Chapter 3

Abrogation of p53 in MCF-7 breast carcinoma cells positively modulates cell growth and chemosensitivity
Summary

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 cancer, 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 the expression of antisense p53 cDNA. Similar to MCF-7 and simultaneously selected hygromycin resistant MCF-7H cells, MCF-7As53 cell line has a consistent basal epithelial phenotype, morphology, and estrogen receptor expression levels at normal growth conditions. The work documents investigation of morphology, estrogen hormone receptor levels, growth kinetics and cell cycle related studies in relation to absence of wild type p53 protein and its transactivation potential as well. These cells exhibited decreased doubling time due to depletion of G0/G1 cell phase attributed to stabilized cyclinD1 expression. The proliferative phenotype is due to constitutively activated Akt kinase and subsequent upregulation of cyclin D1 expression in these cells. This enhanced proliferation has been shown to be abrogated by wortmannin, an inhibitor of PI3-K/Akt signaling pathway. Eventhough 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 which are p53 null, no alterations in these genes was detected. Moreover, chemosensitivity data for this cell line indicates that p53 is an indispensable component of the enzymatic machinery that is responsible for cellular sensor system to sensitize cells to any stress or DNA damage. Therefore MCF-7As53 provides us with unique experimental tool to facilitate the understanding for the role of p53 in proliferation as well chemosensitivity of human breast cancers cells.
3.1 Introduction

The frequent alterations in human malignancies are mutations of the p53 gene and it is the most commonly altered oncogene in the development of sporadic and hereditary breast cancers (Ozbun and Butel, 1995). The loss of wild type p53 function is an important event in breast tumorigenesis as documented in both human and murine systems (Elledge and Allred, 1994; Donehower et al., 1995). Moreover, p53 is mutated or aberrantly expressed in about 40% of human breast cancers (Elledge and Allred, 1994). Most of these 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 maintaining genome stability (Levine, 1997). However, the exact mechanisms by which such lack of normal gene function leads to cancer formation and progression are only beginning to be unraveled. Moreover, all the downstream signaling pathways influenced by p53 remain to be clearly discovered. In cancer, 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 dysregulated (Zantek et al., 2001; Bernhardt et al., 2002). 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, binding to viral oncoproteins (e.g. HPV18E6, SV40 and E1B-55KD) or cellular factors (e.g. mdm-2), over expression of dominant negative mutant TP53 and post-translational modifications (Wang et al., 2001; Franken et al., 2004). More recently use of small interference RNA or antisense oligonucleotide have been exploited for inhibition of p53 functions (Zhang et al., 2004). On the whole, these models have contributed significantly towards the understanding of p53 functions, however the results from these studies are inconclusive, as they depend on differential modes of abrogation or inactivation of p53 protein or its function.
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. Therefore, difference in modes of 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 have not been definitely established because these studies were based on tumor biopsies and cell lines already lacking wild type p53 (Xu et al., 1995; Hawkins et al., 1996; Franken et al., 2004). Moreover additional properties of p53 are now emerging including modulation of signal transduction pathways and firing of signal cascades that originate at the level of the cell membrane. Thus, delineation of the role that p53 may play in cell has been hampered by the lack of appropriate model. Hence, there is a continuing need for genetically matched cell systems that only differ in p53 protein status.

This report describes the characterization of MCF-7As53 cell line derived from breast carcinoma MCF-7 cells as an isogenic cell system differing only in p53 status. This model provides us with a valuable tool to delineate role of p53 in breast cancers and allows us more systemic approach to decipher both upstream and downstream role of p53 as well as signaling networks and pathways it regulates in cancer cells.
3.2 Materials and Methods

3.2.1 Reagents and antibodies

Sources of materials were as follows: 5-Fluorouracil (5-FU), Carboplatin (Carb), Paclitaxel (PXL), Doxorubicin, Methyl-thiazolyl tetrazolium (MTT), wortmannin, Pifithrin alpha (PFTα) and 5-bromo-4-chloro-3-indolyL-β-D-galactoside (X-Gal) were purchased from Sigma, MO, USA. Drugs were dissolved in sterile water to prepare a stock of 50 mM. MTT was reconstituted as 1 mg/ml in DMEM without phenol red. PFTα and wortmannin were reconstituted in DMSO. Antibodies against p53, estrogen receptor-alpha, Mdm2, Bax, p73, ER-alpha, alpha-fetoprotein, cyclin D1, caveolin-1, phospho-caveolin-1, Akt, phospho-Akt, β-tubulin and β-actin were from Santa Cruz Biotechnology, CA, USA.

3.2.2 Cell cultures and development of 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, Virginia, USA and maintained in our in-house National Cell repository. Cells were routinely 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-7As53 was established by Tet-On stable transfectant selection method as follows. The MCF-7 Tet-On (Clontech, CA, USA) cells were co-transfected with pTRErevp53 (containing human p53 cDNA in reverse orientation under Tetracycline Regulatory Element) and pTK-Hyg plasmid (Clontech, CA, USA) which codes for hygromycin resistance (plasmids’ ratio being 5:1). Cells were selected on hygromycin (200 μg/ml) for 4 weeks. 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. When maintained in normal culture medium these cells exhibited complete abrogation of p53 protein as well as its transactivation activity. When immunobotted for total p53 protein, the levels of p53 in MCF-7As53 were diminished significantly as compared to the levels in parental MCF-7 cells and also in MCF-7H cell line. The MCF-7H is a hygromycin resistant clone selected in
parallel with MCF-7As53. The p53 deficient phenotype was maintained in MCF-7As53 even after being passaged for more than 20 times over a period of six months.

### 3.2.3 CAT Reporter Assays

The p53-CAT reporter construct PG13CAT, which contains 13 repeats of p53 binding site inserted 5' to polyomavirus basal promoter linked to CAT reporter gene (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore) were transiently transfected in MCF-7, MCF-7As53 and MCF-7H cells by lipofectamine 2000 method (Invitrogen Life Technologies, MD, USA). Almost 80% confluent cells in 35 mm culture plate were transfected with 4 µg of DNA including 1 µg pEGFP-N1 plasmids as an internal control to assess the transfection efficiency. Vector plasmids were used as carrier DNA to makeup the final DNA concentration to 4 µg. 1 h 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 was diluted into 250 µl of Opti-MEM (Gibco) 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. 500 µl of LF2000-DNA complex was added drop wise to the plate and mixed gently by rocking the plate. Cells were incubated at 37 °C for 24 h. Thereafter, cells were washed and incubated at 37 °C for further 24 h before harvesting.

pWWP-CAT, which has p53 binding site from p21 promoter, was also used in reporter assays to evaluate p21 specific p53 transactivation potential. For CAT reporter assays, cells were then collected and washed thrice with 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 C14-chloramphenicol (NEN, Boston, MA, USA) and 100 µg of acetyl CoA (Amersham, 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 transfecting green fluorescent protein expressing plasmid pEGFPN1 (Clontech, CA, USA). GFP intensity was directly measured from the cell lysates of pEGFP-N1 transfected cells by fluorometer to confirm equal transfection efficiency as well to normalize the reporter activity. Equal amount (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) with filter set at excitation 485 nm and emission 510 nm.

