Research Article

Abrogation of p53 by its antisense in MCF-7 breast carcinoma cells increases cyclin D1 via activation of Akt and promotion of cell proliferation

Rishi Raj Chhipa, Ratna Kumari, Ankur Kumar Upadhyay, Manoj Kumar Bhat

National Centre for Cell Science, NCCS Complex, Ganeshkhind, Pune-411 007, India

A R T I C L E I N F O R M A T I O N
Article Chronology:
Received 24 January 2007
Revised version received
26 July 2007
Accepted 20 August 2007
Available online 4 September 2007

Keywords:
MCF-7
Antisense p53
Cell proliferation
Akt
Caveolin

A B S T R A C T
The p53 protein has been a subject of intense research interest since its discovery as about 50% of human cancers carry p53 mutations. Mutations in the p53 gene are the most frequent genetic lesions in breast cancers suggesting a critical role of p53 in breast cancer development, growth and chemosensitivity. This report describes the derivation and characterization of MCF-7As53, an isogenic cell line derived from MCF-7 breast carcinoma cells in which p53 was abrogated by antisense p53 cDNA. Similar to MCF-7 and simultaneously selected hygromycin resistant MCF-7H cells, MCF-7As53 cells have consistent basal epithelial phenotype, morphology, and estrogen receptor expression levels at normal growth conditions. Present work documents investigation of molecular variations, growth kinetics, and cell cycle related studies in relation to absence of wild-type p53 protein and its transactivation potential as well. Even though wild-type tumor suppressor p53 is an activator of cell growth arrest and apoptosis-mediator genes such as p21, Bax, and GADD45 in MCF-7As53 cells, no alterations in expression levels of these genes were detected. The doubling time of these cells decreased due to depletion of G0/G1 cell phase because of constitutive activation of Akt and increase in cyclin D1 protein levels. This proliferative property was abrogated by wortmannin, an inhibitor of PI3-K/Akt signaling pathway. Therefore this p53 null cell line indicates that p53 is an indispensable component of cellular signaling system which is regulated by caveolin-1 expression, involving Akt activation and increase in cyclin D1, thereby promoting proliferation of breast cancer cells.

Introduction

The frequent alterations in human malignancies are mutation of the p53 gene and it is the most commonly altered oncogene in the development of sporadic and hereditary breast cancers [1,2]. The loss of wild-type p53 function is an important event in breast tumorigenesis as documented in both human and murine systems [2,3]. Most of the p53 mutations result in loss of function although activating mutations are also observed. Usually p53 abnormalities are associated with poorer clinical outcome. This, likely, is the consequence of the known critical roles p53 plays in regulating the cell cycle, apoptosis, DNA repair, and maintenance of genome stability [4]. However, the exact mechanisms by which such lack of normal gene function leads to cancer formation and its progression are only beginning to be understood. Moreover
the downstream signaling pathways influenced by p53 remain to be clearly discovered. In cancers, it is clear that not all p53 mutations have equal effects; some have a dominant-negative effect (such as transdominant suppression of wt p53 or oncogenic gain of function) or loss of function, whereas others show only a partial loss of function where, for example, only a fraction of p53 target genes are deregulated [5,6]. Therefore elucidation of the role of tumor suppressor p53 by its depletion is vital to rational understanding of its involvement in cell cycle checkpoints, DNA repair, senescence, apoptosis, angiogenesis, and surveillance of genomic integrity as well as signaling network in the cells.

Functional inactivation of p53 can occur by several mechanisms, including direct genetic mutation [7], binding to viral oncoproteins (e.g. HPV18E6, SV40, and E1B-55KD) or cellular factors (e.g. Mdm-2), overexpression of dominant-negative mutant p53, and post-translational modifications [8,9] and more recently by small interference RNA or antisense oligonucleotide targeted inhibition [10]. Overall, these models have contributed significantly towards understanding functions of p53, though the results from these studies are not very conclusive as these depend on differential modes of abrogation or inactivation of p53 protein and its function. Moreover, almost all these modes of abrogation of p53 have been studied in the context of comparing gene expression patterns and identification of transcriptional targets in the cells in response to various cellular stresses such as chemo or radiosensitization and not looking at the role of p53 per se.

For all p53 inactivation studies done, it has been observed that they have some or other inherent drawbacks. It is often taken for granted that intracellular expression of E6 or any viral protein targeting p53 reflects a true p53 null phenotype; the major caveat nonetheless exists that these oncoproteins bind and interfere with the activity of many cellular proteins besides p53. On the other hand use of dominant-negative mutants of p53 for studying the importance of wild-type p53 may lead to erroneous conclusions due to unknown gains of function as well as an ineffective reduction of endogenous p53 function. The other modes of attenuation and abrogation of p53 function are either transient or in non-isogenic (differing in cell types and/or genetic background) model systems or are regulated by extra-cellular signal. Thus, the differences in attenuation and abrogation of p53 function may be either transient or in non-isogenic (differing in cell types and/or genetic background) model systems or are regulated by extra-cellular signal. Thus, the differences in attenuation and abrogation of p53 function will significantly alter functional outcome. Additionally, all information about relationship between loss and mutated p53 or any genetic and biochemical changes has not been definitely established because these studies were based on tumor biopsies and cell lines already lacking wild-type p53 [8,11,12]. In addition to all these, some more novel properties of p53 are now emerging including activation of signal transduction pathways and whether p53 is involved in firing of such pathways that originate at the level of the cell membrane. Since delineation of the role that p53 may play in cells has been hampered by the lack of appropriate model, there is a continuing need for genetically matched cell systems that specifically differ in p53 protein status.

Taken together this report describes the characterization of MCF-7As53 cell line derived from breast carcinoma MCF-7 cells as an isogenic cell system deficient only in p53 protein due to its antisense expression. This model provides a valuable tool to delineate the role of p53 in breast cancers and to facilitate in more systemic approach to decipher both up and downstream roles of p53 in a complex signaling network of cancer cells.

