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Retrovirally Mediated Wild-Type p53 Restores S-Phase Modulation Without Inducing *WAF1* mRNA in Breast Carcinoma Cells Containing Mutant p53

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Abstract The mechanism of negative growth regulation by the nuclear phosphoprotein p53 in breast cancer cells may rely on its role as a transcriptional activator of cell cycle-related genes. We have tested this hypothesis using retrovirally transduced wild-type (wt) p53 in breast cancer cell lines containing homozygously endogenous mutant (mt) p53. Restoring the expression of wt p53, the percentage of cells in S phase was reduced, G1/S transition was slowed, and progression through S was restrained. The fraction of cells with a flattened "Cdk-minus" phenotype increased 5- to 10-fold. High constitutive mRNA expression of the cyclin-Cdk inhibitor *WAF1* in MDAMB231 cells was not induced upon restored wt p53 expression suggesting a p53-independent pathway in the regulation of *WAF1* mRNA expression. Wt p53 acted trans-dominantly in the presence of accumulating mt p53 and installed a modulation of G1/S transition and S phase progression independent of WAF1 expression.

Key words: cell cycle, tumor suppressor gene, p21, Cip1, Sdi1, Pic1, gene transfer

Emerging evidence suggests that the DNA-binding 53-kd nuclear phosphoprotein p53 may exert control of cellular proliferation by transcriptional regulation of genes related to cell cycle and DNA replication [Lin et al., 1992; Mercer et al., 1991]. The tumor suppressor gene *TP53* is frequently found mutated in a wide variety of human cancers including breast cancer [Hollstein et al., 1991]. Such alterations can abrogate the growth modulating function of p53. Transfer of the wild-type (wt) gene is sufficient to suppress some features of the neoplastic phenotype in cultured cells derived from various human tumors including breast cancer [Runnebaum et al., 1994]. These studies demonstrate that p53 can play central and even overriding roles in the control of cell replication and proliferation. A number of p53 mutants analyzed no longer bind to p53-specific DNA binding sites. In non-tumorous cells, p53 is expressed at low levels and has apparently no vital role in embryonic or normal mature cells, since p53 "knock-out" mice develop normally [Donehower et al., 1992]. In stressed cells, however, p53 appears to play an important growth-modulating role. Following radiation-induced DNA damage an increase of p53 expression and inhibition of DNA synthesis has been observed suggesting a cell cycle checkpoint function for p53 regulated as a feedback mechanism [Kastan et al., 1992; Zhan et al., 1993]. Recently, a cyclin-dependent kinase inhibiting 21-kd protein (p21), inducible by wt p53, has been identified independently by three groups. p21, variously known as WAF1 (wild-type p53-activated fragment 1) [El-Deiry et al., 1993], Pic1 (p53-regulated inhibitor of Cdks), Cip1 (Cdk-interacting protein) [Harper et al., 1993], and Sdi1 (senescent cell-derived inhibitor) [Noda et al., 1994] has been found to be induced by DNA damaging agents that trigger G1 arrest or apoptosis in cells with wt p53 but not in tumor cells containing mutant (mt) p53 [El-Deiry et al., 1994]. WAF1 may therefore be one important downstream effector of p53 keeping cells from transition into S phase. In 700 primary human breast carcinomas high S phase percentage and abundance of mt p53 protein have been found to indicate poor prognosis [Allred et al., 1993]. Using breast carcinoma cell lines we have studied the hypothesis whether in the presence of accumulating mt p53 the ret-

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rovirally mediated wt p53 could *trans*-dominantly contribute to the modulation of S phase entry and progression through *WAF1* mRNA induction.

MATERIALS AND METHODS

Cell Culture

Breast cancer cell lines MDAMB231, SKBR3, and HS578T expressing mutant p53 [Runnebaum et al., 1991] as well as the helper cell lines psi2 and PA317 were obtained from American Type Culture Collection (ATCC). Cells were grown under conditions recommended by ATCC.

