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Repression of mammary stem/progenitor cells by p53 is mediated by notch and separable from apoptotic activity

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Abstract

Breast cancer is the most common tumor among women with inherited mutations in the p53 gene (Li-Fraumeni syndrome). The tumors represent the basal-like subtype which has been suggested to originate from mammary stem/progenitor cells. In mouse mammary epithelium, mammosphere-forming potential was increased with decreased dosage of the gene encoding the p53 tumor suppressor protein (*Trp53*). Limiting dilution transplantation also showed a 3.3-fold increase in the frequency of long-term regenerative mammary stem cells in *Trp53*−/− mice. The repression of mammospheres by p53 was apparent despite the absence of apoptotic responses to radiation indicating a dissociation of these two activities of p53. The effects of p53 on progenitor cells were also observed in TM40A cells using both mammosphere-forming assays and the DsRed-let7c-sensor. The frequency of long-term label-retaining epithelial cells (LRECs) was decreased in *Trp53*−/− mammary glands indicating that asymmetric segregation of DNA is diminished and contributes to the expansion of the mammary stem cells. Treatment with an inhibitor of γ-secretase (DAPT) reduced the number of *Trp53*−/− mammospheres to the level found in *Trp53*+/+ cells. These results demonstrate that basal levels of p53 restrict mammary stem/progenitor cells through Notch and that the Notch pathway is a therapeutic target to prevent expansion of this vulnerable pool of cells.

Keywords

Tumor Suppressor Protein p53; Li-Fraumeni Syndrome; Adult Stem Cells; Notch Proteins; apoptosis; Breast Neoplasms

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INTRODUCTION

Mammary stem cells have been identified that are capable of regenerating the entire mammary ductal tree and repopulating the mammary fat pad (1, 2). The mechanisms and pathways regulating self-renewal and differentiation of mammary stem cells are of great interest for its potential application in tissue replacement therapies as well as the prevention and treatment of breast cancer. The Wnt pathway was found to regulate the self-renewal of mammary stem/progenitor cells. Ectopic expression of Wnt1 resulted in an increased population of mammary stem/progenitor cells in the mammary gland and eventually induced mammary tumors (3). Another breast cancer related gene, *BRCA1*, was reported to play a critical role in the differentiation of mammary stem/progenitor cells to luminal cells. Loss of both *BRCA1* alleles resulted in expansion of stem/progenitor cells in the breast epithelium of women and increased breast cancer risk (4). The Notch pathway has also been implicated as regulator of mammary stem/progenitor cells self-renewal and differentiation but its function is controversial. Dontu et al reported that the activation of Notch signaling with DSL peptide resulted in 10-fold increase of mammosphere-forming activity (5). Conversely, Bouras et al showed that the inhibition of Notch pathway by knockdown Cbf-1 in $CD29^{hi}CD24^{+}$ cells resulted in increased transplantation efficiency, suggesting that the Notch pathway may restrict mammary stem/progenitor cells expansion (6).

The p53 protein is a central regulator for multiple tumor suppressor pathways. The role of activated p53 in mediating cell cycle arrest and apoptosis has been studied extensively (7–9). In response to DNA damage, oncogene activation or other stresses, p53 accumulates in nucleus and transactivates downstream genes, such as p21 and PUMA, and directs the fate of damaged cells resulting in repair or elimination (10, 11). In addition to the importance of activated p53 under stress conditions, the basal level p53 under normal conditions may also play an essential role in tumor suppressor function. In both hematopoietic system and neural system, basal levels of p53 were shown to negatively regulate the self-renewal of tissue-specific stem cells (12, 13). The *Arf-Trp53* pathway was shown to restrict the efficiency of reprogramming of induced pluripotent stem cells (14, 15).