Materials and methods

Reagents and antibodies

Sources of materials were as follows: doxorubicin, methylthiazolyt tetrazolium (MTT), wortmannin, pifithrin alpha (PFTα), methyl-β-cyclodextrin (MCD), and 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-Gal) were purchased from Sigma, MO, USA. Doxorubicin was dissolved in sterile water to prepare a stock of 50 mM. MTT was reconstituted as 1 mg/ml in DMEM without phenol red. PFTα, wortmannin, and X-Gal were reconstituted in DMSO. Antibodies against p53, estrogen receptor-alpha (ERα), Mdm2, Bax, p73, alpha-fetoprotein (α-FP), cyclin D1, caveolin-1 (Cav-1), Akt, pAkt, β-tubulin, and β-actin were purchased from Santa Cruz Biotechnology, CA, USA. Antibody specific to phospho-caveolin (pCav-1) was purchased from BD Bioscience, CA, USA.

Cell cultures and development of MCF-7As53 cell line

Human breast cancer cell lines MCF-7 (ATCC HTB-22), MDA-MB-231 (ATCC HTB-26), and MDA-MB-468 (ATCC HTB-132) were obtained from ATCC (Manassas, VA, USA) and maintained in our in-house National Cell repository. MCF-7 cells were routinely cultured in DMEM, MDA-MB-231 and MDA-MB-468 were cultured in DMEM and F12K (1:1), supplemented with 10% heat inactivated fetal bovine serum (HyClone, UH, USA), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Invitrogen, MD, USA) at 37 °C with 5% CO2. The MCF-7 Tet-On (Clontech, CA, USA) cells were co-transfected with pTRE2 and pTK-Hyg plasmid expression vector containing human p53 cDNA which was excised from p53 plasmid expression vector pC35-SN3 (a kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore) and cloned in reverse orientation in plasmid’s ratio being 5:1. Cells were selected on hygromycin (200 μg/ml) for 4 weeks. MCF-7H cells were derived from MCF-7 Tet-On cells which were co-transfected with pTRE2 and pTK-Hyg constructs and selected for hygromycin resistance. After screening several clones, we succeeded in developing few individual clones which expressed antisense p53. These clones were subsequently pooled together and designated as MCF-7As53. The p53 deficient phenotype was maintained in MCF-7As53 even after being passaged for more than 20 times over a period of 6 months. We observed that Tet-On expression system functions in cells grown in media supplemented with normal fetal bovine serum (data not shown). Therefore, we choose to propagate cells in media supplemented with normal fetal bovine serum instead of under conditions in which addition of exogenous doxycycline would be necessary. It is likely that levels of expression of antisense RNA in cells grown in media containing normal fetal bovine serum are sufficient to cause abrogation of p53 in MCF-7As53 cells and it does not warrant addition of exogenous doxycycline. When maintained in normal culture medium, these
cells exhibited complete abrogation of p53 protein as well as its transactivation activity.

**CAT reporter assays**

The p53-CAT reporter construct pG13-CAT, which contains 13 repeats of p53 binding site inserted 5′ to poliovirus basal promoter linked to CAT reporter gene (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore), was transiently transfected in MCF-7, MCF-7As53, and MCF-7H cells by lipofectamine 2000 (LF2000) method (Invitrogen, MD, USA). Almost 80% confluent cells in 35 mm culture plate were transfected with 4 μg of DNA including 1 μg either pEGFP-N1 or pCMVβ plasmid (Clontech, CA, USA) as an internal control to assess the transfection efficiency. Vector plasmids were used as carrier DNA to make up the final DNA concentration to 4 μg. One hour before transfection, 1 ml of fresh medium was added to each plate. For each plate to be transfected, each of 4 μg of DNA and 4 μl of LF2000 reagent were diluted into 250 μl of Opti-MEM (Invitrogen, MD, USA) separately and incubated for 5 min at room temperature. Diluted DNA was mixed with diluted LF2000 reagent and incubated at room temperature for 40–45 min to allow LF2000–DNA complex formation. Five hundred microliters of LF2000–DNA complex was added dropwise to the plate and mixed gently by rocking. Cells were incubated at 37 °C for 24 h. Thereafter, cells were washed and incubated at 37 °C for further 24 h before harvesting. pWWPCAT, which has p53 binding site from p21 promoter, was also used in reporter assays to evaluate p21 specific p53 transactivation potential.

To assay CAT activity, cells were collected and washed thrice with ice-cold PBS and resuspended in 0.25 M Tris–Cl (pH 7.5) buffer. Cells were lysed by four cycles of rapid freeze–thaw. CAT assay was performed by taking equal amounts of lysate protein in presence of 1 μCi [14C]-chloramphenicol (NEN, Boston, MA, USA) and 100 μg of acetyl CoA (Amerham, Aylesbury, UK) in 0.25 M Tris–Cl (pH 7.5) in a total reaction volume of 100 μl. Reaction mixture was incubated at 37 °C for 6 h and terminated by adding ethyl acetate to the sample tubes. Products were resolved by thin layer chromatography (TLC), using mixture of chloroform and methanol (19:1 ratio). TLC plates were analyzed by autoradiography and scanning on a phosphor imager (Bio-Rad, USA). The specific CAT activity was calculated by determining the fraction of chloramphenicol that had been acetylated during the reaction. Transfection efficiency was determined simultaneously by measuring GFP intensity directly from the cell lysates of transfected cells by fluorometer to confirm equal transfection efficiency as well to normalize the reporter activity. Equal amounts (50 μg) of cell lysate from pEGFP-N1 transfected cells were taken in the wells of 96-black-well plates. The fluorescence intensity of GFP was recorded on plate reading fluorometer (Fluoroskan Ascent FL, Labsystems, MA, USA) with filter set at excitation 485 nm and emission 510 nm. In experiments where pCMVβ was also used as internal control for normalization of transfection efficiencies, the activity of β-galactosidase was assayed in pCMVβ transfected cells by using chloro phenol-red-β-D-galactopyranoside (CPRG) purchased from Sigma, MO, USA as substrate. Twenty micrograms of cell lysates and one millimolar of CPRG were added to each well and incubated at 37 °C for 6 h. Absorbance was taken in microplate reader (Multiskan Ascent, Labsystems, MA, USA) at 570 nm.