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

In separate experiments involving overexpression of wild type p53, pC53-SN3 plasmid vector (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore) was transiently transfected in MCF-7As53 cells by lipofectamine method (Invitrogen Life Technologies, MD, USA) as described in methods for CAT reporter assay. After transfection, cells were washed and fresh media was 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 as an internal control for normalization of transfection efficiencies. In some experiments pCMVβ (Clontech, CA, USA) was also used as internal control for normalization of transfection efficiencies by quantifying activity of β-galactosidase in the transfected cells. The activity of β-galactosidase was assayed in pCMVβ transfected cells by using CPRG (chlorophenoled-β-D-galactopyranoside) as substrate. Principally, CPRG is cleaved by β-galactosidase to yield chlorophenol red and absorbance of this red colour can be measured at 570 nm. Twenty micrograms of cell lysates from each sample was taken in the wells of flat bottom 96-well plate and diluted to 100 µl with PBS to estimate the β-galactosidase activity in the cells. One millimolar of CPRG was added to each well and incubated at 37 °C for 6 h. Absorbance was taken in microplate reader (Multiskan Ascent, Labsystems) at 570 nm.
3.2.5 siRNA transfection in MCF-7 cells

Almost 80% confluent cells in 60 mm culture plate were transfected with 18 μl of 10 μM siRNA (stock) made in siRNA dilution buffer. Fluorescein conjugated control siRNA was utilized as an internal control to assess the transfection efficiency. For each plate, 18 μl of siRNA from the stock 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 drop wise 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. The siRNAs, transfection medium, transfection buffer and transfection reagent were obtained from Santa Cruz Biotechnology, CA, USA.

3.2.6 In vitro growth rate analysis

Cells were seeded at a density of 2×10^4 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 was decanted and 50 μl of MTT (1 mg/ml) in DMEM (without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals were solubilized in 50 μl of iso-propanol 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 2×10^4 cells taken at 0 hour point.

3.2.7 Flow cytometry for cell cycle analysis

Cells were plated at a density of approximately 8 × 10^4 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 cell pellet and incubated in the dark for 30 minutes 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 $10^6$ cells.

### 3.2.8 Western blot analysis

Following treatments, cells were washed thrice with ice cold PBS 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 Na$_3$VO$_4$ and protease inhibitor cocktail tablet) per $1\times10^6$ 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) were 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).

### 3.2.9 Stripping of the membrane

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.

### 3.2.10 RNA extraction, cDNA synthesis and RT-PCR

Total cellular RNA was extracted using TRizol™ reagent (Invitrogen Life Technologies, MD, USA), according to the manufacturer’s instructions. Five micrograms of total RNA and oligo(dT)$_{12-18}$ primer or random hexamers were taken in diethyl pyrocarbonate (DEPC)-treated water. cDNA synthesis was initiated using 200 units of M-MLV reverse transcriptase (Invitrogen Life Technologies, MD, USA), under conditions recommended by 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-HCl (pH 8.4) containing 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTP mix,
and 1 unit of platinum Tag DNA polymerase (Invitrogen Life Technologies, 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.

For extraction of genomic DNA cells were lysed in TE with 10% SDS followed by proteinase K treatment for 2 h. Tris-saturated phenol and chloroform were mixed in 1:1 ratio, added to the samples which were then centrifuged at 14000 rpm. The aqueous layer was aspirated from the samples and equal amount of chloroform was added. After centrifugation, the supernatants of these samples were mixed with 100% ethanol for precipitation of genomic DNA. The genomic DNA was stored at 4°C. The PCR was performed on 250 ng each of genomic DNA for 30 cycles using TRE (F) and p53 (F) primers. pTRErevp53 (50 ng) was taken as positive control for antisense p53 reading frame.

The primer pairs and their respective annealing temperatures as well as product size are tabulated below:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Annealing Temp</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>(F) 5'-CTG AGG TTG GCT CTG ACT GTA CCA CCA TCC-3' (R) 5'-CTC ATT CAG CTC TCG GAA CAT CTG GAA GCG-3'</td>
<td>55°C</td>
<td>371</td>
</tr>
<tr>
<td>p21</td>
<td>(F) 5'-GAC ACC ACT GGA GGG TGA CT-3' (R) 5'-GGC GTT TGG AGT GGT AGA AA-3'</td>
<td>59°C</td>
<td>299</td>
</tr>
<tr>
<td>hGadd45</td>
<td>(F) 5'-AGA GCA GAA GAC CGA AAG GAT G-3' (R) 5'-ACG CGC AGG ATG TTG ATG TCG-3'</td>
<td>55°C</td>
<td>248</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>(F) 5'-TTC GGG CGA GAT GTC CGC C-3' (R) 5'-TCA CTT GTG GCC CAG ATA GG-3'</td>
<td>61°C</td>
<td>373</td>
</tr>
<tr>
<td>Bax</td>
<td>(F) 5'-TTC ATG GAC GGG TCC GGG GA-3' (R) 5'-TGT CCA GCC CAT GAT GGT TCT-3'</td>
<td>59°C</td>
<td>419</td>
</tr>
<tr>
<td>TRE rev p53</td>
<td>(F) 5'-CGC CTG GAG ACC CCA TCC-3' (R) 5'-CTG AGG TTG GCT CTG ACT GTA CCA CCA TCC-3'</td>
<td>59°C</td>
<td>1227</td>
</tr>
<tr>
<td>β-actin</td>
<td>(F) 5'-ATC TGG CAC CAC TAC TAC AAT GAG CTG CG-3' (R) 5'-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3'</td>
<td>55°C</td>
<td>838</td>
</tr>
</tbody>
</table>
3.2.11 Drug interaction studies in vitro

To assay the cell viability after drug treatments, cells were seeded at a density of 7500 cells per well into 96 well plates and allowed to adhere for 24 h at 37°C. Cells were then treated with Carb (100 μM), 5-FU (100 μM) and PXL (10 nM) for 24 h in triplicates and further incubated for additional 48 h in absence of drugs. Thereafter, media was decanted and 50 μl of MTT (1mg/ml) in MEM (without phenol red) was added to each well and incubated for 4 h at 37°C. Formazan crystals were solubilized in 50 μl of iso-propanol by incubating with shaking at room temperature for 10 min. Absorbance was measured at 570 nm using 630 nm as reference filter. Absorbance given by untreated cells was taken as 100% cell growth. For senescence study, MCF-7 cells were treated with 1μM Doxorubicin for 2 h and stained for β-galactosidase activity after 2 days as a positive control.

