

**p53 Mediated Suppression of Normal and Malignant Mammary Stem Cell Self-Renewal**

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**ABSTRACT**

Accumulating evidence from a number of malignancies including breast cancer suggests that tumorigenesis is driven by a subset of tumor cells which retain stem cell properties. The cancer stem cell concept proposes that cancer originates from malignant transformation of stem/progenitor cells. A number of pathways have been implicated in maintenance of tissue specific stem/progenitor cells, including the p53 pathway. Inactivating mutations or deletions of *TP53* have been reported in 50% of human malignancies. Germline mutations of p53 greatly increase the risk of breast tumors in animal models. Moreover, alterations of the p53 pathway in premalignant breast lesions have been reported and increase the risk of developing invasive ductal carcinoma. Meletis et al., demonstrated a role for p53 in suppressing self-renewal of adult neuronal stem cells. We utilized *in vitro* and mouse systems to examine the role of p53 in mammary stem/progenitor cells. p53 was down regulated by using a lentiviral vector carrying *TP53*-specific short hairpin sequences. Down regulation of p53 in both normal and malignant human mammary epithelial cells increased mammosphere formation and stem/progenitor cell population that expressed the stem cell marker aldehyde dehydrogenase as detected by the Aldefluor assay. Furthermore, down regulation of p53 compared to the control cells in normal mammary epithelial cells resulted in a 5 fold increase in the amount of ductal structures (outgrowths) generated in humanized NOD/SCID mammary fat pads. Outgrowths generated from control cells were characterized by luminal and myoepithelial layers of epithelial cells. However, outgrowths generated by p53 down regulated cells displayed areas of hyperplasia and/or ductal carcinoma in situ resembling human lesions,

which were proliferative and characterized by a basal phenotype. Using a small molecule inhibitor, MI63, that targets the mdm2-p53 complex, we further confirmed that activation of p53 inhibits mammosphere formation and results in reduction of the stem/progenitor cell population. MI63 had no effect on sphere formation of normal and malignant mammary epithelial cells with deleted p53, confirming the specificity of compound. These studies suggest that deregulation of the p53 pathway plays a significant role in malignant transformation of mammary stem/progenitor cells which may generate and subsequently maintain mammary tumors.

## INTRODUCTION

The cancer stem cell hypothesis maintains that tumors are organized in a hierarchical fashion, similar to normal tissue. Tumorigenesis and maintenance is driven by a small subset of cells termed 'cancer stem cells' (CSCs) that retain stem like properties such as the ability to self renew, an increased proliferative capacity, and the ability to differentiate into different lineages. In cancer stem cells, altered differentiation may also account for malignant transformation. CSCs were first identified in the hematopoietic system and subsequently in a variety of solid tumors including brain, breast, colon, prostate, and others (1-5). Cancer stem cells in the breast have been identified by CD44<sup>+</sup>/CD24<sup>-</sup>/lin<sup>-</sup> phenotype and more recently by the expression of aldehyde dehydrogenase 1 (ALDH) (6). Properties of CSCs include the ability to form mammospheres in suspension culture *in vitro* and the ability to generate tumors in serial passages regenerating the original tumor heterogeneity in a mouse model (6).

Although the cancer stem cell concept suggests that cancer originates from malignant transformation of tissue specific stem cells, early genetic and epigenetic events are poorly understood. Malignant transformation is a multi step process in breast and other tumors and alterations in tumor suppressor genes have been implicated as one of the early genetic events in transformation (7, 8). The p53 gene is a well documented tumor suppressor. Normally, p53 is inactive and bound to its inhibitor MDM2, which facilitates ubiquitin-mediated degradation of p53. It is activated in response to stress signals and DNA damage causing agents, regulating cell cycle arrest and DNA repair (9). Based on the signal, p53 can induce differentiation, senescence or apoptosis (9). The p53 gene is mutated or

deleted in 50% of human cancers but only in 20-35% of breast cancers (10). Inactivating mutations of p53 have been reported in patients with premalignant lesions such as ductal carcinoma in situ or atypical ductal hyperplasia (11-13).

In addition, animal models of tumor recurrence have recently revealed that the p53 pathway plays an important role. A doxycycline-inducible *Wnt1* transgenic mouse model (MTB/TWNT) of mammary adenocarcinomas depends on continued Wnt signaling. Down regulation of the Wnt pathway results in rapid disappearance of primary mammary tumors as well as pulmonary metastases (14). However, a significant fraction of tumors progress to a Wnt-independent state. Studies to further investigate molecular pathways involved in the re-growth of residual tumors show that the majority of regressed tumors exhibit complete or partial LOH at the *p53* locus, implying a selective loss of the wild type *p53* allele. Furthermore, almost all tumors with MTB/TWNT/*p53*<sup>+/+</sup> regressed to non-palpable state following doxycycline withdrawal, whereas 40% of tumors arising from MTB/TWNT/*p53*<sup>+/-</sup> mice failed to regress suggesting a specific role for p53 (14). Most recently two different studies have demonstrated that in p53 deficient tumors the restoration of p53 results in tumor regression or arrest of tumor growth (15, 16).