**Transient expression of sense p53 in MCF-7As53 cells**

In separate experiments involving overexpression of wild-type p53, pCS53-SN3 plasmid vector (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore) was transiently transfected in MCF-7As53 cells by LF2000 method (Invitrogen, MD, USA) as described earlier. After transfection, cells were washed and fresh media were added to the cells in culture plates for an additional 24 h. The cells were lysed and lysates were subjected to immunoblotting. Transfection efficiency was determined simultaneously by transfecting green fluorescent protein expressing plasmid pEGFPN1 (Clontech, CA, USA). It was also used for mock transfections as well as an internal control for comparison of protein expression.

**siRNA transfection in MCF-7 cells and MCF-7As53 cells**

Almost 80% confluent cells in 60 mm culture plate were transfected with siRNA reconstituted in siRNA dilution buffer. Fluorescein conjugated control siRNA was utilized as an internal control to assess the transfection efficiency. The siRNAs, transfection medium, transfection buffer, and transfection reagent were obtained from Santa Cruz Biotechnology, CA, USA. For each plate, 18 μl of siRNA from the stock (10 μM) was diluted into 200 μl of transfection medium and 12 μl of transfection reagent was diluted into 200 μl transfection medium in separate tubes. After incubating for 5 min at room temperature, the diluted siRNA was mixed with diluted transfection reagent and further incubated at room temperature for 20–25 min to allow complex formation. The complex was added dropwise to the plate containing cells with 1600 μl transfection medium. Cells were incubated at 37 °C for 7 h. Thereafter, cells were washed and incubated with medium containing 20% serum at 37 °C for further 24 h before harvesting.

**In vitro growth rate analysis**

Cells were seeded at a density of 2×10³ cells per well in triplicates into 96 well microtiter plate and allowed to adhere at 37 °C. After that, cells were cultured for further 24 h, 48 h, 72 h, and 96 h respectively. After each time period, media were decanted and 50 μl of MTT (1 mg/ml) in DMEM (without phenol red) was added to each well and further incubated for 4 h at 37 °C. Formazan crystals were solubilized in 50 μl of isopropanol by incubating with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter. Absorbance was converted to number of cells with 2×10⁵ cells taken at 0 h point.

**Flowcytometry for cell cycle analysis**

Cells were plated at a density of approximately 8×10⁵ cells in 60 mm tissue culture plates and allowed to grow for 24 h. Cells were harvested by trypsinization and subsequently processed
for flow cytometric analysis. In brief, cells were washed twice in chilled PBS and fixed in 70% ethanol on ice. After RNase A (200 µg/ml) treatment for 30 min at 37 °C, 50 µg/ml propidium iodide (PI) was added to the cell pellet and incubated in the dark for 30 min on ice. The fluorescence of PI was collected through a 585 nm filter in FACScan flow cytometer (Becton Dickinson GmbH, Heidelberg, Germany). The data were analyzed using the Cell Quest Software, for 10⁶ cells. Experiments were repeated three times.

Western blot analysis

As required for the experiments, untreated or PFTα (20 µM), DMSO, wortmannin (50 nM) or MCD (5 mM for 4 h) treated MCF-7 or MCF-7As53 cells and MDA-MB-231 cells or MDA-MB-468 cells were washed thrice with ice-cold PBS after 24 h of treatment and lysed in 100 µl of ice-cold lysis buffer (20 mM HEPES pH 7.4 containing 1% NP-40, 2 mM EGTA, 2 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 mM TPCK, 2 mM Na3VO4, and protease inhibitor cocktail table1) per 1×10⁶ cells. Samples were boiled in SDS sample buffer for 10 min followed by separation on an SDS–PAGE. An equal amount of protein samples (75 µg) was resolved on 10–12% SDS–polyacrylamide gel and then transferred onto nitrocellulose membranes (Amersham, Aylesbury, UK). The membranes were probed with respective primary antibodies followed by HRP conjugated secondary antibodies. Immunoblots were detected by enhanced chemiluminescence (ECL) reagent (New England Biolabs, MA, USA). Whenever required, the blots were stripped by incubating the membrane at 50 °C for 30 min in stripping buffer (62.5 mM Tris–Cl pH 6.7, 100 mM mercaptoethanol, 2% SDS) with intermittent shaking. Membranes were washed thoroughly with TBS and reprobed with required antibodies accordingly wherever possible. Otherwise gels run in duplicates were probed for the desired proteins by western blotting.

RNA extraction, cDNA synthesis, and RT-PCR

Total cellular RNA, from treated and untreated cells, was extracted using TRIZol™ reagent (Invitrogen, MD, USA), according to the manufacturer’s instructions. Five micrograms of total RNA and oligo(dT)12-18 primer or random hexamers was taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 units of M-MLV reverse transcriptase (Invitrogen, MD, USA), under conditions recommended by the manufacturer and the reaction was allowed to proceed at 37 °C for 50 min. Reaction was terminated by heating at 70 °C for 15 min. Each RT-PCR contained 10% of cDNA, 20 µM of each primer in 20 mM Tris–Cl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTP mix, and 1 unit of platinum Taq DNA polymerase (Invitrogen, MD, USA) in a final volume of 20 µl. After an initial denaturation for 2 min at 95 °C, 30 cycles of denaturation (94 °C for 1 min), annealing (for 1 min), and extension (72 °C for 1 min) were performed on a DNA thermal cycler (Techne, Cambridge, UK) with a final extension for 10 min at 72 °C. The primer pairs used were as follows: p53 5′-CTG AGG TTG GTT CAT TGG CCA CCA TCC-3′ (F) 5′-TCG TCT CTC TGG GAA CAT CAT GGA GGG-3′ (R), Bax 5′-TTC ATG GAC GGG TCC GGA GAA-3′ (F) 5′-TGT CCA CAT GAT GGT TCT-3′ (R), p21 5′-GAC ACC ACT GGA GGG TGA CT-3′ (F), 5′-GGC GTT TGG AGT AGT AGA AA-3′ (R) hGADD45 5′-AGA GCA CAA GAC CGA AAG GAT-3′(F) 5′-AGC CGG AGG ATG TTT AGT TGC-3′(R) and β-actin 5′-ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3′ (F) 5′-CGT CAT ACT CCC TGC TGT CTT ATC CAC ATC TGC-3′ (R). The annealing temperatures for p53, Bax, p21, GADD45, and β-actin PCR were 55 °C, 59 °C, 59 °C, 55 °C, and 55 °C respectively.