Preparation of Vectors

The derivation and organization of p53 retroviral vector Lhp53RNL used in these studies have been described elsewhere [Cheng et al., 1992]. It expresses a wt p53 cDNA obtained from Dr. M. Oren. The vector Lhp53RNL expresses the human p53 cDNA from the Moloney murine leukemia virus 5′ LTR and the bacterial neomycin phosphotransferase gene from the Rous sarcoma virus LTR. The similar retrovirus construct pLLRNL containing the firefly luciferase gene instead of the p53 cDNA was used as a control for the effect of wt p53 expression in infected cells. Control vector LZRNL has been described elsewhere [Xu et al., 1989]. Techniques used for production and titration of retroviral vectors were similar to those previously reported [Cheng et al., 1992]. Clones producing high titers were identified by titration of producer cell supernatant on 208F cells and the production of p53 was determined by immunoprecipitation.

Retroviral Infection, Morphological Studies

For infection of breast carcinoma cells, supernatant medium from producer cells containing 4 μg/ml of polybrene was used. The supernatant medium was exchanged every four hours until a multiplicity of infection (MOI) of 2 was reached (bulk infection). Cells were then kept in regular nonselective medium. The susceptibility of MDAMB231 cells to infection was determined using LZRNL at a MOI of 2 followed by histochemical detection of β-galactosidase activity [Xu et al., 1989]. Morphological effects of mock infection and of infection with LLRNL or with Lhp53RNL were determined by light microscopy before and after Giemsa staining.

Growth Rate

Growth rate experiments were carried out as described previously to confirm observed changes of the growth rate on the population of Lhp53RNL-, LLRNL-, and mock-infected MDAMB231 cells (MOI 2) subjected to flow cytometry [Runnebaum et al., 1994].

Immunoprecipitation of p53

Immunoprecipitation of wt and mt p53 from 10^6 or 5×10^6 cells was carried out with PAb1801 and PAb421 (Oncogene Science, New York) and G59-12 (Pharmingen, San Diego) as described previously [Runnebaum et al., 1991]. Supernatants of hybridoma cells MOPC21 and MOPC11 served as controls.

Transfection and Luciferase Reporter Gene Assays

Cells were grown to 75% confluence on 60-mm petri dishes. The medium was changed 24 h before transfection. Ten μg of reporter plasmid pWWP-Luc and 2 μg of pC53-SN3 or the parental vector pCMV (CMV-Neo-Bam) were transfected with the calcium phosphate method [Baker et al., 1990; El-Deiry et al., 1992]. Cells were harvested for luciferase activity 36 h after transfection and light production was measured in a luminometer (counts per second, cps) using the Promega luciferase assay kit (Madison, WI). The assays were repeated twice and the results were noted as the means of triplicate experiments.

Differential RT-PCR

Reverse transcription (RT) was prepared from 1 μg of total RNA followed by PCR as previously described [Runnebaum et al., 1991, 1994]. cDNA from p53-transduced and LLRNL-infected MDAMB231 cells was used for PCR amplification of *WAF1* and of β_2-microglobulin for reference. Primer pairs were chosen to span intronic sequence such that only cDNA could be amplified. The primer sequences *WAF1* sense O-122 5′AGGATCCATGTCAGAACCGGCTGG3′ and *WAF1* antisense O-123 5′CAGGATCCTGTGGGCGGATTAGGGCT3′ resulted in a 520-bp fragment, the β_2-microglobulin sense O-239 5′TTAGCTGTGCTCGCGCTACT 3′ and β_2-microglobulin antisense O-240 5′ACACGGCAGGCATACTCATC3′ primer resulted in a 287-bp fragment. One fortieth of the RT product and further dilutions thereof were used for PCR. Four different dilutions (1:40, 1:80, 1:160, and

1:320) of the cDNA template were tested to ensure that the amount of PCR product reflects the quantity of template. Oligonucleotide sense primers for *WAF1* and β_2-microglobulin were 5′ end-labeled by a standard protocol. To amplify sequences of the more abundantly expressed β_2-microglobulin only 50% of the primer concentration (12.5 picomoles per 100 μl reaction mix) was used. After initial denaturation for 2 min at 94°C, 30 cycles consisting each of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min were applied. Ten μl of the PCR product was loaded on a 10% polyacrylamide gel and electrophoresed for 4 h at 400 V. The gel was dried and loaded in the Betascope blot analyzer (Betagen, Waltham, MA) for 2 hours for direct quantification of the counts in each band.

