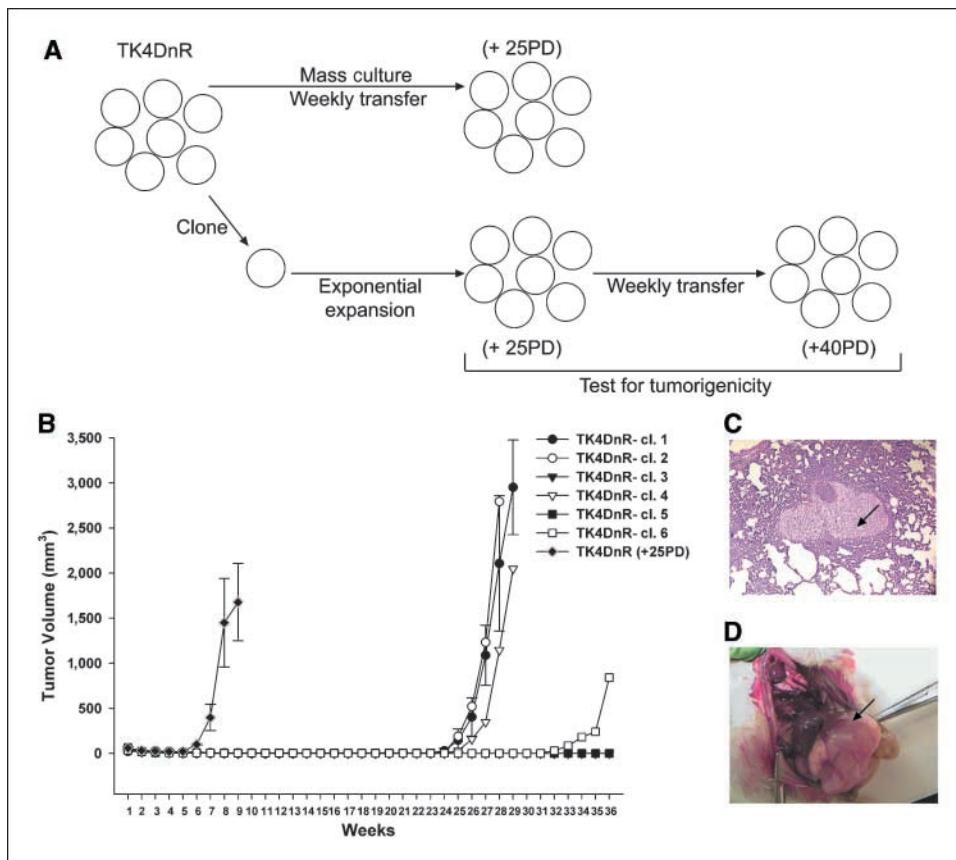
Retroviral integration is known to activate cellular oncogenes. Therefore, we investigated whether LTR-mediated recombination played a role in the acquisition of a highly tumorigenic phenotype. Cellular DNAs from TK4DnR, TK4DnR (+50PD), and TK4DnR (T1) were digested with EcoRI and analyzed by Southern blotting with a LTR probe. These experiments failed to show evidence of any new integration sites in the highly malignant *in vitro* derived TK4DnR (+50PD) or *in vivo* derived TK4DnR (T1) cells that were not already present in parental TK4DnR cells. These results indicated that oncogene activation by retroviral integration was unlikely to be responsible for acquisition of the highly malignant phenotype by these cells (Fig. 2C).

#### Culture conditions that select for highly malignant variants.

Next, we investigated the role of culture conditions in facilitating the emergence of malignant variants from within the TK4DnR mass population. We isolated single cell-derived colonies from the parental culture by limiting dilution in microtiter wells. Each clone was then expanded under exponential growth conditions to around 50 million cells required for *in vivo* tumorigenicity testing (~25PD; Fig. 3A). For comparison, we transferred the parental TK4DnR culture in parallel for an additional 25 PDs by using the weekly transfer schedule (~2 PDs per transfer; Fig. 3A). Upon inoculation into immunocompromised mice, four of six clones induced late-appearing tumors (25–34 weeks), whereas two failed to induce any detectable tumors (Fig. 3B). In contrast to the clonally derived cultures, the parental TK4DnR (+25PD) cells, which had undergone the same number of estimated PD, induced tumors much more rapidly (6–7 weeks; Fig. 3B). There were no differences in the levels



**Figure 2.** Southern analysis for retroviral integration sites shows clonal selection *in vitro*. *A*, DNA samples of the indicated cultures were digested with HindIII (left) or EcoRI (right) and probed with hTERT as described in Materials and Methods. Arrows, major endogenous fragments (~10 kb in left and >12 kb in right). \*, predominant retroviral hTERT integration sites. *B*, DNA samples of the indicated cultures were digested with HindIII (left) or EcoRI (right) and probed with hygromycin as described in Materials and Methods. \*, predominant retroviral ras and MEK integration sites. *C*, DNA samples of the indicated cultures were digested with EcoRI and probed with MMLV LTR as described in Materials and Methods. *hygro*, hygromycin.