Senescence-associated β-galactosidase (SA-β-Gal) staining

MCF-7 and MCF-7As53 cells were plated at a density of 2.5×10⁴ cells per 35 mm culture dish and allowed to grow for 2 weeks. For senescence-associated β-galactosidase staining [14], cells were washed twice with PBS and fixed with 2% formaldehyde and 0.2% glutaraldehyde for 5 min. The cells were then washed again with PBS and incubated at 37 °C (without CO₂) with fresh 1 mg/ml of X-Gal made as 40 mg/ml stock in diethylformamide with 5 mM potassium ferrocyanide, 150 mM NaCl, 40 mM citric acid/sodium phosphate, pH 6.0, and 2 mM MgCl₂. Cells were then examined for the development of blue color, which was evident after 12–16 h of incubation with X-Gal. Doxorubicin (1 µM for 2 h) treated MCF-7 cells were taken as positive control for SA-β-Gal staining done after 2 days of the drug removal. Cells were finally rinsed with PBS and photomicrographs were taken with Olympus digital camera (Olympus, Tokyo, Japan).

Immunofluorescence studies in MCF-7 and MCF-7As53 cell lines

The cells were grown on glass coverslips coated with poly-l-lysine, or multiwell microslides (ICN Pharmaceuticals, CA, USA) until 70% confluency. Media were removed and cells were washed with ice-cold PBS twice. The cells were fixed with cold 4% paraformaldehyde (made freshly in PBS) for 20 min at room temperature (RT). Cells were again washed thoroughly with PBS after fixating. Cells were permeabilized with PBS containing 0.1% Triton-X 100 for 10 min at RT, wherever required. After washing thoroughly with PBS, cells were blocked with 5% fetal bovine serum made in PBS for 1–2 h at RT. Subsequently cells were incubated with antigen specific primary antibodies at 1:100 dilutions in PBS for 2 h at RT. After washing thoroughly cells were incubated with FITC conjugated secondary antibody at 1:200 dilution for 1 h at RT. For negative control cells were incubated with secondary antibody alone. After washing the cells thoroughly they were overlaid with mounting medium containing antifade (Vector laboratories, CA, USA) and with mounting medium containing antifade and DAPI (Santa Cruz Biotechnology, CA, USA). The slides were then subjected to immunofluorescence or confocal microscopy (Zeiss LSM510, Heidelberg, Germany) analysis. Images were subsequently processed by Adobe Photoshop software.

Statistical analysis

Data are expressed as the mean of three independent results. Statistical comparisons are made using Student’s t test and P value <0.05 was considered as significant.
Results

Derivation of antisense p53 expressing breast cancer MCF-7AS53 cells

The MCF-7 Tet-On cells were co-transfected with pTRErevp53 and pTK-Hyg constructs as described in the Materials and methods section. Numbers of individual clones were screened for p53 expression by western blotting. As shown in Fig. 1A, we obtained two clones, MCF-7As3 and MCF-7As6, in which p53 expression was significantly downregulated compared to that in parental MCF-7 cells as well as in parallelly selected control MCF-7H cells. Moreover, when assayed for p53 dependent CAT reporter assays, MCF-7 and MCF-7H cells exhibited higher p53 dependent transactivation potential characteristic of the presence of wild-type p53 protein. The clones designated as MCF-7As3 and MCF-7As6 demonstrated lack of p53-CAT reporter activity due to abrogated p53 protein expression as detected by western blots. Fig. 1Ba shows CAT activity autoradiogram and Fig. 1Bb represents an intensity plot in which CAT activity was normalized with β-galactosidase activity. The antibiotic doxycycline, an inducer for Tetracycline Regulatory Element (TRE), is also a potential anticancer agent known to have effect on p53 in conjunction with chemotherapeutic drugs [15–18]. Since not much is known about the side effects associated with long time exposure of doxycycline on the properties of cells and to avoid possible toxicity, we propagated MCF-7AS53 cells under normal culture conditions in the absence of exogenously added doxycycline. The protein levels for p53 illustrated in Fig. 1C and p53 transcript levels in Fig. 1D are for clones As3 and As6 maintained in the presence of normal serum after 20 passages. The abrogation of p53 due to the stable genomic integration of its antisense fragment was also confirmed in both MCF-7As3 and MCF-7As6 as molecular message for p53 was barely detected. Furthermore, to investigate the status of p53 regulated genes p21, Bax, and GADD45, we carried out RT-PCR analysis under similar growth

Fig. 1 – Establishment of the stable clones expressing p53 antisense cDNA. (A) The clones which were selected to verify antisense p53 expression were investigated for p53 protein levels. The numbers indicate relative levels of p53 which were normalized to β-actin levels. (B) p53 dependent CAT reporter activity (showing (a) as CAT chromatogram and (b) as CAT activity plot). Clones 3 and 6 indicated diminished p53 specific transactivation potential. (C) p53 protein expression after 20 passages, as analyzed by western blot. As3 and As6 being indicative of two selected clones which exhibited null p53 status. Protein loading has been normalized with β-actin levels. (D) MCF-7 (M) and MCF-7H (H) cells were compared for p53 at gene expression levels with both the antisense p53 expressing clones of MCF-7 (As3 and As6). (E) MCF-7 and MCF-7H cells were compared with the MCF-7 derived clones (As3 and As6) expressing antisense p53 for immediate downstream molecules at gene expression level. Bax, p21, and GADD45 transcripts are illustrated. Normalization was done with β-actin levels. All the experiments were performed under the condition where normal fetal bovine serum was used for cell culture.
conditions. As can be seen in Fig. 1E, no significant alteration in the expression pattern of these genes was detected in MCF-7As3 and MCF-7As6 clones in comparison with the expression in parental MCF-7 as well as control MCF-7H cells. These genes may be utilizing p53 independent pathways for their expression [19]. Because both As3 and As6 clones were characteristically similar, for further studies and investigations, MCF-7As3 and MCF-7As6 were pooled together and termed as MCF-7As53 cell line.