The p53 tumor suppressor pathway has also been implicated in the regulation of stem cell self-renewal (17-20). In response to stress signals, such as UV irradiation and DNA damaging agents, p53 becomes activated and promotes cell-cycle arrest or apoptosis depending on the signal. In embryonic stem cells (ESC), however, the p53 cascade appears to play a different role. Despite abundant accumulation of p53 in response to DNA damage, ESCs from wild type mice do not activate p53-dependent stress responses (21). Lin et al, suggested that activated p53 binds to the promoter of *Nanog*, a gene required for ESC self-

renewal (22, 23), and suppresses *Nanog* expression after DNA damage. The rapid down regulation of *Nanog* expression during differentiation correlates with the induction of p53 transcriptional activity and Ser 315 phosphorylation (24). Meletis et al, recently reported that p53 suppresses self-renewal of adult neural stem cells as demonstrated by increased neural stem cell proliferation *in vivo* and increased neurosphere formation of cells *in vitro* from p53 null mouse brain as compared to that of wild type mouse (25). Most recently, Stecca et al., reported that the p53 exerts its effect on neural stem cell self-renewal by inhibiting the activity and nuclear localization of GLI1, a downstream target for Hedgehog (Hh) signaling (26). p53 also regulates hematopoietic stem cell (HSC) quiescence by mediating two target genes, *Gfi-1* and *Necdin* (27).

Furthermore, germline mutations of p53 have been identified in families with Li-Fraumeni syndrome (28). 50% of affected individuals develop tumors by age 30, as compared to 1% in the general population (28, 29). In mouse models, all mice with homozygous p53 deletion (p53<sup>-/-</sup>) develop tumors within 4-5 months, while half of the heterozygous (p53<sup>+/-</sup>) mice develop tumors by 18 months (30).

All together these studies strongly suggest that p53 plays an important role in malignant transformation of tumors and its deregulation appears to be a relatively early event. Utilizing gain of function and loss of function approaches, we demonstrate here that the tumor suppressor p53 is necessary for mammary stem cell maintenance. While activation of the p53 pathway results in inhibition of mammary stem cell self-renewal, down regulation of this pathway by lentiviral shRNA-mediated p53 knock down expands mammary stem/progenitor cell population. These preclinical studies not only enhance our

understanding of p53 pathway but may also provide alternative approaches for targeting the tumorigenic CSC population.

## **MATERIALS AND METHODS**

### ***Dissociation of normal mammary tissue and mammosphere assay***

Mammary tissue from reduction mammoplasties was dissociated as previously described (31). For mammosphere experiments, single cell suspensions of NMECs were plated on 1% agarose coated plates at a density of  $1 \times 10^5$  cells/ml and grown for 7–10 days. Mammosphere cultures were grown in a serum-free mammary epithelium basal medium as previously described (32). Subsequent cultures after dissociation of primary spheres were plated on ultra-low attachment plates at a density of  $5 \times 10^3$  to  $1 \times 10^4$  cells/ml.

### ***Lentiviral construction and cell infections***

All lentiviral constructs were prepared by the University of Michigan Vector Core facility. The primers targeting the human P53 short hairpin sequences were purchased from Integrated DNA technologies, Inc. The forward primer “TGACTCCAGTGGTAATCTACTTCAA GAGAGTAGATTACCACTGGAGTCATTTTTTC” and the reverse primer “TCGAGAAAAAATGAC TCCAGTGGTAATCTACTCTCTTGAAGTAGATTACCACTGGAGTCA” were annealed and cloned into both pLentilox 3.7-GFP or DsRed vectors digested with the HpaI and XhoI enzymes. After confirmation of DNA sequences, 293 host cells were transfected with these plasmids to produce the viruses at the University of Michigan Vector Core facility. Resulting lentiviral p53 shRNA GFP or DsRed constructs were used to transfect NMECs and breast cancer cell lines.



### ***Implantation of cells in NOD/SCID mice***

The fat pads of three week old NOD/SCID mice were cleared and replaced with a 1:1 mixture of irradiated and non-irradiated human fibroblasts. Within two to three weeks, human fibroblasts generated a humanized mammary fat pad in the mice. These resulting fat pads were injected with NMECs infected with DsRed or p53 shRNA lentivirus. An estrogen pellet was also implanted subcutaneously in each mouse. The mice were sacrificed after 4 to 8 weeks and the fat pads were analyzed for outgrowths.

### ***Aldefluor Assay and Flow cytometry***

To measure and isolate cells with high ALDH activity, the Aldefluor assay was performed according to manufacturer's (Stemcell Technologies, Durham, NC) guidelines. Dissociated single cells were resuspended in Aldefluor assay buffer containing the ALDH substrate, Bodipy-aminoacetaldehyde (BAAA) at 1.5  $\mu$ M and incubated for 40 minutes at 37 °C. To distinguish between ALDH-positive and -negative cells, a fraction of cells was incubated under identical condition in the presence of a 10-fold molar excess of the ALDH inhibitor, diethylamino benzaldehyde (DEAB). This results in a significant decrease in the fluorescence intensity of ALDH-positive cells and was used to compensate the flow cytometer.