**Figure 3.** Comparison of tumorigenicity of parental TK4DnR and exponentially expanded TK4DnR clones after similar PDs. *A*, scheme depicting transfer methods used. Cultures as indicated were tested for tumorigenicity as described in Table 2. *B*, comparison of tumor growth after subcutaneous inoculation in immunocompromised mice of exponentially expanded TK4DnR clones (cl. 1–6) and parental TK4DnR (+25PD) cells subjected to a weekly transfer schedule (~2 PDs per transfer). Points, mean tumor volume; bars, SE versus time (wk). Cells ( $2 \times 10^6$ ) were inoculated per site. *C*, H&E-stained lung section showing metastasis (arrow) after subcutaneous inoculation of cl. 2 (+40PD) grown for an additional 40 PDs according to the weekly transfer schedule (see Table 2). *D*, large liver metastasis observed at autopsy (arrow) after subcutaneous inoculation of cl. 3 (+40PD) grown for additional 40 PDs according to the weekly transfer schedule (see Table 2).

of expression of the transduced genes that could account for these striking variations (data not shown).

Table 2 shows that when the clonally derived lines were shifted to the weekly transfer schedule, each clone acquired increased tumor-forming ability. Moreover, histopathologic analysis of tumor-bearing mice revealed that in at least two cases, clonal lines also acquired the ability to form distant metastases (Table 2; Fig. 3C–D). Similarly, we observed tumor formation at a much higher frequency and with shorter latency upon a second *in vivo* passage of cells explanted from late-appearing tumors (Table 2). All of these results showed that the weekly transfer schedule provided a powerful pressure for selection of malignant variants and reinforced the conclusion that *in vitro* and/or *in vivo* pressures selected for additional transforming alterations. Of note, human fibroblast that expressed hTERT, CDK4, DNp53, and Ras showed malignant properties under conditions in which the necessary pressures existed for selection of additional oncogenic events *in vitro* and/or *in vivo*.

**Addition of SV40 ST results in selection of predominant tumor clones *in vivo*.** SV40 ST has been reported to induce polyclonal tumors in combination with the four pathway aberrations used in the present study (7, 8). The genetic instability of HDFs transduced with cellular genes inducing these same pathway alterations and the selective pressures identified above led us to reexamine these observations. Thus, we infected three TK4DnR clones (cl. 2, cl. 4, and cl. 6), which induced only late-appearing tumors, with vector control or ST retroviruses, confirmed ST expression, and tested tumorigenicity. Tumor latency decreased from 25 to 34 weeks to 7 to 15 weeks during

the period required to expand control vector-transduced TK4DnR cells for analysis using the weekly transfer schedule (Fig. 4). Expression of ST was associated with shortened tumor latencies for two of the three clones analyzed (cl. 2 and cl. 6; Fig. 4). However, Southern blot analysis revealed that although each transduced cell population was initially polyclonal for ST integration sites, all of the derived tumors showed a predominant ST DNA fragment (Fig. 4). Thus, although ST in some cases seemed to contribute to decreased tumor latency, the added expression of ST to cells transduced with hTERT, CDK4, DNp53, and Ras was not sufficient for generation of a polyclonal tumor. These results implied that even with ST addition, the cells underwent *in vivo* selection for additional alteration(s).

**Chromosomal instability and the acquisition of malignancy by HDFs.** It has been proposed that chromosomal instability facilitates a more rapid acquisition of events leading to tumorigenesis and may be necessary and sufficient for tumorigenesis (28, 29). Thus, we investigated the relationship of chromosomal instability (ploidy and structural changes) to the selection of malignant clones. The parental HDFs exhibited a normal karyotype, and around 90% of the hTERT transduced–cells remained diploid with  $\leq 5$  mean abnormalities per cell by SKY analysis (Supplementary Fig. S1A and B). An independent hTERT-expressing culture was established (T-puromycin), maintained for 300 PDs [T-puromycin (+300PD)], and analyzed. T-puromycin and T-puromycin (+300PD) cultures remained predominantly diploid (100% and 80%, respectively) with  $\leq 4$  mean abnormalities (data not shown). These results indicate that hTERT-immortalized human fibroblasts maintained a low level of chromosomal

instability over an extended number of PDs in agreement with previous studies (10, 30).