Disruption of the gene encoding p53 (designated *TP53* in human and *Trp53* in mouse) predisposes normal mammary epithelium to tumorigenesis. Women with Li-Fraumeni syndrome, which is most commonly associated with germline heterozygous mutations of *TP53*, have significantly increased risk of breast cancer (16, 17). Mutations and deletions of *TP53* are the most common alterations in cancers. The rate of p53 mutation is as high as 82% in the basal-like subtype of breast cancer, whereas in luminal A subtype, p53 mutations are found in only 13% patients suggesting that p53 mutation promotes basal-like breast cancer (18). This class of aggressive tumors express gene signatures enriched in embryonic stem cells, and thus, have been proposed to originate from progenitor cells (19). Mammary tumors from p53 heterozygous mouse models mimic Li-Fraumeni syndrome in women and the tumors share gene expression patterns with tumors from *Brca1*-deficient and Wnt1 transgenic mice and human basal-like breast cancer, suggesting that mammary tumors from p53-deficient mice may also originate from the stem/progenitor cells (18, 20–22).

In this study, BALB/c-*Trp53*+/+, *Trp53*+/− and *Trp53*−/− mice were used to test the role of p53 in regulating the mammary stem/progenitor cells. We found that decreased p53 dosage resulted in increased frequency of mammary stem/progenitor cells, suggesting that basal levels of p53 inhibited self-renewal of mammary stem/progenitor cells. As the mammosphere-initiating cells of different *Trp53* genotypes were resistant to ionizing radiation (IR), p53-mediated apoptosis is comprised in these cells. Therefore, expansion of the mammary stem/progenitor cells population cannot be attributed to differences in apoptosis. Similarly, the decrease in the pool of label-retaining cells in *Trp53*−/− mammary

epithelium also suggest that survival is not increased, but rather asymmetric segregation of DNA is diminished in the absence of p53 leading to dilution of the label during expansion of mammary stem/progenitor cells. We also showed that γ-secretase inhibitors (GSI) can be used to inhibit the expansion of *Trp53−/−* mammary stem/progenitor cells. The results demonstrate that p53 regulates self-renewal of mammary stem/progenitor cells and that insufficient basal levels of p53 can lead to expansion of the pool of mammary stem/progenitor cells, which are especially vulnerable to tumorigenesis without the proper surveillance of p53. Therefore, the Notch pathway is a potential therapeutic target to inhibit expansion of mammary stem/progenitor cells and reduce breast cancer risk.

MATERIALS AND METHODS

Animals

BALB/c-*Trp53+/+*, *Trp53+/−* and *Trp53−/−* mice were generated by backcrossing (C57BL/c x 129/Sv) *Trp53−/−* mice onto the BALB/cMed strain as described before (23). Wild type 3 weeks old BALB/c recipient mice for transplantation were purchased from Jackson lab.

Isolation of primary mouse mammary cells

Mammary gland harvested from 8–10 weeks old virgin mice were minced and dissociated in DMEM:F12 (Sigma, St. Louis, MO) supplemented with 5% Fetal Bovine serum (Gibco, Paisley, UK), 2mg/ml collagenase (Worthington Biochemicals, Freehold, NJ), 100u/ml hyaluronidase (Sigma), 100u/ml pen/strep (Gibco) and 100μg/ml gentamicin (Gibco) for 6 hours. The cell pellet was collected and further dissociated with 1ml pre-warmed 0.05% Trypsin-EDTA (Gibco) and 200μl 1mg/ml DNase I (Roche, Mannheim, Germany). Cell suspensions were sieved through a 40μm cell strainer to obtain single cell suspension.

Mammosphere culture

Primary single cells were seeded into ultra-low attachment dishes or plates at a density of 20,000 viable cells/ml. Cells were grown in a serum-free mammary epithelial growth medium (HuMEC, Gibco) supplemented with B27 (Gibco), 20ng/ml EGF (Sigma), 20ng/ml bFGF (Sigma), 4μg/ml heparin (Sigma), 100u/ml Pen/Strep, 5μg/ml gentamicin (24). For the GSI treatment, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma) was added into culture medium to the final concentration of 5μM (final DMSO concentration 0.1%). To passage mammospheres, mammospheres were collected with gentle centrifugation 800rpm for 5min 7 days after culture and dissociated with 1ml pre-warmed 0.05% Trypsin-EDTA and 60μl 1mg/ml DNase I for 5–8min. Cell suspensions obtained from dissociation were sieved through 40μm cell strainer and seeded at a density of 1,000 viable cells/ml. To test the IR responses of mammosphere-initiating cells, single cell suspensions received 0-Gy (control group) or 5-Gy dose (radiation group) of γ-irradiation from a cesium-137 source before being plated.