3.2.12 Senescence-associated β-Galactosidase (SA-β-Gal) staining

MCF-7 and MCF-7As53 cells were plated at a density of 25x10^3 cells per 35mm culture dish and allowed to grow for 2 weeks. For senescence-associated β-galactosidase staining, 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 5-bromo-4-chloro-3-indolyl-β-D-galactoside (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 (Elmore et al., 2002). Cells were then examined for the development of blue colour, which was evident after 12-16 h of incubation with X-Gal. Doxorubicin (1 μM for 2h) 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).

3.2.13 Mycoplasma detection using DNA staining

One of the most important procedures often overlooked in establishing a cell line is testing cultures for microbial contamination, especially mycoplasma. It is critical for every cell culture laboratory to only use cell lines that have been
carefully screened for mycoplasma. There is simple fluorochrome DNA staining test that can detect both mycoplasma and virtually any other prokaryote contaminants. Briefly, cells were plated onto coverslips and air dried preparations were immediately fixed in acetic acid and methanol at 1:4 (v/v) for 15 minutes. Then, Hoechst 33258 (0.25 mg/ml in Hanks balanced salt solution) was applied for 30 min in dark environment. Washed and air-dried preparations were then embedded in glycerol and examined under fluorescence microscope.

3.2.14 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) till 70% confluency. Media was removed and cells were washed with PBS twice. The cells were fixed with cold 4% paraformaldehyde (made freshly in PBS) for 20 minutes at room temperature (RT). Cells were again washed thoroughly with PBS after fixing. Cells were permeabilised with PBS containing 0.1% Triton-X 100 for 10 minutes at RT, wherever required. After washing thoroughly with PBS, cells were blocked with 5% fetal bovine serum made in PBS, for 1-2 hours at RT. Subsequently cells were incubated with antigen specific primary antibodies at 1:100 dilution in PBS for 2 hours at RT. After washing thoroughly cells were incubated with secondary antibody (2° Ab with FITC conjugates) at 1:200 dilution for 1 hour at RT. For negative control cells were incubated with 2° Ab alone. After washing the cells thoroughly they were overlayered with mounting medium containing antifade (Vectashield, Vector Laboratories). The slides were then subjected to Immunofluorescence or Confocal microscopy (LSM510, Carl Zeiss). Images were subsequently processed by Adobe Photoshop software.

3.2.15 Animals, animal-keeping conditions and in vivo tumorigenicity assay

Eight weeks old female homozygous (nu+/nu+) nude athymic NIH mice (NIV, Pune, India), 18-22 g, were used as human cancer xenograft models. The animals were housed in specific pathogen-free isolators (Harlan Isotec, USA) in an environmentally controlled Animal Care facility of our institute. They were caged (4 per cage) in polypropylene boxes and had free access to sterilized pelleted
laboratory rodent chow and water. All animal experiments have been performed following the requirement of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, and after permission of the Institute's Animal Care and Use Committee (IACUC). Five consecutive subcutaneous (sc) estradiol valerate (30 μg/day) injections were administered in each mouse prior to sc injection of MCF-7 and MCF-7As53 cells in the exponential growth phase (2 x 10^7 cells in 0.1 ml of PBS), at right flank of each mouse. The mice were randomized into six groups for each cell type injected, with four mice per group (n=4). The mice were supplied with doxycycline in drinking water (2 mg/L) throughout the experimental duration to maintain minimal doxycycline level in their serum eventhough antisense p53 is constitutively expressed in MCF-7As cells. Each group was sacrificed after every 10 days period for weight quantification of surgically excised tumors from the animals. Tumor growth was determined by their weight measurements.

3.2.16 Protein extraction from tumor tissues

From fresh cancer tissues, protein was extracted by the conventional method (Ikeda et al., 1998) in which, 100 mg of fresh tissue was homogenized with a syringe type, hand-held homogenizer and incubated in 1 ml of the lysis buffer (described in western blot analysis section) containing 0.1% SDS at 0°C. The tissue lysates were also centrifuged at 15000 X g for 20 min at 4°C and supernatants were taken for protein estimations.
3.3 RESULTS

3.3.1 Derivation of MCF-7As53 and effect of p53 depletion on downstream molecules

The MCF-7 Tet-On cells were co-transfected with pTRErevp53 and pTK-Hyg constructs as described in materials and methods section.

Fig. 1. Construction of pTRErevp53 vector. pTRErevp53 vector was constructed to express p53 in antisense orientation. (A) Wild-type p53 expression vector pC53-SN3 (obtained from...
Dr. Bert Vogelstein. (B) From pC53-SN3, p53-cDNA was obtained via BamHI digestion, as shown here as 1.8 kb insert. The insert was cloned into BamHI site (unique cloning site) of pTRE vector to obtain pTRErevp53. The reverse orientation of insert was confirmed by DNA sequencing as well as restriction digestion pattern.

Fig. 1A illustrates wild type p53 plasmid expression vector pC53-SN3 (kind gift of Dr. Bert Vogelstein, John Hopkins, Baltimore) and Fig 1B shows schematic illustration for construction of pTRErevp53 (containing human p53 cDNA in reverse orientation under Tetracycline Regulatory Element). The insertion of p53 fragment in reverse orientation was verified by sequencing of resultant construct. The MCF-7 Tet-On cells were co-transfected with pTRErevp53 (containing human p53 cDNA in reverse orientation under Tetracycline Regulatory Element) and pTK-Hyg plasmid which codes for hygromycin resistance as given in methods. Subsequently, the cotransfected cells were selected for hygromycin resistance.

Fig. 2. p53 protein levels in the clones in which p53 antisense cDNA is expressed stably. The clones which were selected to verify antisense p53 expression were investigated for p53 protein levels. As can be seen in clone 3 and clone 6 under normal growth conditions and in the presence of regular FBS, p53 expression is significantly diminished. The numbers indicate relative levels of p53 which were normalized to β-actin levels.

Number of individual clones were picked and screened for p53 expression by western blotting. As shown in Fig. 2, we obtained two clones MCF-7As3 and MCF-7As6 in which p53 expression was significantly downregulated compared to that of parental MCF-7 cells as well as control MCF-7H cells which were selected as hygromycin resistant MCF-7 Tet-On 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 established in these clone (Fig. 3). Fig. 3A is CAT histogram and 3B is corresponding intensity plot where CAT activity was normalized with β-galactosidase activity.