WAF1 Sequence Analysis

Mutational analysis of the *WAF1* gene based on nucleotide sequencing. For PCR amplification of the coding region, 3 sets of primers (sequences generously provided by Dr. Sukumar, Johns Hopkins University, Baltimore, MD) were used: Sense O-241 CTT GTA TCT CTG CTG CAG GC, antisense O-242 CGT GGG AAG GTA GAG CTT G, sense O-243 ACT TCG CCT GGG AGC GTG T, antisense O-244 CGT GCA CAT GTC CGC ACC T, sense O-245 TCC TCT TCT TCT TGG CCT GG, antisense O-246 AGG ACT GCA GGC TTC GTG T. PCR fragments were subcloned and sequenced on an automated A.L.F. DNA Sequencer (Pharmacia LKB, Uppsala, Sweden).

Cell Cycle Analysis

Bulk-infected MDAMB231 cells were synchronized by a double thymidine block [Mudryi et al., 1991]. The cells were released from the second thymidine block and immediately pulse-labeled with 10 μM bromodeoxyuridine (BrdU) in regular cell culture medium for 45 min, for 5 h, 11 h, or 27 h [Sasaki et al., 1987]. For fluorescence-activated cell scan (FACScan) cells were harvested and fixed in 70% ethanol for 30 min. After pelleting 10^6 cells at 1,000*g* for 10 min at room temperature, the cell pellet was incubated on ice in 0.1 M HCl/0.5% Triton X-100, and washed with water. Cellular DNA was denatured in a boiling water bath for 10 minutes and rapidly cooled on ice. After an additional wash with PBS-A/0.5% Triton X-100, the cells were incubated with 0.5 μg anti-BrdU fluorescein-isothiocyanate conjugate at 25°C in the dark for 30 min and rewashed. Finally, cells were resuspended in 6 ml PBS-A containing 6 μl propidium iodide (PI) at 5 mg/ml and 120 μl RNAse at 10 mg/ml. Green (BrdU) and red (PI) fluorescence was analyzed on a Becton Dickinson FACScan and a Ortho Cytofluorograph 50-H. The percentage of cells in the G1/G0, S, and G2/M phases of the cell cycle labeled with BrdU and PI was determined.

RESULTS

To investigate the effect of restored wt p53 expression on cell cycling of breast carcinoma cells that express only endogenous mt p53, MDAMB231 (Lys^{280}), SKBR3 (His^{175}), and HS578T (Phe^{157}), cells were bulk-infected with the retroviral vector Lhp53RNL or with the luciferase control vector LLRNL [Runnebaum et al., 1994; Xu et al., 1989]. The virus titers were determined by infection of rat 208F fibroblast cells. 208F cells carry wt p53 as analyzed by rat-specific PCR-single-strand conformation polymorphism (SSCP) screening. The introduction of an extra functioning wt allele of p53 into this rat cell line had no apparent effect on the morphology or the growth properties of these cells (data not shown). Bulk infection of the cell lines with Lhp53RNL or control vector LLRNL was carried out with MOI of 2 to avoid exposure to G418. Trypan blue cell exclusion test revealed no differences in the viability of the cells after infection with the two vectors compared with mock-infected cells. Two days after infection, the number of cells exhibiting a more flattened and enlarged phenotype had increased by 5- to 10-fold in all three cell lines (data not shown).

Preceding the FACScan analysis of MDAMB231, fractions of the infected cells were subjected to expression assays: Successful transduction after infection with Lhp53RNL was tested by determining the expression of the proviral wt p53 cDNA using RFLP analysis of the RT-PCR product as reported previously [Runnebaum et al., 1994]. By immunoprecipitation it was possible to detect transduced protein as a faint band in addition to abundant endogenous mt gene product (Fig. 1).