Abrogation of either p53 or Rb pathways in hTERT-immortalized cultures resulted in the emergence of a hypertriploid subpopulation (16%; Supplementary Fig. S1A). The hypertriploidy increased significantly ( $P < 0.005$ ) with abrogation of both the pathways in TK4DnV (60%) and TK4DnR (80%) cultures, and both cultures were 100% aneuploid after 50 PDs *in vitro* passage (Supplementary Fig. S1A). Supplementary Fig. S1B shows that structural aberrations (mean breaks) and/or total aberrations (mean abnormalities) in TK4DnV, TK4DnR cells, and their respective *in vitro* derivatives were also significantly higher ( $P < 0.005$ ). These findings indicate that chromosomal instability increased in hTERT-immortalized HDFs in which both Rb and p53 pathways were inactivated. Of note, the highest degree of chromosomal instability was observed in TK4DnV (+50PD). Thus, although chromosomal instability may be necessary, it was not sufficient for acquisition of malignant properties by these cells.

## Discussion

Increased understanding of genetic alterations that commonly occur in human tumors has led to efforts to define the minimum number of such alterations required to cause normal human cells to acquire malignant properties. Transduction of viral elements, which have more than one function, in concert with various cellular genes or in some cases only cellular genes,

has been reported to cause malignant transformation of a variety of normal human cell types (31, 32). Most such studies have concluded that inactivation of p53 and Rb tumor suppressor pathways in combination with activated ras and hTERT immortalization is not sufficient to induce the malignant phenotype (8, 9). However, the addition of elements, such as SV40 ST, siRNA for the PP2A B56 $\gamma$  subunit, or stabilized myc, has been reported as both necessary and sufficient to convert such cells to malignancy (14). The fact that a combination of specific genes can result in tumor formation has led a number of groups to claim the establishment of genetically defined models of human cell transformation (7, 33–38).

In the present study, we observed that retroviral transduction of four cellular genes, hTERT, CDK4, Dnp53, together with activated ras led primary HDFs to reproducibly acquire malignant properties. However, such tumors only formed with long latency, and continued *in vitro* passage of the transduced cells resulted in their acquiring increasingly transformed properties as measured both *in vitro* and *in vivo*. Moreover, cells explanted from tumors that formed with long latency exhibited much greater malignant potency upon a second passage *in vivo*. All of these results strongly implied that selection pressures both *in vitro* and *in vivo* resulted in the emergence of increasingly tumorigenic variants after transduction of this set of genes. Detailed analysis of integration sites for sequentially transduced genes confirmed clonal selection both *in vitro* and *in vivo*. Of note, clonal selection occurred even during the course of sequential gene transduction in tissue culture.

We showed that culture conditions rather than number of cell generations were responsible for the clonal selection observed *in vitro*. To allow ready calculation of the number of passage doublings as well as to retain a “mass cell population,” most investigators have used a conservative transfer schedule involving a weekly split at confluence (7, 18, 39). Our evidence indicates that this transfer protocol provides a strong selection pressure for overgrowth by increasingly transformed variants because transduced cells remained only poorly tumorigenic when cloned and expanded exponentially for a comparable number of populations doublings and reproducibly acquired increased malignant potency when switched to the weekly transfer schedule. These results may help to explain earlier findings, which implicated a conservative transfer schedule in determining the malignant potential of mouse embryo fibroblasts established as Balb3T3 and Balb3T12 lines (40), and the spontaneous emergence of tumorigenic fibroblasts as a consequence of extended passaging of a telomerase-immortalized human fibroblast cell line (41).