Limiting dilution and transplantation

Primary mammary epithelial cells (MECs) were freshly isolated as described above and resuspended in DMEM:F12 with 5%FBS. Six different cell concentrations were used: 50,000/10μl, 10,000/10μl, 5,000/10μl, 2,500/10μl, 1,000/10μl, 100/10μl. *Trp53+/+* cell suspensions were injected into right side of #4 cleared fat pads of 3 weeks old recipient mice and the same concentration of *Trp53−/−* cell suspensions were injected into the contralateral left side fat pad. The transplanted fat pads were harvested and stained with Carmine Alum solution 8 weeks after transplantation (25). Outgrowths that occupied >5% of the fat pad were regarded as a successful outgrowth (26). Two methods were used to estimate the frequency of long-term regenerative mammary stem cells. The L-Calc software (Stemcell

Tech, Vancouver, Canada) has been reported previously (27). We also used a generalized linear model approach assuming an underlying Poisson distribution of stem cell frequency to model the limiting dilution data using Stata (Stata Corp, College Station, TX). The regression model included a term for the multiplicative effect of *Trp53+/+* (relative to *Trp53−/−*) and model adequacy was assessed using the link test. The Wilcoxon signed-rank test was used to compare the percentage of filled fat pad between *Trp53+/+* and *Trp53−/−* epithelium. The model was not adjusted for the paired design where *Trp53+/+* and *Trp53−/−* transplants are tested within each animal. The paired design would bias results in the direction of the null hypothesis resulting in a conservative estimate of statistically significant findings.

TM40A cell culture and retroviral infection

TM40A cells were maintained in regular MECL media: DMEM:F12 supplemented with 2% adult bovine serum (Gibco), 10μg/ml Insulin (Sigma), 20ng/ml mEGF, and 100u/ml Pen/Strep. Oligos coding for the p53 knockdown or scramble shRNA were annealed and cloned into pSicoR-PGK-puro vector (Addgene, Cambridge, MA). The p53 target sequence was GTACTCTCCTCCCCTCAAT and the scramble sequence was CGCTACACACTTCTTCTCC. The infection of TM40A cells with let-7c sensor plasmid, pSicoR-PGK-puro-p53KD plasmid or the control plasmids were performed as described previously (28, 29).

Flow cytometry and cell sorting

Cells were freshly collected and resuspended in DMEM:F12 supplemented with 1mM EDTA, 25mM HEPES, 1%FBS and 100u/ml Pen/Strep. The FACS data were collected using LSRII (Becton Dickinson, San Jose CA). A total 100,000 events were collected and analyzed using BD FACSDiva software (Becton Dickinson). Cell sorting was performed using a FACSVantage SE (Becton Dickinson).

Western blot

TM40A cell protein lysates were harvested 1 hour after 0-Gy or 10-Gy of γ-irradiation using RIPA buffer (50mM Tris, 150mM NaCl, 1% TritonX-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 1x protease inhibitor (Sigma, p8340) and 1x phosphatase inhibitor (Sigma, P5726)). Protein lysates (80μg) were separated by 10% SDS-PAGE and transferred to PVDF membrane (Millipore, Billerica, MA). The membrane was incubated with anti-phospho p53 (1:1000, Cell Signaling, 9284), or anti-β-actin (1:4000, Sigma, A1978), followed by incubation with hoseradish peroxidase conjugated secondary antibodies (1:5000, GE Healthcare, Little Chalfont, Buckinghamshire, UK), and developed using enhanced chemiluminescence (ECL) solution (GE Healthcare) in G:Box imaging system (Syngene, Cambridge, UK).