Molecular characterization of MCF-7As53

The antisense p53 expressing MCF-7As53 cells, parental MCF-7 cells, and resistant clone MCF-7H were further characterized and compared for breast carcinoma specific marker molecules as well as for other p53 associated proteins. ERα plays an essential role in breast cancer development and MCF-7 cells are ER positive breast cancer model [20]. As illustrated in Fig. 2A, no difference in ER expression levels was detected in the three cell lines and the level of ER expression was identical. Apart from ER status MCF-7As53 cells exhibited normal α-FP levels, which is a well known carcinoembryonic antigen expressed in breast carcinoma [21]. Bax, a well-known p53 regulated apoptotic protein, was also not altered very significantly. No differences were detected in the expression of Mdm2 oncoprotein, the key upstream regulator of p53, which inhibits its transactivation properties and targets it to proteasome mediated degradation. Mdm2 is amplified or over-expressed in many human cancers, including breast cancer, ovarian cancer, osteosarcoma, and lymphoma [22,23]. Another important molecule is p73, which is a p53 family protein with structural and functional homology and shares similarities with the tumor suppressor gene with respect to activation of transcription from p53-responsive promoters, along with directly or indirectly affecting either p53 activity or expression levels [24,12]. The steady-state p73 protein levels in the MCF-7As53 cell line were equal when compared with those in parental cells (Fig. 2A). These results imply that MCF-7As53 exhibited no gross variability at molecular level except for the p53 expression. The house keeping proteins such as β-tubulin and β-actin were used as internal controls for protein loading as well as for comparing changes in the protein expression pattern in the cells. In some experiments comparative profile of molecules were compiled from various duplicate gels.

Further to verify that indeed p53 downregulation also results in decrease in p53 dependent transactivation activity, we performed CAT reporter assay. MCF-7 and MCF-7As53 cells were separately transfected with either pG13-CAT or pWWP-CAT constructs as described in Materials and methods. As expected CAT reporter activity is barely detected in MCF-7As53 cells when compared with CAT reporter activity

Fig. 2 – Relative expression levels of p53 associated molecules in MCF-7 (M), MCF-7As53 (As), and MCF-7H (H) cell lines. (A) Molecules such as Mdm2 oncoprotein, p73, Bax, ERα, and α-FP are compared in these cells. Both β-actin and β-tubulin were used as loading controls. Blots were analyzed densitometrically for quantification and the values were normalized to β-actin. (B) MCF-7 (M) and MCF-7As53 (As) cells were transiently transfected with pG13-CAT and pWWP-CAT (with p21 promoter element) reporter plasmids and harvested for estimation of basal level of CAT reporter activity. CAT assay was performed as described in Materials and methods. As shown, chromatogram (a) and CAT reporter activity plot (b) with the of EGFP intensities which were estimated for comparison of transfection efficiency under similar experimental conditions.
in MCF-7 cells (Figs. 2Ba and Bb). The decreased p53 reporter activity is indeed due to lack of functional p53. In all the transfection experiments EGFP was used as an internal control for transfection efficiency and EGFP intensity was more or less identical in all the samples.

**Morphology, growth, apoptotic, and senescence studies on MCF-7As53**

MCF-7As53 cells have uniform and basal epithelial morphology, size, and shape (Figs. 3Aa and Ab) at normal and identical growth conditions. Data also imply normal anchorage dependent growth of these cells in tissue culture dishes. Despite p53 being a regulator of senescence and differentiation [25] and MCF-7As53 cells having negligible total p53, these do not express cellular senescence-associated β-galactosidase and therefore are not senescent even after being in culture for 2 weeks (Figs. 3Aa and Ab). The doxorubicin treated MCF-7 cells are shown as positive control (Fig. 3Ac) for the method employed [26,14]. We further investigated the growth pattern by performing MTT proliferation assay as described in Materials and methods. As shown in Fig. 3B, MCF-7As53 cells grow more rapidly than parental MCF-7 cells. The doubling time of MCF-7As53 was about 24 h compared to >36 h for MCF-7.

**MCF-7As53 cells have proliferative phenotype due to upregulated cyclin D1 and overexpression of p53 downregulates cyclin D1**

MCF-7As53 cells were identical to MCF-7 cells except for the growth pattern as indicated by MTT proliferation assay (Fig. 3B). As shown in Fig. 3C, the altered growth rate of MCF-7As53 is due to variations in distribution of cells in different phases of cell cycle. The cell cycle analysis by flowcytometry revealed that in MCF-7As53 cells G0/G1 was significantly depleted ("P<0.05 vs. MCF-7 cells) and more cells accumulated in S/G2M phases within 24 h of normal growth conditions. Also, no change in sub-G0/G1 population that designates apoptotic phenotype [17,27] was detected in MCF-7As53 cells. Furthermore, to investigate whether there is any alteration in the status of cyclins that control cell cycle phase transitions and also regulate its progression, we investigated the status of cyclin D1 and cyclin E. Both MCF-7As53 and MCF-7 cells were serum starved for 24 h. As shown in Fig. 4A, cyclin D1 was barely detectable in MCF-7 cells whereas in MCF-7As53 cells