### ***Cell lines and p53 shRNA, DsRed lentiviral infections***

The MCF7 cell line was maintained in RPMI supplemented with 5% Fetal Bovine Serum (FBS), 5  $\mu$ g/ml insulin and Antibiotic-Antimycotic (10,000 units/ml penicillin G sodium, 10,000  $\mu$ g/ml streptomycin sulfate, Gentamycin 4 $\mu$ g/ml, HEPES 10mM and 25  $\mu$ g/ml

amphotericin B). The SUM159 and SUM149 cell lines were maintained in Ham's F12 medium supplemented with 5% FBS, 5 µg/ml insulin, 1µg/ml hydrocortisone and antibiotic/antimycotic. SUM159, SUM149 and MCF7 cells were infected with the lentivirus expressing p53 shRNA or empty vector expressing either GFP or DsRed. Following 12–16 hours of incubation, the viruses were removed and replaced with fresh medium.

### ***Immunoblotting***

Cells were lysed in RIPA Lysis Buffer and the amount of protein was estimated using the Bio Rad Protein Assay (Bio Rad Laboratories, Hercules, CA). Equal amounts of protein were mixed 1:1 with Laemmli buffer, boiled and loaded onto SDS-polyacrylamide gels. Following electrophoresis, proteins were transferred onto nitrocellulose membranes (Pierce, Rockford, IL) using a semidry Trans-Blot (Bio Rad Laboratories, Hercules, CA). Blots were first incubated in TBS blocking buffer containing either 2% Milk or 2% BSA (for phospho-specific antibodies) for 1–2 hours at room temperature and then with the respective primary antibodies diluted in TBST (containing 0.1% Tween20 and 2% BSA) overnight at 4°. Subsequently, blots were washed and incubated with appropriate secondary antibodies (GE Healthcare, UK) in TBST and detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Antibody to human p53, Anti-p53 (Ab-6) (Pantropic) mouse monoclonal antibody (DO-1) was from Calbiochem (San Diego, CA).

### ***Immunohistochemistry***

For immunohistochemistry, paraffin-embedded sections were deparaffinized in xylene and rehydrated in graded alcohol. Antigen enhancement was done by incubating the sections in

citrate buffer pH6 (Dakocytomation, Denmark) as recommended. Staining was done using Peroxidase Histostain-Plus Kit (Zymed) according to the manufacturer's protocol. Sections were incubated with primary antibodies for 1 hour. Following incubation with broad spectrum secondary antibody and HRP-conjugated streptavidin, AEC (Zymed) was used as substrate for peroxidase. Slides were counter-stained with hematoxylin, and coverslipped using glycerin. The primary antibodies for human smooth muscle actin, Ki67, CK5/6, SMA and CK18 were purchased from Zymed laboratories Inc. (Invitrogen, Carlsbad, CA). ER is from Neomarkers (Fremont, CA), and antibody for ALDH was from BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ).

### ***MDM2 small molecule inhibitor MI63 and the inactive analog MI61***

For activation of p53, a small molecule inhibitor of MDM2 was used. MI63, and its inactive analog MI61 were generous gifts from Dr. Shaomeng Wang. Cells were plated in suspension culture as described and MI61 or MI63 was added to media at a final concentration of 5 $\mu$ M. After 7 days the spheres were counted and dissociated and replated in suspension culture with fresh media containing 5 $\mu$ M MI61 or MI63.

## RESULTS

### ***Knockdown of p53 expands the number of mammospheres and the Aldefluor-positive population in normal mammary epithelial cells***

It has previously been shown that human normal mammary epithelial cells (NMECs) can be propagated in suspension culture as floating colonies called mammospheres and that these mammospheres have a higher proportion of stem and progenitor cells as opposed to mammary epithelial cells in adherent culture (32). To test the effects of p53 deletion on the normal mammary stem/progenitor cell population, NMECs were infected with a pLentiLox 3.7 lentivirus containing *TP53*-specific short hairpin sequences and a DsRed marker, or just the DsRed marker as a control. The infected NMECs were maintained in mammosphere culture to enrich for stem and progenitor cells. As shown in Figure 1A, western blotting for p53 protein levels shows more than 90% reduction of p53 protein. Figure 1B shows primary mammospheres positive for DsRed, confirming efficient transfection of the cells with both lentiviruses. Consequent experiments were performed with transfected NMECs flow sorted to select for DsRed positivity.

After seven days in suspension culture, the number and size of mammospheres from p53 deleted NMECs was increased more than two fold as compared to the DsRed controls. The number of mammospheres in serial passages has been shown to be an indirect assay of the approximate number of stem cells in the population (32). The increase in size and number of spheres was maintained in a serial passage; the p53 deleted NMECs maintained a higher fraction of mammosphere forming cells (data not shown), indicating a p53 dependent suppression of mammary stem cell self-renewal.