In FACScan analysis after propidium iodide stain, the distribution of Lhp53RNL-infected MDAMB231 cells in the phases of the cell cycle was changed when compared to the control cells that were mock-infected or infected by LLRNL. A higher number of Lhp53RNL-infected cells resided in G1/G0 phase, less of these cells were

found in S phase when compared with LLRNL or mock controls (Table 1).

MDAMB231 cell growth was synchronized by a double thymidine block at the G1/S transition point to study the effect of wt p53 on S entry and progression. After removing the second thymidine-containing medium the synchronized cells were exposed to BrdU. Incorporation of BrdU during a pulse of 45 min revealed a slower entry of Lhp53RNL-infected cells into S phase (Fig. 2). The majority of the LLRNL-infected control cells had moved significantly further into the S phase. De novo DNA synthesis was analyzed by continued BrdU-labeling. BrdU exposure for 5 h showed that the entire S phase fraction of LLRNL-infected cells had incorporated BrdU (Fig. 3C) whereas only 60% of the S phase fraction of Lhp53RNL-infected cells were BrdU-labeled (Fig. 3A): S fraction 27.5%, 16.6% labeled, 10.9% unlabeled. 11 h of continuous exposure to BrdU increased the percentage of labeled Lhp53RNL-infected cells in S to 70% (S fraction 28.3%, 19.9% labeled, 8.4% unlabeled) (Fig. 3B) and 27 h to 94% (27.7% labeled, 1.8% unlabeled, S fraction 29.5%).

WAF1 mRNA expression was compared in Lhp53RNL- and LLRNL-infected MDAMB231 cells. Expression of the reference β_2-microglobulin mRNA was found at similar levels in both, Lhp53RNL-infected and control virus-infected cells, indicating similar amounts of cDNA template present in the PCR. Results for *WAF1* expression were slightly different. In control virus-infected cells the basal expression level of *WAF1* was assessed (Fig. 4). The dilution factor was directly reflected by the number of counts in the specific PCR bands of both genes in the Betascope analyzer. Comparing the RT-PCR counts from Lhp53RNL- and LLRNL-infected cells, no p53-induced increase of *WAF1* expression was found. In the differential RT-PCR, *WAF1* mRNA levels in Lhp53RNL-infected cells were only half of the control cells in three independent experiments.

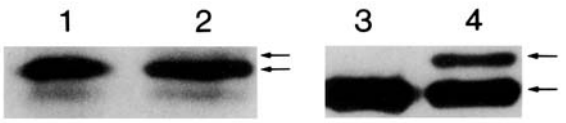

Fig. 1. Expression of p53 protein. 1, MDAMB231 infected with LLRNL; 2, MDAMB231 infected with Lhp53RNL expressing wt p53 (*upper arrow*, partly fused with the faster migrating mt p53) and endogenous mt p53 (*lower arrow*); 3, PA317-LLRNL mouse fibroblasts; 4, PA317-Lhp53RNL mouse fibroblasts expressing endogenous mouse wt p53 (*lower arrow*) and human wt p53 (*upper arrow*).

The entire coding sequence including exons 2 and 3 of the *WAF1* gene of the MDAMB231 cell line was analyzed to exclude the possibility of expression of a mutant *WAF1*. A heterozygous polymorphic third-base substitution (AGC to AGA) was identified at codon 31. The *WAF1* sequence was found to be wild type.

To investigate further the p53-independent *WAF1* mRNA expression, we used a transient transfection assay analyzing WAF1 transcription with the construct pWWP-Luc carrying the WAF1 promoter plus the luciferase reporter gene. The luciferase activities from a representative experiment were 4052 cps of mock-transfected, 28539 cps of pC53-SN3 (wt p53) and pWWP-Luc cotransfected, and 76310 cps of pWWP-Luc transfected MDAMB231 cells. The luciferase activity was found to be consistently 2- to 2.5-fold higher in cells that had not been transfected with the wt p53 cDNA.