Label retaining cells

3 weeks old BALB/c-*Trp53+/+* and BALB/c-*Trp53−/−* mice were injected with BrdU (Sigma) 300μg/10g body weight for 7 days. Mammary glands were harvested 9 weeks after the final injection. 5 *Trp53+/+* mice and 3 *Trp53−/−* mice were used in this experiment. BrdU staining was done using the BrdU staining kit (Invitrogen, Carlsbad, CA) and the whole slides were counted for the total epithelial cells and LRECs.

RESULTS

p53 inhibits the expansion of mammary stem/progenitor cells

In order to examine the effect of p53 on mammary stem/progenitor cells, mammosphere formation capacity was compared among BALB/c-*Trp53+/+*, *Trp53+/−* and *Trp53−/−*mice. During serial passages, *Trp53−/−* epithelial cells gave rise to significantly higher numbers of secondary and tertiary mammospheres than wild type epithelium ($p<0.01$) (Fig. 1A), suggesting that p53 restricts expansion of mammary stem/progenitor cells. *Trp53+/−* epithelium also gave rise to higher numbers of mammmospheres than *Trp53*+/+, indicating the importance of p53 dosage with respect to regulation of mammary stem/progenitor cells. Furthermore, the *Trp53−/−* mammospheres are also larger than *Trp53+/+* mammospheres, suggesting more extensive proliferation (Fig. 1B).

To estimate the frequency of long-term regenerative mammary stem cells, we performed limiting dilution and transplantation to test the ability of cells to reconstitute the mammary gland. Total mammary cells were isolated from 8–10 week old aged-matched BALB/c-*Trp53+/+* and *Trp53−/−* donor mice. The cells were transplanted into cleared mammary fat pads of 3-week old wild type BALB/c recipients. Both *Trp53+/+* and *Trp53−/−* outgrowths showed normal ductal structure in both whole mounts (Fig.1D, F) and HE staining (Fig.1E, G). UsingL-Calc Software, the frequency of mammary stem cell in BALB/c-*Trp53+/+* epithelium was estimated to be 1 in 8,085 (± 1S.E. 6,508 −10,045) compared to 1 in 2,445 (± 1S.E. 2,033 − 2,940) in BALB/c-*Trp53−/−* epithelium (Fig. 1C, H). The frequency of long-term regenerative mammary stem cells in *Trp53−/−*epithelium was 3.3-fold higher than the *Trp53+/+* epithelium ($p<0.001$), suggesting that basal levels of p53 inhibits the expansion of mammary stem cells and that insufficient p53 dosage results in increased numbers of mammary stem cells between these genotypes. A generalized linear model approach was also applied and produced similar estimates of the difference in frequency of mammary stem cells. It is also noticeable that *Trp53−/−*outgrowths occupied a significantly higher percentage of the gland than the *Trp53+/+* outgrowth ($p<0.01$), suggesting increased regenerative capacity of the *Trp53−/−* mammary stem cells (Fig. 1H).

p53-mediated apoptosis pathway is compromised in mammary stem/progenitor cells

Ionizing radiation (IR) causes DNA double strand breaks, which induces preferentially p53-dependent cell cycle arrest and apoptosis. To test whether the different number of mammospheres may be an artifact of defective apoptosis in *Trp53*−/− cells, we used IR to trigger DNA damage and apoptosis. Upon serial passages, mammosphere cell suspensions were treated with either 0-Gy or 5-Gy γ-irradiation then seeded in parallel. Surprisingly, the number of secondary or tertiary mammospheres was not affected by IR in any of the genotypes ($p>0.05$) (Fig. 2A, B), suggesting that the mammosphere-initiating cells are resistant to IR and that the p53-mediated apoptosis pathway is compromised in these cells. These results indicate that the increase in mammary stem/progenitor cells is not attributed to differences in apoptosis or survival in *Trp53−/−* cells and that p53 acts by a distinct mechanism to limit the mammary stem/progenitor cells.