Fig. 3. p53-CAT reporter activity in the antisense clones. The clones which were selected to verify antisense p53 expression were investigated for p53 dependent CAT reporter activity. The p53-CAT activity, as seen for clones 3 and 6, under the condition where normal fetal bovine serum was used for cell culture, indicated diminished p53 specific transactivation potential.

Since our target was to completely abrogate p53 expression in the breast cancer MCF-7 cell line, and having succeeded in obtaining such clones, they were maintained under normal growth conditions. The protein levels for p53 illustrated in Fig. 4A are for clones 3 and 6 maintained in presence of normal/regular serum for 20 passages. Doxycycline (water soluble analogue of Tetracycline) is a potential anticancer agent and is known to have effect on p53 in conjunction with chemotherapeutic drugs (Grimberg et al., 2002; Fujioka et al., 2004; Chhipa et al., 2005). To avoid the chronic exposure of MCF-7As clones to doxycycline and since
not much is know as to what effect this antibiotic will have on the properties of cells over long period of time. we propagated cells in normal culture conditions without further exogenous addition of doxycycline.

Similarly the abrogation of p53 expression was further verified by carrying out RT-PCR analysis of RNA obtained from cells after 20 passages (Fig. 4B).

In both MCF-7As3 and MCF-7As6, p53 mRNA was barely detectable. These results confirmed abrogation of p53 in the stable clones. The results indicated that p53 status remained unchanged irrespective of passages undergone by these clones. Further, to investigate the status of p53 regulated genes p21, Bax and GADD45, we carried out RT-PCR analysis. As can be seen in the Fig. 5, no significant alteration in the expression pattern of these genes was detected in MCF-7As3 and MCF-7As6 clones, when compared with the expression in parental MCF-7 as well as control MCF-7H cells.
Fig. 5. Molecular characterization. MCF-7 and MCF-7H cells were compared with the MCF-7 derived clones (As3 and As6) constitutively 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.

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 (It has been denoted as MCF-7As or As in the further provided figures).

The genomic integration of antisense p53 in MCF-7As53 was verified by PCR and the resultant product length matched with the amplified antisense p53 as per calculations by primer specificity and extension (Fig.6).
After confirming genomic integration of the antisense p53 fragment in the MCF-7As53 cells, we further examined immediate p53 related molecules as well as characteristic molecules in the cell lines to verify any changes that might have occurred in antisense p53 expressing cells in comparison with parental MCF-7 or MCF-7H cells (Fig. 7). The expression levels of MDM2, which is the key regulator upstream to p53, p73 (a p53 family protein with shared structural and functional homology with p53), Bax, ERz (Estrogen receptor alpha) which plays a critical role in breast cancer development and as MCF-7 cells are ER positive (Ali and Coombes, 2000), alpha FP (alpha fetoprotein), a well known carcinoembryonic antigen expressed in breast carcinoma (Sarcione and Hart, 1985), β-tubulin and β-actin were determined in the cell lysates.
Fig. 7. Blots showing the relative expression levels of p53 associated molecules in MCF-7 (M), MCF-7As53 (As) and MCF-7H (H) cell lines. Molecules such as MDM2 oncoprotein, p73, Bax, ERα (Estrogen receptor alpha) and alpha FP (alpha fetoprotein) are compared in these cells. Both β-actin and β-tubulin being taken as loading controls. Blots were analysed densitometrically for quantification and the values were normalized to β-actin.

No detectable difference was seen in MCF-7As53 cells in comparison with parental MCF-7 as well as control MCF-7H. The housekeeping proteins such as β-tubulin and β-actin were taken as internal controls for protein loading as well as for the detection of any significant changes in basic protein expression patterns in these cells.

Further to verify whether p53 downregulation also indeed results in decrease in its specific transactivation activity, we performed CAT reporter assays. MCF-7 and MCF-7As53 cells were separately transfected with either pG13-CAT or pWWP-CAT constructs.
Fig.8. 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. Intensities in chromatogram were measured by phosphorimager densitometry scanner analysis (Bio-rad, USA). (B) The intensity of CAT reporter activity was plotted along with the of EGFP intensities which was estimated for comparison of transfection efficiency under similar experimental conditions.

As shown in Fig. 8A and 8B, and as expected CAT reporter activity is barely detected in MCF-7As53 cells when compared with CAT reporter activity in MCF-7 cells. The decreased p53 reporter activity is indeed due to lack of functional p53. In the transfection experiments EGFP was used as an internal control and EGFP expression is more or less identical in all the samples.

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

MCF-7As53 cells have normal size, shape and uniform basal epithelial morphology which are comparable to that of parental MCF-7 cells at normal growth conditions defined in materials and methods. Both the cell lines exhibited normal anchorage dependent growth in culture dishes, as can be visualized in Fig. 9. We
next investigated whether abrogation of p53 induced senescence since it is a known regulator of senescence (Lacroix et al., 2006). Expression of cellular β-galactosidase is a good marker for detection of cellular senescence. As shown in Fig. 9 (A, B and C), the β-galactosidase positive MCF-7As53 cells were barely detected and were in fact comparable with those in MCF-7 cells even after being in culture for 2 weeks. The doxorubicin treated MCF-7 cells (as in methods section) were used as a positive control for senescent phenotype (Bodnar et al., 1998; Elmore et al., 2002).

Fig. 9. MCF-7(A) and MCF-7As53 (B) were cultured as monolayer on tissue culture dish for two weeks and photographed using phase-contrast microscope to show comparative epithelial morphology of the cells and anchorage dependent growth characteristic. 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). Shown are representative microscopic fields with X20 original magnification.

Additionally, the MCF-7As53 cells were analyzed for any mycoplasma contamination in them by conventional nuclear staining method with Hoechst 33258 dye (Fig. 10). The culture was tested as negative for any mycoplasma presence. Since there was no extranuclear staining with fluorescent Hoechst 33258 dye, this indicated that no mycoplasma infection was present in the cultures.
3.3.3 MCF-7As53 cells have proliferative phenotype due to stabilized cyclin D1 and overexpression of p53 in these cells downregulates cyclin D1

Having demonstrated that MCF-7As53 cells were identical to MCF-7 cells except in p53 status, we further investigated the growth pattern by performing MTT proliferation assay. MCF-7As53 and MCF-7 cells were plated in triplicate in 96 well culture plate as described in materials and methods. After every 24 h (followed for 4 days) cells were withdrawn by trypsinization and counted. As shown in Fig. 11, MCF-7As53 cells grow more rapidly than parental MCF-7 cells. The doubling time of MCF-7As53 is about 24 h compared to > 36 h for MCF-7. MCF-7As53 cells have approximately half the doubling time as indicated by comparative growth curve plotted for parental cells (Fig. 11A). This prompted us to investigate whether MCF-7As53 cells have altered cell cycle. The cell cycle analysis by flow cytometry revealed that MCF-7As53 cells have depletion of G0/G1 phase and progressive accumulation of cells in S/G2M phases within 24 h of normal growth conditions (Fig. 11B).