To confirm that the increase in the size and number of mammospheres in p53 deleted NMECs is directly correlated with stem cell number, we quantified the stem cell number using FACS. We have recently shown that aldehyde dehydrogenase 1 (ALDH), as detected by the Aldefluor assay, is a marker of normal and malignant human mammary stem and progenitor cells (6). Quantification of stem cell number was confirmed by FACS analysis using the Aldefluor assay, as outlined in Materials and Methods. The percentage of ALDH-positive NMECs was increased approximately twofold in p53-shRNA infected NMECs as compared to the DsRed controls, confirming our hypothesis that p53 deletion leads to an increase in stem/progenitor cell number (Figure 2). All of these experiments suggest that active p53 may suppress mammary stem cell self-renewal, while deleted p53 appears to release this suppression, expanding stem/progenitor population.

***Knockdown of p53 in NMECs generates outgrowths resembling hyperplastic lesions in humanized NOD-SCID mice***

A mouse model to study normal human mammary development has been generated by Proia and Kuperwasser (33). In order to investigate the effect of p53 deletion in an *in vivo* setting, we injected control and p53 deleted NMECs into the fat pads of NOD-SCID mice. Utilizing this model we examined the *in vivo* morphological changes in ductal structures generated by p53 knockdown cells. Consistent with our *in vitro* findings, an increased number of ALDH positive cells was observed in the p53 down regulated outgrowths (Figure 3A). The outgrowths generated by control NMECs were composed of luminal and myoepithelial cells (Figure 3B). p53 knockdown NMECs gave rise to an increased number of outgrowths which appear to be hyperplastic as seen in Figure 3B. Control NMECs

generated normal ductal structures/outgrowths characterized by a single layer of myoepithelial cells expressing smooth muscle actin (Figure 3C), recapitulating the architecture of normal mammary ducts in women. In contrast, outgrowths generated by p53 knock down NMECs are larger and morphologically distinct (Figure 3C), resembling ductal carcinoma in situ. Figure 3C also shows increased disorganization in outgrowths generated from p53 downregulated NMECs as shown by smooth muscle actin staining of the outer myoepithelial layer. In addition, there was a significantly higher number of proliferating cells in outgrowths generated by p53 downregulated NMECs as compared to controls (Figure 3D) assessed by Ki-67 staining.

We also utilized immunohistochemical staining for markers of basal and luminal epithelial cells to characterize outgrowths generated from both control and p53 knock down cells. Examination of epithelial markers demonstrated significant differences between structures derived from p53 knock down and control cells. In control outgrowths, the majority of the epithelial cells expressed the luminal marker CK18, while only a small fraction of p53 knock down cells expressed this marker (Figure 3E). Outgrowths produced by control cells contained only a small number of cells expressing the primitive cytokeratins 5/6, whereas the frequency of these cells was greatly increased in p53 down regulated structures (Figure 3F). Estrogen receptor (ER) is expressed in luminal cells in structures generated from DsRed control cells, but only a small fraction of cells in structures derived from p53 down regulated cells (Figure 3G). These experiments confirm and extend the *in vitro* findings suggesting that the down regulation of p53 results in enrichment of the more primitive stem/progenitor cells in humanized NOD-SCID mice indicating that p53 plays a role in mediating lineage differentiation of the mammary stem/progenitor cells.

### ***p53 signaling suppresses cancer stem cell self-renewal***

Recent studies have suggested that established breast cancer cell lines may contain subpopulations that display stem cell characteristics (34, 35). We previously demonstrated that normal mammary stem/progenitor cells can be enriched in mammosphere cultures (32). Furthermore, breast cancer cell lines generate spheres in suspension culture called “tumorspheres” enriched in CSCs (36). The malignant characteristics and increased stem cell population seen in p53 deleted NMECs led us to reason whether p53 also mediates the breast cancer stem cell population and also whether this pathway can be utilized as a therapeutic approach.

A small molecule inhibitor of the MDM2-p53 complex, MI63, was used in breast cancer cell lines to investigate the effects of p53 activation on the cancer stem cell population. p53 is generally inactive and complexed with MDM2, which promotes its ubiquitin-mediated degradation (9). MI63 is a competitive inhibitor that binds MDM2. Unbound, activated p53 is then free to activate its myriad tumor suppressing pathways.

Breast cancer cell lines MCF7, SUM159, and SUM149 were treated with MI63 to stimulate p53 activity. The MCF7 cell line harbors wild type p53 responding to apoptosis inducing agents (37). SUM159 and SUM149 cell lines both have non-functional p53 due to mutations in its gene. An inactive analog of MI63 called MI61 was used as a control and then the cells were analyzed by western blot for levels of p53. The resulting effect of MI63 on MCF7 and SUM159 cells is demonstrated in Figure 4. MCF7 shows a robust increase in p53 levels upon MI63 treatment, while p53 is undetectable in control and MI61 treated MCF7 cells. SUM159 shows no difference in levels of p53 regardless of treatment. SUM149 cells harbor inactive p53 and therefore also failed to respond to MI63 treatment (data not shown).