Trp53−/− mammary epithelium contained fewer label retaining epithelial cells (LRECs)

Non-random segregation of chromatids was reported in both embryonic stem cells and multilineage progenitor cells (30). It has been postulated that the tissue specific stem cells maintain their “stemness” and protect themselves from mutation through asymmetric segregation of their template DNA strands (31, 32). LRECs have been reported in mammary gland by using either [^{3}H]-thymidine or BrdU labeling (33). We labeled BALB/c-*Trp53+/+* and *Trp53−/−* mice with BrdU when 3 weeks old and chased for 9 weeks. Both genotypes exhibited similar incorporation of BrdU immediately after the labeling period. After 9 weeks

of chasing, the BrdU-retaining epithelial cells were found in both luminal and basal compartments and the distribution of LRECs was similar among both genotypes (Fig. 3A, B). However, *Trp53−/−* mammary glands contained significantly fewer LRECs (1.26±0.09%) than the *Trp53+/+* mammary glands (2.56±0.18%) ($p<0.01$, Fig. 3C), indicating that asymmetric segregation of DNA is impaired in the absence of p53 resulting in dilution of the BrdU label.

TM40A cells as an *in vitro* model to test the function of basal level p53

The role of p53 in regulation of mammary stem/progenitor cells was further confirmed *in vitro* using the TM40A cell line, a mammary epithelial cell line derived from BALB/c mice and retains wild type p53 mRNA (unpublished data, DJJ). TM40A cells form hyperplastic outgrowths when transplanted *in vivo* (Fig. 4A) but have undetectable tumorigenicity through 20 weeks. The let-7 microRNA family was shown to be depleted in the mammary progenitor cells and highly expressed in the more differentiated cell types and the let-7c sensor plasmid (let7^s) has been used to label mammary progenitor population *in vitro* (28). The TM40A-let7^s cells contained 0.8% of DsRed positive (DsR$^+$) progenitor cells (Fig. 4B). The mammosphere-forming capacity of DsR$^+$ cells is 3.8-fold greater than DsR$^-$ cells ($p<0.01$) (Fig. 4C), confirming their progenitor feature. Interestingly, most of the cells in the DsR$^+$ mammospheres remained DsR$^+$ (Fig. 4D).

We proceeded to determine whether the knockdown of p53 can change the proportion of DsR$^+$ progenitor cells. TM40A-let7^s cells were infected with a p53 shRNA plasmid (TM40A-let7^s-p53KD) or control plasmid (TM40A-let7^s-ctrl) as described previously (29). The p53 shRNA decreased p53 protein to less than 24% of the original level (Fig. 4E). The TM40A-let7^s-p53KD contained increased numbers of DsR$^+$ cells (2.5%) compared to the TM40A-let7^s-ctrl cells (0.8%) (Fig. 5A and B). The number of secondary mammospheres formed by TM40A-let7^s-p53KD cells was also 2.2-fold higher than the control cells ($p<0.01$) (Fig. 5E), further proving that the basal level of p53 inhibits the expansion of mammary progenitor cells.

Notch inhibitor reduced the number of mammary stem/progenitor cells

Increased mammary stem/progenitor cells could be vulnerable targets for carcinogenesis, especially in Li-Fraumeni patients in whom p53-mediated genome surveillance is compromised. We proceeded to test the potential of pharmacological methods to inhibit the expansion of mammary stem/progenitor cells. The Notch pathway has been reported to both promote and limit progenitor cells (5, 6), therefore we tested whether γ-secretase inhibitors (GSI), could affect the expansion of p53-deficient mammary stem/progenitor cells. The number of TM40A-let7^s-p53KD mammospheres decreased to baseline levels after the treatment with DAPT, a GSI (Fig. 5E; $p<0.01$) indicating that Notch pathway could be a potential therapeutic target for downregulation of mammary stem/progenitor cells. The number of mammospheres in control cells with wild type p53 was not changed with DAPT treatment showing that inhibition of Notch was not a general effect, but specifically reversed the effect of p53-deficiency (Fig. 5E). DAPT treatment also restricted the expansion of progenitors measured by DsR$^+$ sensor after p53 knockdown. In TM40A-let7^s-p53KD cells, DAPT decreased the DsR$^+$ cells from 2.5% to 1.4% (compare Fig. 5B and D), whereas the proportion of DsR$^+$ cells was not changed by DAPT in the control group (compare Fig. 5A and C). *Trp53−/−* and *Trp53+/+* primary mammary epithelial cells were also treated with 5μM DAPT or DMSO. Similarly, DAPT significantly inhibited the expansion of *Trp53−/−* mammospheres. The number of *Trp53−/−*secondary mammospheres decreased significantly from 250±13/10,000 cells to 168±11/10,000 cells after the DAPT treatment ($p<0.01$), but the number of *Trp53+/+* mammospheres were not affected (Fig. 5F).