Since cyclins are the regulators of cell cycle, 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. 12, cyclin D1 is not detectable in MCF-7 cells whereas in MCF-7As53 cells significant level of cyclin D1 expression was detected when
probed immediately after (at 0 h) serum starvation. Following 24 h serum starvation the cells were further grown in media supplemented with serum for 12 and 24 h and as shown in Fig. 12A, cyclin D1 is detected in MCF-7 as well as MCF-7As53 cells. However, at any given time point cyclin D1 levels in MCF-7As53 cells were much higher than those in MCF-7 cells. Under similar experiment condition, 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/stabilized, it is likely that this difference could be attributed to enhanced growth of the cells.

![Graph showing cell proliferation](image1.png)

![Graph showing cell cycle distribution](image2.png)

Fig.11. (A) MCF-7 and MCF-7As53 cells were cultured for 24 h, 48 h, 72 h, 96 h and MTT assay was performed and subsequently the growth curves were plotted. The growth curves clearly indicate enhanced proliferation in MCF-7As53 cells when compared with MCF-7 cells. (B) 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.

Since cyclin D1 was overexpressed in MCF-7As53, it was of interest to study the involvement of p53. MCF-7As53 cells were mock transfected or transfected with p53 expression vector pC53-SN3. Interestingly, expression of p53 resulted in decrease in cyclin D1 levels (Fig. 12B). These results clearly demonstrate direct correlation between p53 levels and cyclin D1 expression. 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 (Lopez-Beltran et al., 2004).
3.3.4 Akt/PKB is activated in MCF-7As53 cells and p53 overexpression downregulates activity of Akt/PKB

To investigate the interrelationship between p53, cyclin D1 and proliferation, we explored PI3-K/Akt signaling pathway in MCF-7As53 cells, since it is well established pathway shown to be involved in cell growth and survival (Sheng et al., 2003). Downstream to PI3-K is Akt (Liang and Slingerland, 2003) and in MCF-7As53 cells, interestingly Akt was found to be constitutively phosphorylated (activated) under normal growth conditions (Fig. 13). Moreover, when p53 was expressed exogenously in this p53 null cell line, phospho-Akt levels were down regulated. These results are in agreement with the reports where p53 has been shown to have repressive activity for PI3-K pathway (Ostrakhovitch and Cherian, 2004; Zheng et al., 2004).
Fig. 13. Constitutively enhanced Akt activity can be abrogated by overexpressed p53. Shown here (1) MCF-7 cells, (2) MCF-7As cells, (3) MCF-7As cells subjected to mock transfection and (4) MCF-7As cells transfected with p53 expression vector. Densitometry was normalized with beta tubulin.

3.3.5 Inhibition of Akt/PKB downregulates cyclin D1 and decreases proliferation

Along with the results obtained from p53 overexpression studies, the treatment with wortmannin (a pharmacological inhibitor of PI3-K pathway) also down regulates phospho-Akt levels in MCF-7As53 cells (Fig. 14). Activated PI3-K/Akt is critically involved in determining the proliferation, growth and survival of cancer cells (Liang and Slingerland, 2003).

Fig. 14. (A) Cells were evaluated for proliferation and Akt phosphorylation after wortmannin treatment (50 nM). (A) After treating with wortmannin, growth curve was made following MTT method, as described in materials and methods. (B) Akt phosphorylation and cyclin D1 levels in (1) MCF-7As cells, (2) MCF-7As cells after wortmannin treatment, (3) MCF-7 cells, (4) MCF-7 cells after wortmannin treatment.
Collectively, we have demonstrated that the inhibition of Akt/PKB activity downregulates cyclin D1 levels in MCF-7As53 cells (Fig. 14B). Moreover, when the exponentially growing cells were treated with wortmannin and examined for growth rate, as shown in Fig. 14A, MCF-7As53 cells growth was comparable to the growth characteristic of untreated MCF-7 cells. Thus, it can be concluded that constitutive activation of PI3-K/Akt results in faster G1 to S cell cycle entry due to stabilized cyclin D1 (Musgrove et al., 1994) in MCF-7As53 cells, under in vitro conditions. Also, the pharmacological inhibitor of PI3-K could inhibit Akt activation resulting in decreased cell proliferation rate.

### 3.3.6 p53 is a negative regulator of caveolin-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 caveolin-1 (Cav-1 in figures) levels in these cells as previous studies have indicated that it is the most potent activator of PI3-K/Akt pathway (Li et al., 2003; Gonzalez et al., 2004).

![Fig. 15. p53 is upstream to caveolin-1 (Cav-1) expression in MCF-7As breast cancer cells. MCF-7As cells were either mock transfected or transfected with p53 expression vector. The lysates were prepared and immunoblotted. The transfer membranes were probed with p53, phosphocaveolin-1 and caveolin-1 specific antibodies. Protein levels were normalized with β-actin.](image)

Caveolin-1 is the structural component of caveolae which are invaginations of plasma membrane. Caveolae participate in vesicular trafficking and signal transduction events. Caveolin-1 is an integral membrane protein known to play an important role in regulating cell growth through its ability to modulate the activities
of molecules involved in growth factor signaling (Shack et al., 2003). Interestingly, we found upregulated expression of caveolin-1 in these antisense p53 expressing cells, as shown in Fig. 15. Moreover, we derived a negative correlation between p53 and caveolin-1 expression at protein levels. When p53 was overexpressed in these cells, the caveolin-1 levels decreased and correspondingly its phosphorylated levels also decreased (Fig. 15).

Additionally, we also confirmed by immunofluorescent studies that caveolin-1 is overexpressed and its enhanced localization could be detected on the cell membrane in MCF-7As53 cells as compared to MCF-7 cells. This underscored its possible signaling activities in these cells (Fig. 16).

To investigate whether this constitutively upregulated caveolin-1 message is responsible for constitutively active Akt, we treated these cells with cholesterol-depleting agent β-methyl cyclodextrin (MCD) which downregulates phosphocaveolin-1 levels without affecting its basal expression (Podar et al., 2003). We observed that the decrease in Akt activity correlated directly with the decrease of caveolin-1 phosphorylation (Fig. 17).
Fig. 17. Immunoblot analysis on lysates/extracts from cells treated with cholesterol depleting agent methyl beta cyclodextrin (MCD) was performed as described in text. β-actin was used as protein loading control.