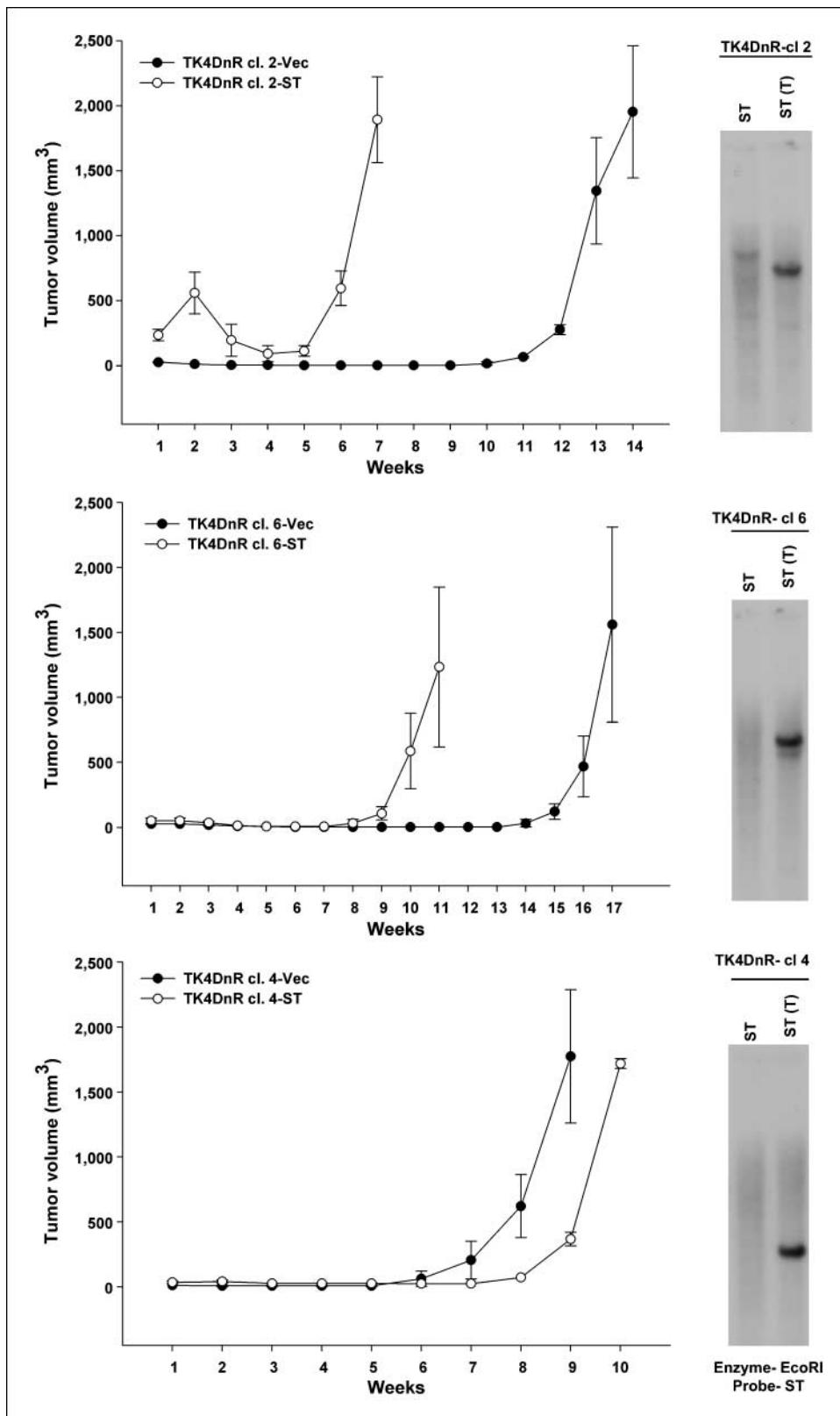
We showed that addition of SV40 ST to poorly tumorigenic HDF clonal lines expressing hTERT, DNp53, CDK4, and activated ras was associated in the majority of cases with increased efficiency of tumor formation over that of identically passaged vector control cells. However, tumors that formed showed predominant ST integration sites, implying clonal selection *in vivo*. Moreover, none of these tumors formed as rapidly as those induced after long-term passage of “mass cultures” transduced by the same initial four genes or by naturally occurring human fibrosarcoma cells. All of these findings strongly argue that even with the addition of ST, further alterations were selected *in vivo* to create a clonal tumor cell.

Drayton et al. (19) suggested the requirement for additional alterations based on the long latency observed for tumor formation after transduction of hTERT, myc, and activated Ras

**Table 2.** Weekly transfer schedule enhances tumorigenic potency of TK4DnR clones

Clones	Tumors/sites inoculated	Latency (wk)
cl. 1	2/4	24, 25
cl. 1 (+40PD)	4/4	9, 11, 13, 16
cl. 2	1/4	24
cl. 2 (+40PD)	9/9	1, 1, 1, 1, 1, 2, 2, 2, 7
cl. 3	0/4	>50
cl. 3 (+40PD)	4/4	1, 7, 8, 8
cl. 4	2/8	25, 33
cl. 4 (+40PD)	2/4	6, 21
cl. 5	0/4	>50
cl. 5 (+40PD)	2/4	23, 32
cl. 6	1/4	32
cl. 6 (+40PD)	3/4	9, 14, 15
Explanted tumor cells		
cl. 1 (T1)	4/4	4, 4, 5, 5
cl. 5 (+40PD)(T1)	4/4	1, 1, 2, 4
cl. 6 (+40PD)(T1)	4/4	6, 6, 7, 8