DISCUSSION

The importance of p53 in breast cancer is highlighted by the dramatic increase of breast cancer risk among women with Li-Fraumeni syndrome (16, 17). Although the function of activated p53 in mammary epithelium has been extensively studied, its role at basal levels under normal conditions is not fully understood. Both the mammosphere and limiting dilution data showed that insufficient basal levels of p53 resulted in increased numbers of mammary stem/progenitor cells. A gene dosage effect was also detected with the frequency of mammosphere-forming activity being intermediate for *Trp53*+/− mammary epithelium compared to the *Trp53*+/+ and *Trp53*−/−.

Label-retaining cell assays provide a measure of the asymmetric divisions of stem cells. Smith *et al* reported that by using [^{3}H]-thymidine as the first label for LRECs and BrdU as secondary label for recently proliferating cells, most LRECs were actively synthesizing DNA yet retained their [^{3}H]-thymidine labeled strands, suggesting that asymmetrically dividing cells contribute to most of LRECs (33). Organ specific stem cells could also be static and divide less frequently, which may also contribute to their label-retaining feature (31, 34). Recently, reports showed that p53 is essential for maintaining quiescence of mammary stem cells as well as hematopoietic stem cells (34, 35). In our experiment, the frequency of LRECs in wild type mice was 2.56±0.18%, which is close to that reported by Smith *et al* using [^{3}H]-thymidine. We showed that *Trp53*−/− epithelium contained fewer LRECs than wild type epithelium. This could be explained by increased proliferation of *Trp53*−/− mammary stem cells, which dilute the BrdU after 9 weeks of chasing. Alternatively, p53 may regulate the asymmetric segregation of sister chromatids during mitosis, which could be vital for the fate decision of daughter cells. Loss of p53 may result in the disruption of this asymmetric segregation, leading to the loss of BrdU labeling after several rounds of division.

As p53 plays a prominent role in apoptosis, it was possible that differences in cell survival could contribute to the apparent increase in mammary stem/progenitor cells. However, radiation treatment failed to alter the number of secondary or tertiary mammospheres in any *Trp53* genotype suggesting that p53-mediated apoptosis is compromised in the mammary stem/progenitor cell population. Previous studies have also demonstrated the resistance of mammary progenitors to therapeutic doses of ionizing radiation (36, 37). Furthermore, if differences in apoptosis were responsible for the apparent expansion of mammary stem/progenitor cells, the frequency of LRECs would be expected to be increased, but were in fact decreased significantly. Therefore, the expansion of mammary stem/progenitor cells cannot be attributed to altered survival of p53-deficient cells.

These results highlight the disparate functions and roles of p53 in different cell types. It was reported that ES cells could not activate p53-dependent responses to ionizing radiation because p53 protein was sequestered in the cytoplasm (38). Nonetheless, under basal conditions p53 was found to suppress expression of Nanog and induce differentiation of mouse ES cells (39). In mammary gland, irradiation triggers p53-mediated apoptosis in ductal epithelium (40), but this surveillance activity of p53 was not detectable in mammary stem/progenitor cells (Fig. 2). Nonetheless, the ability of basal levels of p53 to restrict the pool of progenitors was retained (Fig. 1). Therefore, the tumor suppressor function of p53 can be divided into two different aspects. In differentiated cells, p53 can be activated due to various genotoxic or cellular stresses so that the damaged cells will either be repaired or eliminated through apoptosis, depending on the extent of damage. In contrast, the apoptosis-inducing function of p53 is compromised in the mammary stem/progenitor cells, which prevents the loss of tissue-specific stem cells and the premature aging process due to DNA damage or other cellular challenges. In these cells, the major tumor suppressor function of

p53 is to restrict self-renewal and inhibit inappropriate expansion of mammary stem/progenitor cells, a function that is independent of the pro-apoptotic function of p53 (Fig. 5G).