Fig. 18. (A) A panel of breast cancer cells were assessed for caveolin-1 levels and correlated with wild type p53 transactivity. A representative western blot showing levels of caveolin-1 expression on (1) MCF-7 cells, (2) MCF-7 treated with PFTα, (3) MCF-7 Δ53, (4) MDA-MB-231 and (5) MDA-MB-468. β-actin was used as a control for loading. (B) Immunoblot for p53, caveolin-1 and activated Akt in (1) MCF-7 cells, (2) MCF-7 cells treated with PFTα and (3) MCF-7 cells transfected with p53 siRNA.

To verify whether p53 regulates caveolin-1 expression, we compared the expression levels of caveolin-1 in MCF-7 cells as such, in MCF-7 cells treated with PFTα, MCF-7 Δ53 cells, and in other breast cancer cells such as MDA-MB-231 or MDA-MB-468 which express mutant p53. Inhibition of p53 activity by PFTα in
MCF-7 cells, abrogation of p53 in MCF-7As53 cells or presence of mutant p53 in MDA-MB-231 or MDA-MB-468, all results indicated upregulation of caveolin-1 as compared to its level in parental MCF-7 cells (Fig. 18A). We also performed experiments by silencing p53 message in MCF-7 cells using siRNA. We observed that caveolin-1 levels also diminished in the cells which were transfected with p53 siRNA as compared with MCF-7 which were mock transfected (Fig. 18B). All these results imply that p53 status regulates caveolin-1 expression and dysfunctional p53 leads to overexpression of caveolin-1.

3.3.7 In vivo growth assay and molecular characterization

Nude mice bearing tumor derived by injecting MCF-7 and MCF-7As53 cells, were observed for comparing in vivo growth properties of these breast cancer cells. The tumor growth analysis over a period of 60 days indicated a higher rate of proliferation for MCF-7As53 cells in vivo also. As represented by tumor photographs, it can be concluded that the size of the tumors in animals which were injected with parental MCF-7 cells were about fifty percent of tumors which were derived from injecting MCF-7As53 cells (Fig. 19A and 19B).

![Graphical representation of tumor formation in nude mice showing tumor weight. Tumors were surgically excised from the](attachment://Fig.19)
mice injected with MCF-7 and MCF-7As53 cells after every ten days over a period of two months. The tumors were weighed and values are expressed as means (standard deviation, n=3). (B) The representative photograph of tumors excised after 60 days of in vivo growth. (C) Immunoblot for p53 and caveolin-1 messages in lysates prepared from tumor tissues.

To ascertain that the in vivo growth of cells in form of tumors does not alter the characteristic p53 and caveolin-1 expression signatures in these cell lines as established by in vitro studies, western blot analysis was carried out to monitor expression of these proteins in the tumor tissues (Fig. 19C). The protein level of p53 for MCF-7As53 derived tumors was coherent with our in vitro data and p53 protein message was not present in them, as compared to tumors derived from MCF-7 cells. Concurrently, the level of caveolin-1 was found increased in MCF-7As53 derived tumors. These results are in accordance with those obtained with in vitro cell culture studies.

3.3.8 MCF-7As53 has enhanced chemosensitivity

MCF-7As53 cells, parental MCF-7 and resistant clone MCF-7H were separately treated with Carb, 5-FU or PXL and resultant cytotoxicities were evaluated by MTT assays as described in materials and methods.

Fig. 20. MCF-7As cell line has chemosensitive phenotype. (A) For cell viability assay in drug treated cells, MTT assay was performed as documented in materials and methods. MCF-7 and MCF-7As cells were incubated in the presence of drugs for 24 h, and cell survival was determined after 48 h of post drug treatment by MTT assay. Data represents
cell survival (mean±standard deviation, n=5; P<0.005) as a fraction of viability of untreated control cells set as 100%.

The cell viability plot (Fig. 20) is based on one dose which was found to be least toxic to MCF-7 cells. Cytotoxic effects of Carb, 5-FU and PXL have been shown to be mediated via p53 dependent pathways. The graph shows that there is 30%-40% increase in cytotoxic cell death in MCF-753As when compared with parental cell lines. Thus it can be concluded that MCF-7 cells have become more sensitive to p53 dependent cytotoxic agents in absence of p53 protein. It is consistent with other studies done for chemosensitivity and downregulated or null p53 systems (Ramachandran and You, 1999; Chhipa et al., 2005). Thus, this cell line can be a tool to derive initial conclusions regarding p53 status and chemosensitivity in cell type specific context.

3.3.8 Enhanced chemosensitivity of MCF-7As53 cells is independent of activated Akt

The enhanced drug induced cell death in MCF-7As53 cells compared to parental MCF-7 cells was evaluated for its dependency on constitutively activated Akt.

![Graph showing cell viability](image)

*Fig. 21. PI3-Kinase/Akt pathway inhibitor wortmannin does not inhibit chemosensitivity of the antisense p53 expressing cell line, MCF-7As53. Shown here (1) control MCF-7As53 cells, (2) MCF-7As53 cells treated with wortmannin, (3) MCF-7As53 cells treated with Carb, (4) MCF-7As53 cells treated with Carb in presence of wortmannin, (5) MCF-7As53...*
cells treated with 5-FU and (6) MCF-7As53 cells treated with 5-FU in presence of wortmannin.

Since Akt activity has been shown to be involved in cellular sensitivities in several studies and chemosensitivity is based on assumption that rapidly growing cells are more prone to the chemotherapeutic drugs than non-dividing one, we observed that when the MCF-7As53 cells were treated with Carb and 5-FU in presence of wortmannin, the chemosensitive phenotype could not be reverted and cells were equally sensitive to these drugs. The results obtained indicated that chemosensitivity of MCF-7As53 cells remained unaltered in the presence of wortmannin even though it inhibits Akt activation. These results clearly demonstrated that activation of Akt due to abrogation of p53 does not play a role in the outcome to chemotherapeutic treatment of cancer cells.
3.4 DISCUSSION

Progress in breast cancer research has been greatly limited by the 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 (Lacroix and Leclercq, 2004). Although, MCF-7 is a well-characterized and established wild type p53 expressing breast cancer model (Okumura et al., 2002), there are not enough reports on genetically matched breast cancer cell system which differ in the status of p53 only. Different cell lines, experimental protocols, cell growth states or genetic backgrounds may have contributed to the conflicting conclusions (Zantek et al., 2001; Bernhardt et al., 2002) and it is generally believed that divergence in cancer cell line is the consequence of differences in culture conditions and passages undergone by a cell line (Osborne et al., 1987; Nugoli et al., 2003). Thus a genetically matched cell system with similarity in everything but p53 expression will be of great importance in understanding the functions of p53. We report here the development of a breast cancer epithelial cell line, MCF-7As53, derived from MCF-7 cells, in which p53 protein as well as its activity is abrogated due to constitutive and stable expression of antisense p53 cDNA.