All three cell lines were serially passaged in suspension culture and treated with MI61 or MI63 to ascertain the effects of p53 activation on the cancer stem cell population. Activation of p53 using MI63 reduced primary sphere formation in MCF7 cells by 80% and completely eliminated secondary sphere forming cells (Figure 5A). SUM159 cells were unaffected by the MI63 treatment. Secondary sphere formation was not affected and sphere numbers between MI61 and MI63 treated SUM159 cells did not change significantly (Figure 6A and B). Similarly, SUM149 sphere growth was also unaffected and primary and secondary sphere counts were not significantly different between MI61 and MI63 treatments (Figure 6C and D). The observation that SUM159 and SUM149 cells were unaffected by the MDM2 inhibitor is due to mutations in the p53 pathways of these two cell lines, and this data implies a p53-specific effect on sphere formation and mammary stem cell self-renewal.

In order to study the effect of MI63 treatment on p53 downregulated MCF7 cells, MCF7 cells were transfected with the p53 shRNA or DsRed control lentivirus and serially passaged in suspension culture and treated with the MDM2 inhibitor. Western blot analysis confirmed the deletion of p53 and the specificity of the MI63 compound; in control MCF7 cells there was a robust increase in p53 levels upon MI63 treatment but in MCF7-p53 shRNA cells p53 levels were undetectable and even with MI63 treatment there was only a very modest increase in p53, indicating almost 100% knockdown of p53 (Figure 7A). The MI63 compound had no effect on sphere formation in MCF7-p53 shRNA cells (Figure 7B). As compared to control MCF7 spheres, the number of MCF7-p53 shRNA spheres was significantly increased in both the primary and secondary spheres, as seen in Figure 7C.



The failed response of MCF7-p53 shRNA spheres to MI63 indicated a p53-specific mediation of sphere formation and mammary stem cell self-renewal.

## DISCUSSION

The cancer stem cell model suggests that tumors are organized in a cellular hierarchy and are driven by a subset of cells capable of self-renewal as well as differentiation generating the bulk of the tumor (38, 39). Despite the progress that has been made in identifying CSCs in a variety of human malignancies, the pathways which drive transformation of these cells are poorly understood. We and others have suggested that carcinogenesis may involve deregulation of the normally tightly regulated process of stem cell self-renewal coupled to aberrant differentiation of progeny cells (40). Previous studies demonstrate that mutations in the *TP53* gene are one of the early premalignant events in the majority of human malignancies (11-13). Additionally, Meletis et al., previously demonstrated a p53 mediated suppression of neural stem cell proliferation and self-renewal in a mouse model (25). This may suggest a role for p53 in maintenance of mammary stem/progenitor cells.

We hypothesized that p53 suppresses the self-renewal of mammary stem cells and may also induce lineage differentiation, maintaining homeostasis in the mammary gland. We employed both gain of function and loss of function approaches to explore the role of p53 in mammary stem/progenitor cells. We knocked down p53 levels in normal mammary epithelial cells from reduction mammaplasties and tested its effects in mammosphere formation *in vitro*. Down regulation of p53 increased the size and the number of mammospheres from NMECs. This may suggest that removal of p53 suppression on mammary stem cell self-renewal results in expansion of this population due to increased self-renewal. We also activated p53 pathway utilizing an MDM2 inhibitor to further confirm the p53 mediated effect on mammary stem and progenitor cells. Activation of the

p53 pathway in NMECs significantly reduced the number of secondary mammospheres confirming results from knock down experiments that the effect is p53 mediated (data not shown).

Furthermore, we utilized a previously described humanized NOD/SCID mouse model of human mammary gland development (33) to ascertain the effect of p53 on mammary stem/progenitor cells. Implantation of p53 knock down NMECs displayed morphologically distinct outgrowths as compared to controls, and they resembled DCIS, a premalignant lesion. Outgrowths generated from control cells were characterized by low proliferating luminal and myoepithelial layers of cells. In contrast, structures generated from p53 down regulated cells showed a higher number of proliferating cells displaying basal phenotype and increased number of ALDH1 expressing cells. This is in line with a recent study by Manie et al., who reported high frequency of *TP53* mutations in BRCA1 and sporadic basal-like carcinomas but not in luminal tumors of breast (41). In addition, a smaller fraction of cells in outgrowths generated from p53 down regulated cells express estrogen receptor (ER), a luminal marker, as compared to cells in control outgrowths. It has been previously demonstrated that ER-expressing cells show little or no stem cell activity in mouse models of mammary gland development (42). Altogether, these data suggest that down regulation of p53 expands mammary stem cell/progenitor cells by perhaps reversing its inhibitory effect.