The elucidation of mammary stem cells and breast cancer stem cells has stimulated greatly the discussion of the cellular origins of breast cancers (21, 41). In small intestine, the deletion of the adenomatosis polyposis coli gene (*Apc*) in intestinal stem cells showed much higher transformation efficiency than in short-lived transit-amplifying cells, providing direct evidence of the stem cell origin of intestinal cancer (42). In mammary gland, the complexity of breast cancer subtypes and mammary epithelial cell hierarchy makes it hard to identify the cellular origins of breast cancer. Type I human breast epithelial cells (HBECs) express features of luminal stem/progenitor cells and show a greater potential for immortalization and transformation by oncogenes (43–45). The pathologic features of tumors also appear to differ among populations sequentially immortalized and transformed with TERT, SV40-T-antigen and activated Ras (46). These observations suggest that the mammary stem/progenitor cells are sensitive to oncogenic transformation, although the possibility of transformation of differentiated epithelial cells can not be ruled out. Therefore, modest increases in the prevalence of stem/progenitor cells would be anticipated to increase risk of breast cancer and are a likely source of cancer stem cells.

Germline heterozygous mutations in *TP53* or *BRCA1* significantly increase breast cancer risk. *BRCA1* was also shown to regulate self-renewal and cell fate decision of mammary stem/progenitor cells. Loss of heterozygosity (LOH) of *BRCA1* resulted in histologically normal lobules, which are comprised of progenitor cells and have higher transformation risk (4). Similarly, loss of p53 function is associated with basal-like breast cancers that express markers of embryonic stem cells (18, 19). Mammary tumors from p53-deficient mouse models also appear to develop from bipotent progenitor cells and have gene expression patterns similar to embryonic stem cells (22, 41). Our lab showed that 62% of spontaneous mammary tumors from *Trp53+/−* mice contained mixture of cells expressing either K5 or K8/18 (22). It is likely that the expansion of mammary stem/progenitor cells resulting from loss of p53 activity contributes to the great breast cancer risk due to their long life span and the ability to give rise to multiple lineages of differentiated cells. Therefore the inhibition of mammary stem/progenitor cell expansion may be a key target for prevention of hereditary breast cancers.

Expression of Notch pathway members were especially prominent in the gene expression patterns in mammary tumors of p53-deficient mice (22) suggesting that this pathway may contribute to the expansion of mammary stem/progenitor cells. We found that treatment of *Trp53−/−* primary cells and TM40A-let7^{s}-p53KD cells with DAPT significantly downregulated the mammosphere-forming activity and the number of DsR^{+} progenitor cells. However DAPT did not change the mammosphere number of *Trp53+/+* or TM40A-let7^{s}-ctrl cells. This data agreed with the report of Dontu *et al*, which demonstrated that the Notch pathway upregulated the number of mammary stem/progenitor cells (5). The p53 protein has been reported to inhibit activation of the Notch pathway at different levels by either inhibiting the transcription of presenilin-1 (PS1) or competing with Notch-1 intracellular domain for co-activator p300/CBP (47–49). Similar mechanisms may lead to the inhibition of Notch pathway by p53 in mammary stem/progenitor cells. In the absence of p53, this inhibition is released, which allows expansion of mammary stem/progenitor cells. While in the presence of p53, the inhibition mechanism is intact and the treatment of DAPT did not affect the self-renewal of mammary stem/progenitor cells (Fig. 5G). Researchers have tried to apply GSI on breast cancer treatment and showed that GSI is effective in suppression of breast cancer stem cells and inhibition of breast cancer growth (50, 51). The GSI-sensitive signature suggested that pathways, including the Notch pathway and chemokine signaling

pathway may contribute to the sensitivity of breast cancer to GSI (51). However, it remains possible that other targets of γ-secretase are important. Our results suggest that the Notch pathway is a potential therapeutic target to inhibit the expansion of mammary stem/progenitor cells and GSI may be applied to prevent breast cancer in patients with Li-Fraumeni syndrome.