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**Fig. 3** – Cells were cultured as monolayer on tissue culture dish for 2 weeks and photographed using phase-contrast microscope. (A) Epithelial morphology and anchorage dependent growth characteristic of the MCF-7 (a) and MCF-7As53 (b) cells (microscopic fields with ×20 original magnification) are illustrated. Senescence was evaluated by comparing expression of senescence-associated β-galactosidase in MCF-7 and MCF-7As53 cells with doxorubicin treated MCF-7 cells (c) stained blue (positive control). (B) MCF-7 and MCF-7As53 cells were cultured for 24 h, 48 h, 72 h, and 96 h and MTT assay was performed and subsequently the growth curves were plotted. (C) Cell cycle distribution expressed as percentage of cells in any one phase was determined by FACS analysis. The bars represent the proportion of cells in G1, S, and G2M phases respectively. There is significant depletion of G1 population in MCF-7As53 cells ("P<0.05 vs. MCF-7 cells).
significantly increased expression of cyclin D1 was detected. Following 24 h serum starvation, the cells were further grown in media supplemented with serum for 12 and 24 h. As can be seen, cyclin D1 was detected in MCF-7 as well as MCF-7As53 cells (Fig. 4A). However, at any given time point cyclin D1 levels in MCF-7As53 cells are much higher than those in MCF-7 cells. Increase in cyclin D1 expression in MCF-7As53 cells was further reconfirmed by confocal microscopy studies (Fig. 4C). Under similar experimental conditions no significant alterations in either cyclin E or β-actin were detected in both the cell lines. In MCF-7As53 cells since cyclin D1 is overexpressed, it is likely that this difference could be attributed to enhanced growth of these cells. Since cyclin D1 was overexpressed in MCF-7As53, it was of further interest to study the involvement of p53. MCF-7As53 cells were mock transfected or transfected with p53 expression vector pC53-SN3, as described in Materials and methods. Interestingly, expression of p53 resulted in decrease in cyclin D1 levels (Fig. 4B). The direct regulation of cyclin D1 by p53 has been reported [28] and p53 induced cyclin D1 via p21 (WAF1/CIP1) [29] is reported to be involved in p53 induced growth arrest [30]. However, none have demonstrated that cyclin D1 levels can be downregulated by p53. The results presented in this manuscript clearly demonstrate a correlation between p53 levels and cyclin D1 expression. To the best of our knowledge, this is one of the few reports, which directly correlates p53 status with cyclin D1 since both are regulators of G1 to S phase transition [31].

p53 overexpression downregulates Akt which is constitutively active in MCF-7As53 cells

Akt activation which is downstream of PI3-K pathway is known to be involved in cell growth and survival [32]. In our quest to investigate the factors responsible for the proliferative phenotype of MCF-7As53 cells we checked the status of Akt activity. We found that Akt is constitutively activated and pAkt levels are high in MCF-7As53 cells (Figs. 5A and C). Therefore, we next investigated the inter-relationship between p53 and Akt activity. To ascertain that the activation of Akt is a direct consequence of decreased p53 levels, MCF-7As53 cells were either mock transfected or transfected with the wild-type p53 expression vector. Interestingly, expression of p53 results in decrease in pAkt levels whereas basal Akt levels remained unaltered (Fig. 5A, lane 3 vs. lane 4). These results clearly suggest a direct correlation between p53 levels and Akt activation. Our findings are in accordance with the reports in which it has been reported that overexpression of p53 exogenously leads to a decrease in pAkt levels [33,34].

Inhibition of Akt activity downregulates cyclin D1 and decreases proliferation of MCF-7As53 cells

The phosphoinositide 3-kinase (PI3-K) signaling pathway has been shown to play a pivotal role in intracellular signaling involved in cell growth, cellular transformation, and
Akt has been implicated as an intermediate in PI3-K generated survival signals [35]. Activation of this kinase contributes to various malignant phenotypes in human cancers, including breast tumor [36]. Our results already indicated that in MCF-7As53 cells cyclin D1 is significantly upregulated and it plays a role in cell proliferation. Thus, we next probed whether Akt activation and cyclin D1 are interrelated. MCF-7As53 and MCF-7 cells were treated with PI3-K inhibitor wortmannin. As shown, pAkt (Figs. 5B and C) and cyclin D1 (Fig. 5B) levels are elevated in MCF-7As53 cells in comparison with MCF-7 cells. Treatment of cells with wortmannin not only decreases pAkt levels, but also diminishes cyclin D1 levels. Moreover, the pharmacological inhibitor of PI3-K also inhibits Akt activation, resulting in decreased cell proliferation rate (Fig. 5D). Therefore, these data imply that constitutive activation of PI3-K/Akt results in faster G1 to S cell cycle entry due to increase in cyclin D1 levels [37] in MCF-7As53 cells.