In order to examine the role of p53 in cancer stem cell self-renewal, we also manipulated levels of p53 in a number of breast cancer cell lines. Activation of p53 signaling using an MDM2 small molecule inhibitor in MCF7 cells with wild type *TP53* gene (43) inhibited tumorsphere formation *in vitro*. In contrast, Sum149 and Sum159 cells with mutated *TP53*

gene failed to respond to the MDM2 inhibitor treatment, suggesting that inhibition of MCF7 sphere is p53 mediated. This was further confirmed by the increased formation of MCF7 tumorspheres when the *TP53* gene is down regulated by lentiviral shRNA. Furthermore, p53 down regulation rendered MCF7 cells non-responsive to the MDM2 inhibitor, mimicking the scenario found in cell lines with mutated *TP53* gene. These data strongly suggest that p53 plays a role in mammary stem cell self-renewal and imply that this may be utilized for therapeutic use.

The cancer stem cell hypothesis has the potential to revolutionize the treatment of cancer. Traditionally, therapies have been aimed at the bulk of cycling cells within a tumor, and may fail to target the CSCs, which can explain why some patients relapse following initial response (44). CSCs have also been implicated in therapeutic resistance, metastasis and chemoresistance (45, 46). It is now widely acknowledged that new therapeutic approaches are required for targeting and elimination of CSCs (38). This will also provide an opportunity for less deleterious side effects of broad spectrum chemotherapeutic agents that kill healthy as well as tumor cells. The p53 gene is mutated or deleted in 50% of human cancers but only in 20-35% of breast cancers (10). Our results here show that targeting the cancer stem cell population through activation of p53 could be an effective therapy in patients with wild type p53.

In addition to its direct effect on mammary stem cells, p53 has been shown to interact with numerous other proteins and signaling pathways that are involved with regulation of the mammary stem/progenitor population. *NUMB* is implicated to regulate the switch between self-renewal and differentiation in normal and cancerous cells through its target gene *Notch* (47). Colaluca et al., showed that NUMB, p53 and MDM2 form a tricomplex that

inhibits p53 degradation, resulting in increased p53 activity independent of Notch activity. The authors also suggest that deregulation of this interaction between NUMB and p53 could be one mechanism of tumorigenesis due to a shift towards symmetric self renewal in the stem cell population (47). Recently, Secchiero et al., demonstrated in leukemias with wild type p53 that p53 activation through an MDM2 inhibitor increases levels of NOTCH1 through binding of p53 to the *NOTCH1* promoter (48). Notch signaling plays an important role in mammary stem cell maintenance by inhibiting lineage differentiation. Thus this suggests another downstream effect of p53 activation on the mammary stem cell compartment. p53 has also been shown to affect levels of PTEN and therefore could influence the PTEN/PI3K/AKT pathway and its effects on mammary stem cells (49). These findings indicate that p53 plays a role within a larger network of signaling pathways. As such, targeting p53 therapeutically could not only have a direct effect on the cancer stem cell population but also a potentially more widespread effect through its influence on various other signaling pathways.

## FIGURE LEGENDS

Figure 1. Transfection of normal mammary epithelial cells with p53 shRNA results in down regulation of p53 and increased sphere formation.

- A. Western Blot analysis confirms down regulation of p53 upon shRNA infection in normal mammary epithelial cells (NMECs).
- B. NMEC spheres show efficient transfection with pLentiLox 3.7 vector carrying DsRed and *TP53*-specific sequences.

Figure 2. ALDH<sup>+</sup> population increases two-fold upon down regulation of p53. Flow cytometry analysis of control and p53 shRNA-infected NMECs using the Aldefluor assay shows that the stem/progenitor population as marked by aldehyde dehydrogenase activity is increased when p53 is downregulated.

Figure 3. Immunohistochemistry of control and p53 shRNA treated mouse xenografts. p53 downregulation leads to increased stem/progenitor population (A, ALDH), increased outgrowths *in vivo* (B, H&E), increased disorganization in p53 downregulated xenografts (C, SMA), increased proliferation of ductal structures, leading to hyperplasia and DCIS in NOD-SCID mice (D, KI67), loss of double layered ducts (E and F, CK 5/6 and CK18) and decreased differentiation (G, ER).

Figure 4. Effect of MI63 on breast cancer cell lines. Levels of p53 in MCF7 and SUM159 cells by western blot analysis. MCF7 has a functional copy of p53 and is therefore affected by

MI63; SUM159 and SUM149 have mutations in p53. These cells lines are not affected by the MI63 compound.

Figure 5. p53 activation results in decreased sphere formation.

- A. MCF7 cells treated with MI61 or MI63 assayed for the ability to form spheres through serial passages. In the secondary passage for MI63 but not MI61, MCF7 cells cannot form spheres.
- B. Relative to the primary growth, zero secondary spheres were able to grow when the cells were treated with MI63, as compared to an 80% reduction in the cells treated with MI61.

Figure 6. Sphere formation is only affected by MI63 when a functional copy of the p53 gene is present. In both SUM159 (A,B) and SUM149 (C,D) cells there is no functional copy of p53, so sphere formation does not change between primary and secondary passages.