NOTE: Tumor frequency and latency upon subcutaneous inoculation of TK4DnR clones in immunocompromised mice. TK4DnR clones were isolated from the TK4DnR mass culture by limiting dilution in 96-well microtiter plates as described in Materials and Methods. Each clone was exponentially expanded (~25 PDs) before inoculation and also passaged further on a weekly transfer schedule (~2 PDs per transfer) for additional 40 PDs (see Fig. 3A), followed by analysis of tumorigenicity. Explanted cells from long latency tumors (T) were also analyzed for tumor formation. Cells ( $2 \times 10^6$ ) were inoculated per site.



**Figure 4.** *In vivo* selection of SV40 ST integrants. Subcutaneous tumor growth of indicated TK4DnR clones transduced with vector or SV40 ST expressing retroviral constructs. Points, mean tumor volume; bars, SE versus time (wk). Cells ( $2 \times 10^6$ ) were inoculated per site. Corresponding Southern blots probed for ST integration sites in parental cultures and derived tumor explants after digestion with EcoRI are shown on the right.

in Leiden fibroblasts that lack functional p16. Similarly, Akagi et al. (42, 43) showed heterogeneity in transformability among HDFs from different sources and pointed out the presence of as yet unidentified factors in human cell transformation. Of note,

two previous studies found telomerase to be activated in tumors formed by cells transduced with gene combinations including activated ras together with E1A and MDM2, or with CDK4 (17, 18). We infer from these results that there must have

been *in vivo* selection for this additional alteration. All of these findings are consistent with our evidence that after transduction of normal human cells with an appropriate set of complementing oncogenes, additional stochastic events leading to increasing degrees of malignancy are selected for both *in vitro* and *in vivo*. Kendall et al (44) showed that simultaneous transduction of six cellular genes including hTERT, CDK4, Cyclin D1, DNp53, Ras, and mutant myc in primary human embryonic kidney cells, resulted in the ability of these cells to induce tumors. This approach would be expected to minimize the possibility of *in vitro* selection, but there was no clonal analysis of the tumors.

The actual number of molecular lesions selected *in vitro* or *in vivo* beyond those directly attributable to the transduced oncogenes remains to be determined. Our findings indicate that at least two additional steps must be required. One involves the clonal selection of hTERT and another must be associated with the acquisition of increased tumorigenicity with tissue culture passage of cells with Ras or MEK. However, based on our results demonstrating the distinct patterns of tumorigenic progression exhibited by the individual HDF clonal cultures, each possessing the same initially transduced genes, it seems that the number of steps may be greater depending on the nature of the acquired hits. Together, our findings strongly imply that the actual number of cellular alterations required for acquisition of a phenotype comparable with that of malignant human tumor has likely been underestimated by gene transduction analysis. This conclusion is strengthened by a recent study of breast and colon

cancers, which identified ~90 mutated genes in an average tumor, with at least 11 thought to be cancer promoting (45). While our article was in review, two additional reports have further supported the heterogeneity and complexity of human neoplasia and concluded that many cancer-associated genes remain to be identified (46, 47).

We observed chromosomal instability in our transduced HDF cultures, consistent with the possibility that genomic instability associated with the defined genetic alterations introduced, may have accelerated the malignant process. Nonetheless, it should be noted that the highest degree of chromosomal instability was observed in cultures transduced with hTERT, DNp53, and CDK4, which remained nontumorigenic despite evidence of clonal selection using a mass culture transfer protocol. The application of gene chip technology, including genome-wide single nucleotide polymorphism analysis and expression arrays, to this series of tumorigenic HDF variants with both defined and additional undefined transforming alterations may lead to identification of potentially novel candidate cancer genes.

## Acknowledgments

Received 8/6/2007; revised 12/13/2007; accepted 12/20/2007.

**Grant support:** NIH grant CA085214-08 to S.A. Aaronson is gratefully acknowledged.

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We thank R. Weinberg for kindly providing hTERT cDNA; Wu X for dominant-negative p53 cDNA and J. Manfredi for genomic SV40 plasmid; and S. Macip, G. Liu, A. Bafico, C.M. Fontela, and B. Zhao for helpful discussions.

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## Clonal Selection in Malignant Transformation of Human Fibroblasts Transduced with Defined Cellular Oncogenes

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*Cancer Res* 2008;68:1417-1426.

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