DISCUSSION

Recent studies on the action of p53 in regulating cell proliferation have proposed p53 to control the transition through the restriction point in late G1 [Hunter, 1993]. p21 has been identified as the first direct link between the tumor suppressor protein p53 and cell cycle control [El-Deiry et al., 1993]. The ability of p53 to arrest cell growth could be explained by its ability to induce p21 which in turn would inhibit G1 cyclin-Cdk complexes necessary for transition into S.

An association of p53 inactivation and loss of cell cycle regulation has been observed in human breast cancer. In primary breast carcino-

TABLE I. Percentage of MDAMB231 in Phases of the Cell Cycle*

	Lhp53RNL-infected (%)	LLRNL-infected (%)	Mock-infected (%)
G1/G0 phase	50.4	41.9	40.9
S phase	28.9	38.7	39.6
G2/M phase	20.7	19.4	19.5

*Numbers from a representative experiment.

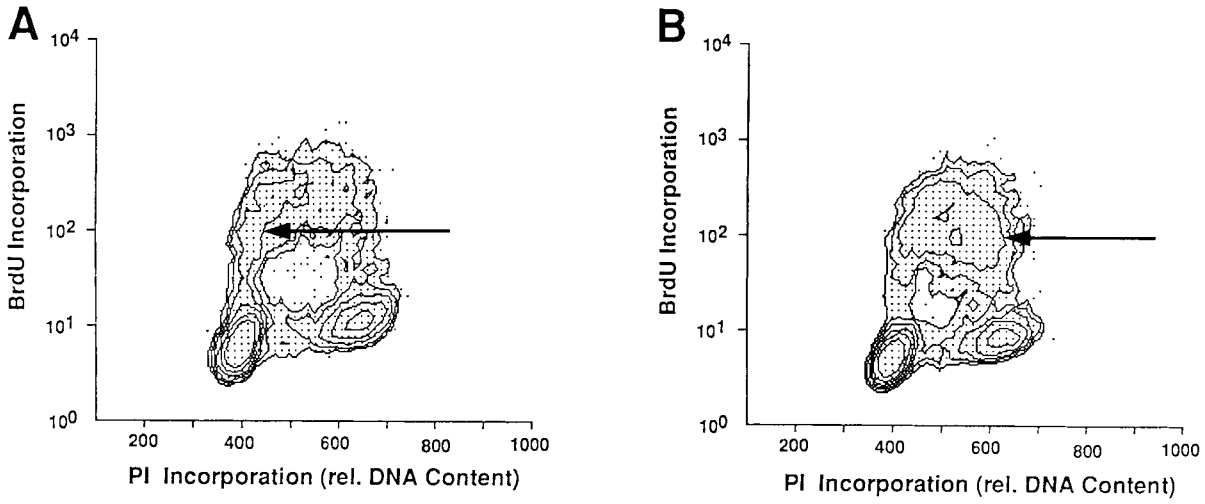

Fig. 2. Cytograms of red fluorescence (total DNA content) versus green fluorescence (BrdU incorporation) for MDAMB231 cells labeled with 10 μM BrdU for 45 min. The inner circles (*arrows*) indicate how far the majority of cells have progressed into S. **A:** Lhp53RNL-infected. **B:** LLRNL-infected.