Figure 7. Effect of p53 downregulation on growth of mammospheres.

- A. Western blot analysis confirms the specificity of the MI63 compound.
- B. MCF7 and MCF7-p53 shRNA spheres treated with control compound MI61 or MDM2-p53 complex inhibitor (p53 activator) MI63.
- C. A significant increase in sphere formation is seen in p53 shRNA spheres as compared to MCF7 control spheres.
- D. A significant increase in sphere formation is seen in p53 shRNA spheres treated with MI63 as compared to MCF7 control spheres.

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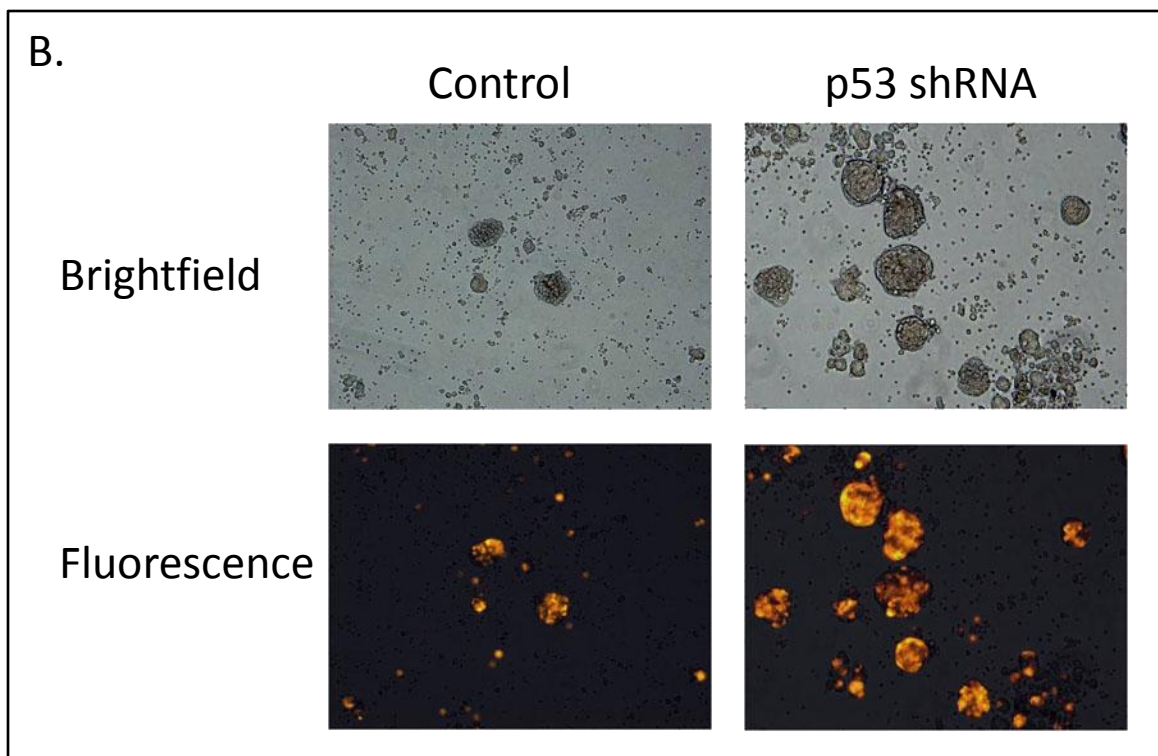
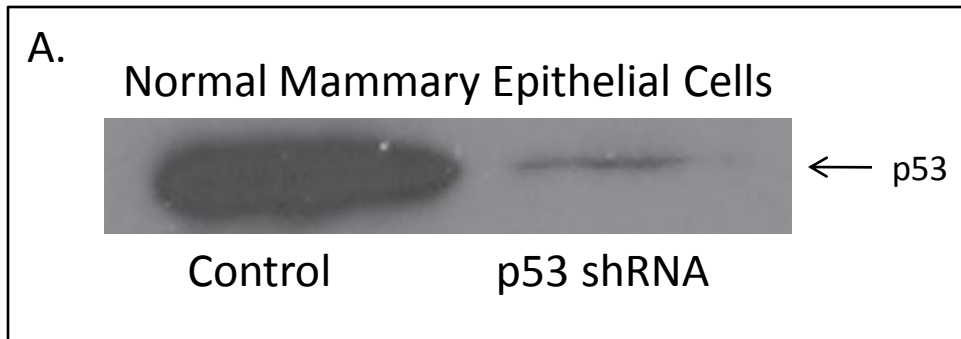