Having developed one such unique isogenic MCF-7As53 cell line, we verified its epithelial morphology, p53 null status and estrogen receptor levels in comparison with parental MCF-7 cells. Our results suggest that these parameters remained unaltered over 20 passages in our laboratory. The p53 null phenotype of MCF-7As53 was routinely confirmed by carrying out western blot analysis. In order to find if there are any changes in molecular characteristics of this antisense p53 expressing cell line, we analysed 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. Furthermore, we are providing experimental evidence that p53 protein does not have any
functional role towards steady state levels of important chemotherapy response mediators such as p21, Bax and GADD45 in regulating cell growth (el Deiry et al., 1993; Miyashita and Reed, 1995; Taylor and Stark, 2001).

Wild type p53 is a negative regulator of cell proliferation (Baker et al., 1990; Michalovitz et al., 1990). The complex process of cell immortalization and transformation is likely to involve the inactivation of growth regulatory genes. Mutations (deletions, missense mutations) in the p53 gene are the most frequently observed genetic alteration in human tumors, making p53 a candidate for a cellular protein involved in the control of cell growth (Finlay, 1992). MCF-7As53 cells have enhanced rate of proliferation and we demonstrate that this proliferative phenotype is due to the depletion of G0-G1 phase as compared to MCF-7 parental cells. Progressively, stabilized cyclin D1 has been correlated with decreased cell doubling time of this clone as stabilized expression of cyclin D1 leads to characteristiclly faster transition from G1 to S phase. Cyclin D1 plays an important role in controlling the cell cycle in mammary tissue, and clinical studies on human breast cancer have confirmed its importance. Mammary tumors exhibiting high levels of cyclin D1 expression show higher rates of proliferation than cyclinD1-negative tumors (Watts et al., 1994; Zhang et al., 2004; Mawson et al., 2005; Zhang et al., 2005). Our studies with MCF-7As53 is one of the few reports in which p53 overexpression has been shown to down regulate cyclin D1 protein level, which may be consequence of direct or indirect molecular interactions. Therefore this cell line provides us with a tool to explore interrelationship between p53 and cyclin D1 which is yet to be clearly understood (Meng et al., 2004).

The PI3-kinase signaling pathway has been shown to play a pivotal role in intracellular signal transduction pathways involved in cell growth, cellular transformation and tumorigenesis. PKB/Akt has been implicated as an intermediate in PI3-K generated survival signals (Burow et al., 2000). Activation of these kinase signaling pathways contributes to various malignant phenotypes in human cancers, including breast tumor (Lin et al., 2005). Therefore we examined the phosphorylation and activation status of PKB/Akt kinase, which is found to be constitutively active in MCF-7As53 cells. When constitutively active PKB/Akt was
inhibited by PI3-K inhibitor wortmannin and cell were assayed for proliferation, it was observed that treatment with wortmannin not only resulted in decrease in growth but also led to downregulation of cyclin D1 protein in MCF-7As53 cells. These data imply that PI 3-kinase/PKB signaling is upstream of cyclin D1 and p53 protein directly controls this pathway. This report is in consistent with several other reports in which either p53 was inhibited or PI 3-kinase/PKB signaling was upregulated (Liang and Slingerland, 2003; Zhang et al., 2004). Activation of PI3-K/Akt pathway triggers a network that positively regulates G1/S cell cycle progression through inactivation of glycogen synthase kinase 3-beta (GSK3-beta) via its phosphorylation leading to stabilized cyclin D1, a key regulator of cell cycle, which is accumulated during the G1 phase (Ostrakhovitch and Cherian, 2004). Additionally, PKB also promotes transcription and translation of cyclin D1 gene (Liang and Slingerland, 2003). Moreover, recent reports suggest that p53 can negatively regulate Akt by repression of the catalytic subunit of PI3-kinase (Singh et al., 2002), as well as via expression of the PTEN tumor-suppressor gene (Stambolic et al., 2001).

In our quest to explore the reason for constitutively activated PI3-kinase/PKB signaling in MCF-7As53 cell line we reviewed the available experimental evidences for the cross talks between signal transduction pathways and cellular plasma membrane structural integrity which regulate growth and survival of the cells. We narrowed down on caveolae, the structural domains in the plasma membrane, which are known modulators of various cell signals from membrane to cytoplasm. Caveolae are 50- to 100-nm omega-shaped invaginations of the plasma membrane and consists of caveolins. Caveolins are a class of oligomeric structural proteins that are both necessary and sufficient for caveolae formation. Interestingly, caveolin-1 has been implicated in the pathogenesis of oncogenic cell transformation, tumorigenesis, and metastasis. Experimental evidences (gleaned from cultured cells, animal models, and human tumor samples) have concluded that caveolin-1 functions as a "tumor and/or metastasis modifier gene" (Williams and Lisanti, 2005). Caveolin-1 is the principal structural protein of caveolae, which are sphingolipid and cholesterol-rich invaginations of the plasma membrane involved in vesicular trafficking and signal transduction. During caveolae-dependent signaling, caveolin-1
acts as a scaffold protein to sequester and organize multi-molecular signaling complexes involved in diverse cellular activities. The hypothesis that caveolin-1 conveys a tumor/transformation suppressor function in the mammary gland is derived from several independent lines of evidence accumulated by genetic, molecular and clinical approaches. The human caveolin-1 gene maps to a suspected tumor suppressor locus (D7S522/7q31.1) frequently deleted in human breast carcinomas. Moreover, caveolin appears to be associated with human prostate cancer progression and is also present in primary and metastatic human breast cancer. In human breast cancer specimens, increased caveolin staining was detected in intraductal and infiltrating ductal carcinoma as well as in nodal disease (Yang et al., 1998). Recent studies have also implicated caveolin-1 in breast cancer pathogenesis, with emphasis on the signaling pathways regulated during these processes (Bouras et al., 2004).