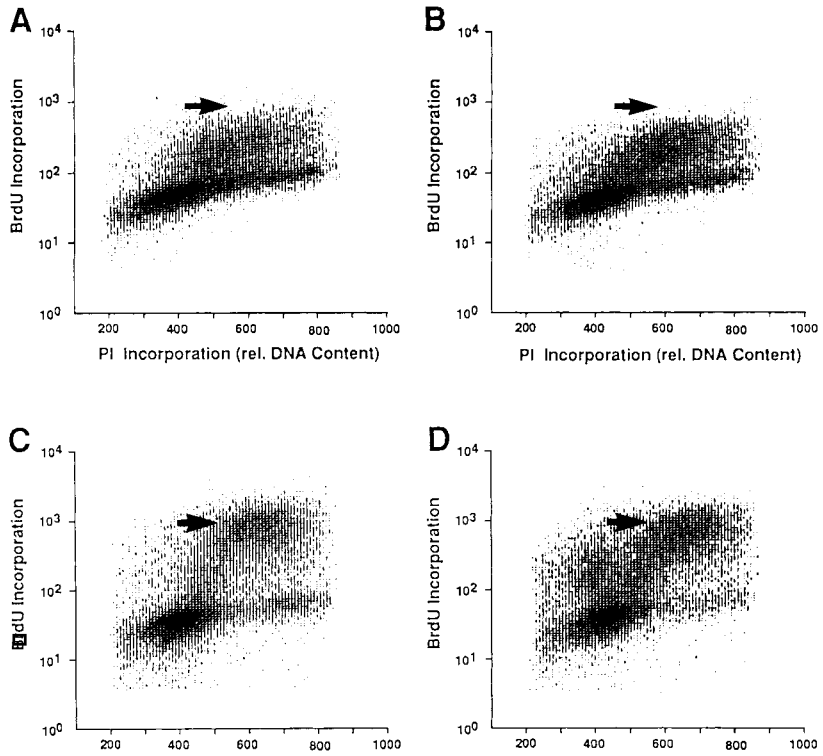

Fig. 3. Cytograms of red fluorescence (total DNA content) versus green fluorescence (BrdU incorporation) for MDAMB231 cells labeled with 10 μM BrdU. *Arrows,* BrdU-labeled cells in S. **A:** Lhp53RNL-infected, labeled for 5 h. **B:** Lhp53RNL-infected, labeled for 11 h. **C:** LLRNL-infected, labeled for 5 h. **D:** LLRNL-infected, labeled for 11 h.

mas, high S phase fraction and accumulation of mt p53 have been found to be strongly correlated, both independently associated with poor prognosis [Isola et al., 1992; Thor et al., 1992; Allred et al., 1993]. We chose three breast carcinoma cell lines expressing only mt p53 to test the effect of restored expression from a wt p53 allele on cell cycle progression and *WAF1* expression in this tumor type. The cell lines show immunodetectable p53 accumulation, HS578T

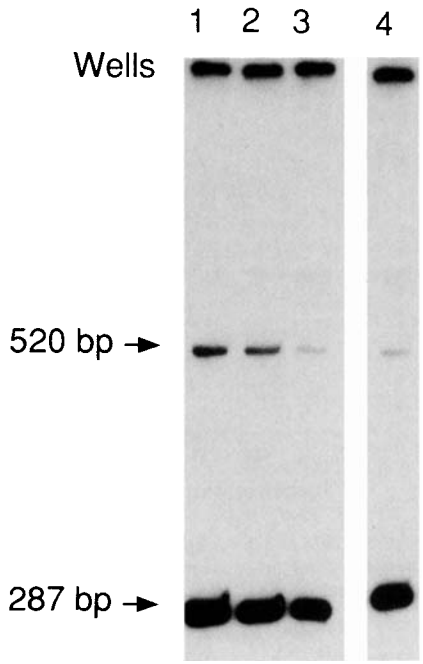

Fig. 4. Differential RT-PCR analysis of mRNA levels of *WAF1* (520 bp) and β_2-microglobulin (287 bp) in MDAMB231 cell using doubling cDNA template dilutions. The wells of the gel are indicated. 1, 1:40 MDAMB231-LLRNL; 2, 1:80 MDAMB231-LLRNL; 3, 1:160 MDAMB231-LLRNL; 4, 1:80 MDAMB231-Lhp53RNL.