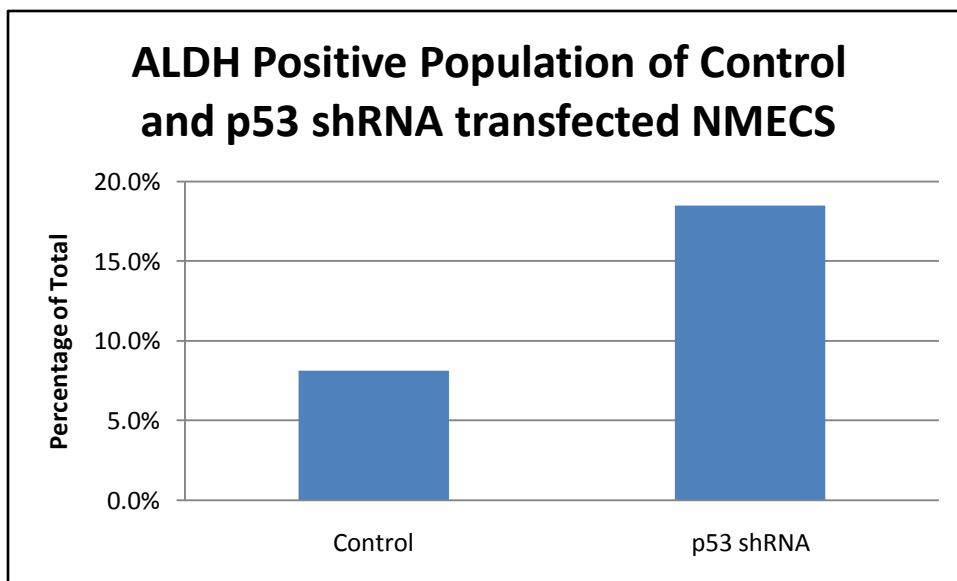
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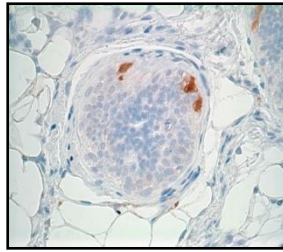


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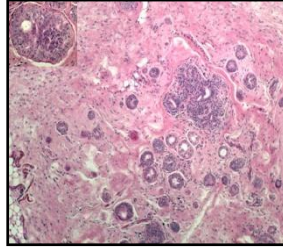
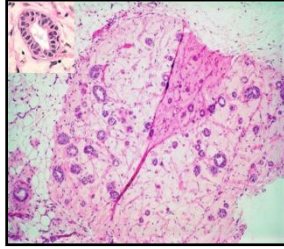
p53 shRNA

Figure 3

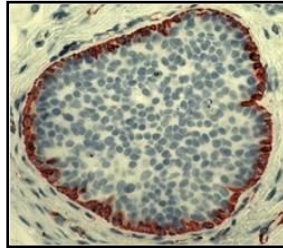
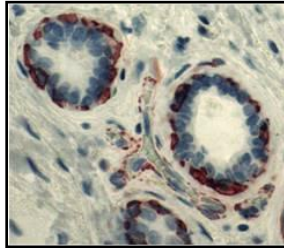
A. ALDH



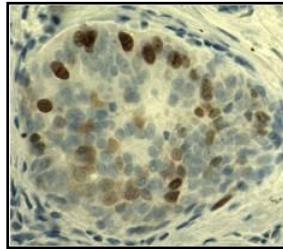
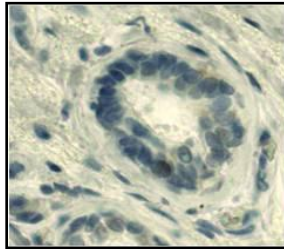
B. H&E



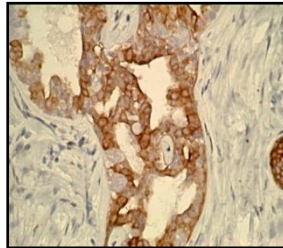
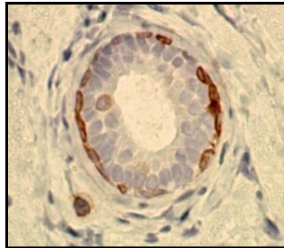
C. SMA



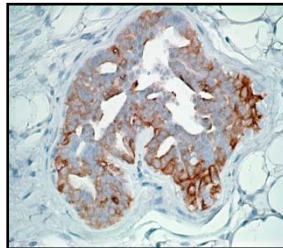
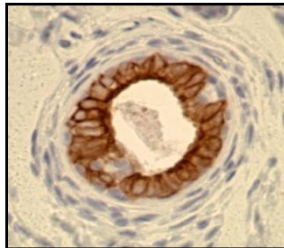
D. KI67



E. CK5/6



F. CK18



G. ER

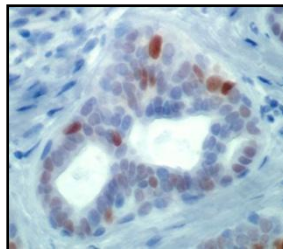
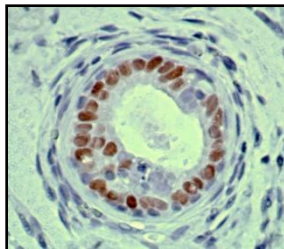


Figure 4