**p53 is a negative regulator of Cav-1/Akt regulated signaling in breast cancer cells**

In our quest to identify the upstream regulator of activated PI3-K/Akt in MCF-7As53 cells, we probed for Cav-1 as well as pCav-1 levels in these cells. Previous studies have indicated that Cav-1 is a potent activator of PI3-K/Akt pathway [38,39]. In MCF-7As53 cells, we detected significantly higher levels of Cav-1 as well as pCav-1 levels in comparison to those present in parental MCF-7 cells (Fig. 6A, lane 1 vs. lane 2). To confirm whether the increase in Cav-1 and pCav-1 is a direct consequence of decreased p53 levels in MCF-7As53 cells, the cells were transfected with the wild-type p53 expression vector. When p53 was overexpressed in these cells, the Cav-1 levels decreased and correspondingly pCav-1 levels also decreased. These results clearly are indicative of a direct correlation between p53 levels and Cav-1 expression, as well as its activation (Fig. 6A, lane 2 vs. lane 3). Additionally, immunofluorescent studies also confirm that Cav-1 is overexpressed and its enhanced localization could be detected on the cell membrane in MCF-7As53 cells, as compared to MCF-7 cells (Fig. 6B). To investigate whether constitutively upregulated Cav-1 activity is indeed responsible for activation of Akt, we treated the cells with cholesterol depleting agent MCD which is known to downregulate pCav-1 levels without affecting its basal expression [40]. Following MCD treatment, we observed that the decrease in Akt activity correlated with the decrease in phosphorylation of Cav-1 (Fig. 6B). Furthermore, to demonstrate a direct correlation between Cav-1 and
Akt activation, we transfected MCF-7As53 cells with Cav-1 siRNA. When Cav-1 siRNA was introduced into the cells, Cav-1 levels decreased and correspondingly pAkt levels also decreased. No decrease in either Cav-1 level or pAkt level was detected in the cells that were transfected with the control siRNA (Fig. 6D). Subsequently, we also performed the experiment in MCF-7 in which p53 activity was inhibited either by PFTα, a specific inhibitor of p53 [41] treatment, or by silencing the p53 message using p53 siRNA. As expected p53 siRNA expression decreases p53 protein levels (Fig. 6E, lane 1 vs. lane 3). We observed that Cav-1 as well as pAkt levels increased in the cells in which p53 was inactivated by PFTα and also in the cells which were transfected with p53 siRNA, as compared with mock transfected MCF-7 cells (Fig. 6E). Further to verify the inter-relationship between p53 status and Cav-1 expression in MCF-7 cells as well as other breast cancer cells, we compared the expression levels of Cav-1 in MCF-7 cells, in MCF-7 cells treated with PFTα, MCF-7As53 cells and in other breast cancer cells such as MDA-MB-231 or MDA-MB-468 which express mutant p53. The results obtained by the inhibition of p53 activity by PFTα in MCF-7 cells, presence of antisense p53 in MCF-7As53 cells, or presence of transactivation mutant of p53 in MDA-MB-231 or MDA-MB-468 clearly are indicative of a inverse correlation between Cav-1 expression and p53 functional status suggesting that p53 tightly regulates Cav-1 expression in a cell (Fig. 7A). Furthermore to ascertain that functional alterations in p53 status resulting in the regulation of Cav-1 expression indeed also affect activation of Akt as well as levels of cyclin D1, the lysates were probed for pAkt, Akt, and Cav-1 on lysates prepared from (1) MCF-7As53 cells, (2) MCF-7As53 cells mock treated, and (3) MCF-7As53 cells treated with cholesterol depleting agent MCD as described in text. β-Actin was used as a control for loading. (D) Immunoblot of pAkt, Akt, and Cav-1 in lysates prepared from (1) MCF-7As53 cells, (2) MCF-7As53 cells transfected with control siRNA, or (3) MCF-7As53 cells transfected Cav-1 siRNA. (E) Immunoblot for p53, Cav-1, pAkt and Akt in (1) MCF-7 cells, (2) MCF-7 cells treated with PFTα, and (3) MCF-7 cells transfected with p53 siRNA. β-Tubulin was used as a control for loading.

**Discussion**

Progress in breast cancer research has been greatly limited by the non-availability of enough suitable, extensively studied, and well-characterized human cancer cell lines which are important research resources for studying cancer cell biology along with developing new therapeutic strategies against breast cancer cell growth and progression [42]. Although MCF-7 is a well-characterized and established wild-type p53...
expressing breast cancer model [43], there are not enough reports on genetically matched breast cancer cell systems which differ in the status of p53 only. Furthermore, different cell lines, experimental protocols, cell growth states, or genetic backgrounds have contributed to the conflicting conclusions [5,6]. Thus, a genetically matched cell system with similarity in everything except in p53 expression will be of great importance in understanding the functions of p53. We report here the development of a breast cancer cell line, MCF-7As53, derived from MCF-7 cells, in which p53 protein as well as its activity is abrogated due to stable expression of antisense p53 cDNA. We verified MCF-7As53 cell line for its epithelial morphology, stable p53 null status, and ERα levels in comparison with parental MCF-7 cells and no alterations were detected even after 20 passages. Furthermore, we provide experimental evidences that abrogation of p53 protein does not alter steady-state levels of important stress response mediators such as p21, Bax, and GADD45 in regulating cell growth [44–46]. We analyzed upstream, downstream, and proteins homologous to p53 in this cell model and compared it with the parental cell line. MCF-7As53 exhibited no variability in Mdm2 oncoprotein level when compared to parental cells. Simultaneously, the p53 family protein p73 was verified in terms of its expression and also to check the specificity of p53 antisense function.

Wild-type p53 is a negative regulator of cell proliferation, and the mutations in the p53 gene are most frequently observed genetic alterations in human tumors, making p53 a candidate for a cellular protein involved in the control of cell growth [47–49]. MCF-7As53 cells have enhanced rate of proliferation, and this proliferative phenotype is due to increased expression of cyclin D1 leading to characteristically faster transition from G1 to S phase as compared to that in MCF-7 parental cells. Cyclin D1 plays an important role in controlling the cell cycle in mammary tissues and clinical studies on human breast cancers have confirmed its importance. Mammary tumors exhibiting high levels of cyclin D1 expression show higher rates of proliferation than cyclin D1-negative tumors [10,50–52]. Our studies with MCF-7As53 are one of the few reports in which p53 overexpression has been shown to downregulate cyclin D1 protein level, which may be a consequence of direct or indirect molecular interactions. Therefore, this cell line provides us with an important tool to explore the interrelationship between p53 and cyclin D1 which is yet to be clearly understood [53]. Our results are in accordance with the fact that p53 regulates cyclin D1 and cyclin D1 being involved in p53 induced G1 block which certainly also implies that loss of p53 could lead to increased cyclin D1 in cancer cells thereby promoting faster G1 to S transition during cell cycle progression, which enhances cellular proliferation.