with a valine to phenylalanine change at position 157, SKBR3 with an arginine to histidine change at position 175, and MDAMB231 with an arginine to lysine change at position 280 [Bartek et al., 1990; Runnebaum et al., 1991; Runnebaum et al., 1993]. To avoid the use of any selective drug in the culture media a bulk infection protocol was applied [Runnebaum et al., 1995]. A similar protocol has been successfully used by others to stably transduce CFT-1 cells (cystic fibrosis epithelial cell line) for at least 1 year following multiple infections with an amphotropic retrovirus in the absence of G418 selection [Olsen et al., 1993]. Upon Lhp53RNL infection all three cell lines showed an increased number of cells with an enlarged and flattened cytoplasm. Such changes may indicate an increase of a so-called Cdk-minus phenotype or of replicative senescence, a pre-crisis phenomenon in which p53 is thought to be involved in [Shay et al., 1991]. Among the three cell lines MDAMB231 showed the highest level of mutant p53 with an extended half-life of 7 h [Runnebaum et al., 1994]. MDAMB231 derived from a highly undifferentiated adenocarcinoma of the breast was used for cell cycle analysis after infection with Lhp53RNL. The decreased number of cells in S phase correlated with the extension of the doubling time from 31 to 40 h upon infection with Lhp53RNL. Restoring of wt p53 expression changed the dynamics of the G1/S transition and the DNA replicative phase of the cell cycle in MDAMB231. After release from the thymidine block in G1 and a 45 min pulse with BrdU the majority of labeled Lhp53RNL-infected cells had only little progressed into S when compared with cells infected by the control virus.

Several lines of evidence have suggested a "checkpoint" role of p53 in the regulation of the transition from G1 into S phase [Kastan et al., 1992; Livingstone et al., 1992; Yin et al., 1992]. Complexes of cyclins and cyclin-dependent kinases which are necessary to surmount the G1 restriction point can be inhibited by the wt p53-inducible factor *WAF1* (for review, see [Hunter, 1993]). The mutant p53 Lys^{280} of MDAMB231 has recently been shown to neither bind to p53 consensus DNA-binding sequences nor to transactivate [Park et al., 1994] and should therefore not be able to induce *WAF1*. Differential RT-PCR analysis of MDAMB231 showed a readily detectable constitutive expression level of *WAF1* mRNA indicating an induction of *WAF1* by p53-independent pathways as shown for fibroblasts from p53 knock-out mice [Michieli et al., 1994]. The level of *WAF1* expression, however, was not increased but even two fold reduced upon transduction of wt p53. This finding was further confirmed by transient transfection with the WAF1 promoter construct pWWP-Luc and suggested that *WAF1* was not the mediator of wt p53-dependent modulation of cell proliferation and tumor suppression previously studied in MDAMB231 breast cancer cells [Runnebaum et al., 1994]. The endogenous *WAF1* sequence proved to be wild-type indicating that WAF1 may well be functionally active in MDAMB231. It is conceivable that p53-dependent pathways different from *WAF1* exist to control the G1 restriction point. The antiproliferative effect of p53 has also been shown to be associated with a decrease of mRNA steady-state levels of a subset of other late G1 genes [Mercer et al., 1991; Lin et al., 1992].

In MDAMB231, the checkpoint function of wt p53 can be dominant over Lys^{280} mutant abundantly present in these breast carcinoma cells. A moderately slowed S phase entry however may not fully explain the prolonged doubling time

and the drastic tumor suppressing effect on MDAMB231 as described previously [Runnebaum et al., 1994]. Since p53 may be implicated in DNA replication we further tested for BrdU incorporation during S phase progression [Sherley, 1991; Wilcock and Lane, 1991]. Significantly more time was required for Lhp53RNL-infected cells until all cells in S were BrdU-labeled. This finding may indicate that wt p53 causes cells to proceed slower through the replicative phase. p53 may install a rate limiting mechanism in S, possibly in nucleotide synthesis, thereby modulating the proliferative potential [Sherley, 1991].

In conclusion, retroviral bulk infection without subsequent marker selection restored a p53-dependent *WAF1*-independent cell cycle modulation during late G1 as well as a rate-limiting mechanism during DNA synthesis associated with tumor suppression. The search for p53-inducible cell cycle-related genes may finally lead to factors other than *WAF1,* explaining the negative effect of p53 on the growth of breast cancer cells.

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