In our investigations with MCF-7As53 cell line which has proliferative phenotype, we detected constitutive upregulation of caveolin-1 message. This result is in contrast to earlier report where utilizing MCF-7 human breast adenocarcinoma cells stably transfected with caveolin-1 (MCF-7/Cavl), it was demonstrated that caveolin-1 expression decreases MCF-7 cell proliferation rate and markedly reduces their capacity to form colonies in soft agar (Fiucci et al., 2002). However, our observation is in agreement with the reports in which it has been demonstrated that Akt activation correlates with caveolin-1 expression in the cells (Li et al., 2003). It has been reported that not only caveolin-1 is more expressed but also Akt-1 is activated in colon cancer tissues, than in normal colon tissues. These results suggest that caveolin-1 affects Akt-1 activation and thus proliferation of cells (Kim et al., 2006). Moreover it has also been demonstrated that disruption of caveolar structures with beta-cyclodextrin or transfection of VSMC with caveolin-1 antisense oligonucleotides (ODN) prevents PI3-K and Akt activation and therefore cell cycle entry. In addition caveolin-1 is also essential for the integrin-mediated activation of PI3-K/Akt (Sedding et al., 2005). Also siRNA-mediated caveolin-1 knockdown enhances basal as well as SIP- and VEGF-induced phosphorylation of the protein kinase Akt without modifying the basal or agonist-induced phosphorylation of extracellular signal-regulated kinases 1/2 (Gonzalez et al., 2004). All these reports
imply a correlation between caveolin-I and Akt activation. We observed that treatment of MCF-7As53 cells with cholesterol depleting agent beta cyclodextrin downregulated the active phosphorylated state of caveolin-1 as well as the levels of phospho-Akt. Subsequently, we established that enhanced phosphocaveolin-1 levels promote constitutive activation of Akt and stabilization of cyclin D1, leading to enhanced growth phenotype in p53 null conditions. It is also in consistency with the study where it was demonstrated that overexpression of caveolin-1 resulted in substantial growth inhibition or cell cycle arrest only through a p53/p21-dependent pathway (Lee et al., 1998; Galbiati et al., 2001). Congruent with the high proliferative nature of MCF-7As53 in monolayer culture conditions, these cells exhibited an enhanced in vivo tumorigenicity (Mukhopadhyay and Roth, 1993) as compared to MCF-7 parental cells as established by using xenograft analysis in nude mice. Western blot analysis on the excised tumor samples was performed to detect the levels of p53 and caveolin-1 and the results obtained were identical to those established in cell cultures.

Interestingly, it was also observed that overexpression of p53 in MCF-7As53 cell line leads to decrease in caveolin-1 protein levels. The expression levels of caveolin-1 was also correlated with functional status of p53 in a panel of breast cancer cells where either parental MCF-7 cells were treated with PFTa, a specific inhibitor of p53 transactivity, or cells expressed transactivation mutant p53. All these results strongly imply that wild type p53 is upstream of caveolin-1 in breast cancer cells and acts as its negative regulator. Therefore, it could be concluded that either deletion by antisense or abrogation of p53 activity due to mutations results in upregulation of caveolin-1 in breast cancer cells.

p53 is also a crucial checkpoint regulator of cytotoxic response after any stress or anticancer treatment (Brown and Wouters, 1999; Gimonet et al., 2004). This can be explained by differences in activation of susceptibility determining genes directly or indirectly regulated by wild type p53 protein. Of particular interest for the regimen of antisense-based therapy, p53 has always been a major target molecule (Wang et al., 2001). We are able to demonstrate that wild type p53 protein
level is major determinant of cells’ response to anticancer agents. These advantages now enable one to address effects of p53 protein levels on p53 dependent apoptosis and chemosensitivity in multiple breast cancer cell types under physiologically relevant conditions (Caelles et al., 1994). Consistent with previous reports on cytotoxicity (Xu et al., 1995; Wahl et al., 1996; Ceraline et al., 1998), MCF-7As53 exhibited significant increase in sensitivity to 5-FU, Carb, and PXL. These observations are consistent with those reported for p53 null cell systems (Hawkins et al., 1996; Ceraline et al., 1998).

Moreover, MCF-7As53 cells being more sensitive to the drugs is in agreement with the fact that chemosensitivity of cancer cells is based on the assumption that rapidly growing cells are more prone to the chemotherapeutic drugs than non-dividing one (Amadori et al., 1997; Rickardson et al., 2005). Also, several related studies have been done where caveolin-1 mediated higher PKB/Akt activity has enhanced the chemosensitivity in different cell lines. In contrast to its extensively documented antiapoptotic influence, the elevated activity of Akt appears to be important in sensitizing caveolin-expressing cells to arsenite-induced toxicity, as both pretreatment of cells with wortmannin and overexpression of a dominant-negative Akt mutant markedly improved the survival of arsenite-treated cells (Shack et al., 2003). As demonstrated earlier that the activation of Akt in MCF-7As53 is responsible for its enhanced growth phenotype, therefore, to determine whether the factors responsible for enhanced growth are also responsible for enhanced cytotoxicity of MCF-7As53 cells to chemotherapeutic drugs, we performed MTT cytotoxicity assays in the presence wortmannin. The results obtained indicated that the inhibition of PKB/Akt activity by wortmannin did not increase cell survival after drug treatments in MCF-7As53 cells; therefore it is unlikely that chemosensitivity of cancer cells could be attributed to caveolin-1/Akt activation in p53 null status. Hence, it could be concluded that loss of p53 associated G1 checkpoint and acquired proliferative potential in the MCF-7As53 cells is not associated with enhanced chemosensitivity of these cells which is consistent with earlier results obtained with colorectal carcinoma cells (Slichenmyer et al., 1993).
Finally, MCF-7As53 cell culture system may be extremely useful to recreate current perception of the importance of p53 levels and functions in breast cancer (Eeles et al., 1993; Vojtesek and Lane, 1993; Levine, 1997) with special emphasis on cell growth behaviour, chemotherapy and tumorigenesis in p53 null conditions. With this cumulative view, the role of p53 protein as a powerful transcriptional factor that binds to as many as 300 different promoter elements in the genome (Zhao et al., 2000), can be evaluated under p53 underexpression systems with reference to MCF-7 representative cancers. Our results strongly suggest that the absence of p53 promotes genomic instability (Livingstone et al., 1992; Harvey et al., 1993), which in turn may result in genetic alterations which directly produce chemosensitive phenotype and provide a unique model to identify secondary events critical for chemosensitivity, after the loss of p53 and upregulation of caveolin-1 in breast cancers represented by MCF-7 cells. Additionally with MCF-7As53, we have established an experimentally amenable system to analyse the possibility of dispensability of p53 transactivation potential at normal growth conditions as well as with anticancer treatments. Our observations thus validate MCF-7As53 as an experimental tool for elucidating cell growth, tumorigenesis and chemosensitization in breast cancers as function of p53 tumor suppressor with its specific, stable and endogenous antisense expression.
3.5 REFERENCES


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cyclin D1, and cyclin D3), proliferation index, and clinicopathologic parameters. *Am J Clin Pathol* 122: 444-452


Musgrove EA, Lee CS, Buckley MF, Sutherland RL (1994) Cyclin D1 induction in breast cancer cells shortens G1 and is sufficient for cells arrested in G1 to complete the cell cycle. *Proc Natl Acad Sci USA* 91: 8022-8026


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