**Fig. 7** A panel of breast cancer cells as well as MCF-7 cells in which p53 activity was abrogated were assessed for Cav-1, Akt, and cyclin D1 levels. (A) Representative western blot showing expression levels of Cav-1 and p53. (B) Representative blot of cyclin D1, pAkt and Akt, in (1) MCF-7 cells, (2) MCF-7 treated with PFTα (20 μM), (3) MCF-7As53, (4) MDA-MB-231 and (5) MDA-MB-468. β-Actin and basal Akt were used as controls for loading. (C) Proposed model for p53 mediated regulation of cyclin D1.
The role played by increased cyclin D1 expression in the enhanced cell growth of MCF-7As53 led to exploration of the status of Akt activity in these cells as Akt is linked to cyclin D1 expression in cancer cells [35]. The Akt has been implicated as an intermediate in PI3-Kinase generated survival signals and the PI3-K signaling pathway has been shown to play a pivotal role in intracellular signal transduction pathways involved in cell growth, cellular transformation, and tumorigenesis [54]. Activation of these kinase signaling pathways contributes to various malignant phenotypes in human cancers, including breast tumor [36]. Therefore, we examined the phosphorylation status of Akt kinase, which was constitutively active in MCF-7As53 cells. Inhibition of constitutively active Akt by wortmannin, an inhibitor of upstream PI3-K, resulted not only in decrease in the growth but also led to downregulation of cyclin D1 protein in MCF-7As53 cells. This implies that PI3-K/Akt signaling is upstream of cyclin D1 and p53 protein directly controls it. These results are consistent with several other studies in which either p53 was inhibited or PI3-K/Akt signaling was upregulated, leading to enhanced proliferation of cancer cells [10,35]. Moreover, the activation of PI3-K/Akt pathway is shown to trigger a network that positively regulates G1/S cell cycle progression through inactivation of glycogen synthase kinase 3-beta (GSK3-β) via its phosphorylation leading to an increase in cyclin D1, a key regulator of cell cycle, which is accumulated during the G1 phase [33]. Additionally, Akt also promotes transcription and translation of cyclin D1 gene [35]. Moreover, recent reports suggest that p53 can negatively regulate Akt by repression of the catalytic subunit of PI3-Kinase [55], as well as via expression of the PTEN tumor-suppressor gene [56].

In our quest to explore the reason for constitutively activated PI3-K/PKB signaling in MCF-7As53 cell line, we investigated the connections between signal transduction pathways and components of cellular plasma membrane needed for the regulation of growth and survival of the cells. We narrowed down on caveolae, which are sphingolipid and cholesterol-rich invaginations of the plasma membrane involved in vesicular trafficking and signal transduction. Caveolins are a class of oligomeric structural proteins that are both necessary and sufficient for caveolae formation and Cav-1 is the principal structural protein of caveolae. Interestingly, Cav-1 has been implicated in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis. Experimental evidences from cultured cells, animal models, and human tumor samples have led to conclusion that Cav-1 functions as a “tumor and/or metastasis modifier gene” [57]. Interestingly, in human breast cancer specimens, increased caveolin staining in intraductal and infiltrating ductal carcinoma as well as in nodal disease has been reported [58]. Recent studies have also implicated Cav-1 in breast cancer pathogenesis, with emphasis on the signaling pathways regulated during these processes [59]. In addition to proliferative phenotype, we also detected constitutive upregulation of Cav-1 and its phosphorylation in MCF-7As53 cell line. This result is in contrast to earlier report where in utilizing MCF-7 human breast adenocarcinoma cells stably transfected with Cav-1 (MCF-7/Cav-1), it was demonstrated that Cav-1 expression decreases cell proliferation rate and markedly reduces their capacity to form colonies in soft agar [60]. However, our observation is in agreement with the report demonstrating correlation between Akt activation and Cav-1 expression in the cells [38] and with the recent findings that not only Cav-1 is overexpressed but also Akt-1 is activated in colon cancer tissues than in normal colon tissues [61]. In addition, Cav-1 is also essential for the integrin-mediated activation of PI3-K/Akt [62]. Collectively, these reports are suggestive of a correlation between Cav-1 regulated Akt activation and proliferation of the cells [61,39]. Depletion of cholesterol by MCD in MCF-7As53 cells not only decreases pCav-1 levels but also downregulates pAkt levels as well. Moreover, knocking down of Cav-1 with Cav-1 siRNA also resulted in a decrease in pAkt levels. Therefore, all these results established a molecular link between enhanced Cav-1 levels and Akt activation, increased cyclin D1, leading to enhanced growth phenotype in MCF-7As53 monolayer cultures, and are identical to other reports [61,62]. It was also observed that overexpression of p53 in MCF-7As53 cell line leads to a decrease in Cav-1 protein levels. Interestingly, not only the expression levels of Cav-1 correlated with the functional status of p53 in a panel of breast cancer cells where either parental MCF-7 cells were treated with PFTα, a specific inhibitor of p53 transactivity, or cells expressed transactivation mutant p53, but it also correlates with the activation state of Akt as well and increased cyclin D1 levels. All these results strongly suggest that wild-type p53 is an upstream negative regulator of Cav-1 in breast cancer cells. Thus, it could be concluded that either deletion by antisense or abrogation of p53 activity due to mutations or by siRNA results in upregulation of Cav-1, activation of Akt, and increased cyclin D1 levels in breast cancer cells, thereby facilitating growth of tumor cells. From all the results presented in this manuscript we propose that p53 under normal conditions keeps Cav-1 gene expression under tight control thereby regulating the activation of Akt and subsequently the cell growth.

In summary, MCF-7As53 cell culture system will be extremely useful to recreate current perception of the importance of p53 levels and functions in breast cancer [4,63,64] with special emphasis on cell growth behavior under p53 null conditions in cancers. Additionally with MCF-7As53, we have established an experimentally amenable system to analyze how the absence of p53 promotes genomic instability [65,66], which in turn may result in molecular alterations in signaling pathways in the breast cancers. Our studies for the first time indicate the significance of p53 in modulation of signaling for cell growth and also points towards the scope for exploring these pathways either to increase cancer cell killing in future therapeutic interventions or for better understanding of factors regulating cancer cell growth.

Acknowledgments

We thank Dr. G.C. Mishra, Director, NCCS for being very supportive and giving all the encouragement to carry out this work. We also thank Department of Biotechnology, Government of India for providing financial support. RC thanks Council for Scientific and Industrial Research (CSIR). RK and AKU thank University Grants Commission (UGC) for providing fellowships.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2007.08.022.

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