SUMMARY

Proper regulation of the pool of progenitor cells is increasingly recognized as a factor in determining risk of breast cancer. We show that decreased p53 gene dosage results in increased numbers of progenitor cells through a mechanism that involves loss of asymmetric divisions and apparent increases in Notch activity, but not alterations in apoptosis. Inhibition of Notch signaling with a γ-secretase inhibitor reversed the effect of p53 loss resulting in restriction of the number of mammary stem/progenitor cells. As inhibition of Notch limited the pool of mammary stem/progenitor cells in p53-deficient mammary epithelium but had no effect in *Trp53*+/+ cells, it appears to be an effective treatment to prevent mammary tumors due to loss of p53 function with minimal consequences to cells with wild type p53.

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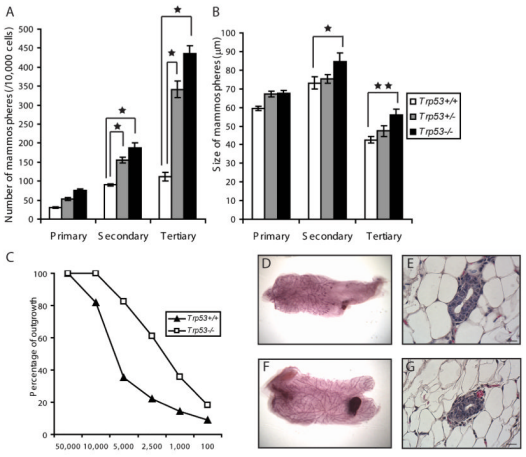

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Number of Cells	Number of Mice	*Trp53+/+* Take Rate	*Trp53+/+* Percent Fat Pad Filled	*Trp53-/-* Take Rate	*Trp53-/-* Percent Fat Pad Filled
50,000	3	3(100%)	●●●	3(100%)	●●●
10,000	11	9(81.8%)	●●●◕◕◔◔◔◔	11(100%)	●●●●●●●●●◕◔
5,000	17	6(35.5%)	●●◓◓◔◔	14(82.4%)	●●●●●●●●◕◕◕◔◔◔
2,500	18	4(22.2%)	●◓◓◔	11(61.1%)	●●●●●●●●●◔
1,000	14	2(14.3%)	◓◔	5(35.7%)	●●●◔◔
100	11	1(9.1%)	◔	2(18.2%)	●◔
Frequency		1 in 8,085		1 in 2,445	
Frequency Range (±1 S.E.)		1 in 6,508 ~ 10,045		1 in 2,033 ~ 2,940	
p value		<0.001			

Fig. 1.

p53 inhibits the expansion of mammary stem/progenitor cells. (A) *Trp53−/−* and *Trp53+/−* mammary epithelial cells (MECs) gave rise to significantly higher number of mammospheres than *Trp53+/+* MECs ($p<0.01$). The data shown represent 12 replicates for each genotype. The results were reproduced in a second independent experiment. (B) *Trp53−/−* MECs gave rise to significantly larger mammospheres than *Trp53+/+* MECs upon serial passages (★$p<0.01$, ★★$p<0.05$). (C) *Trp53−/−* MECs gave a higher outgrowth rate than *Trp53+/+* MECs. (D–F) Both *Trp53+/+* (D, E) and *Trp53−/−* (F, G) outgrowths were histologically normal as shown by whole mount and HE staining. (H) The extent of fat pad filled for each successful outgrowth was recorded and the frequency of mammary stem/progenitor cells was estimated for *Trp53+/+* and *Trp53−/−* using L-Calc software. *Trp53−/−* MECs contained significantly higher frequency of long-term regenerative mammary stem cells than *Trp53+/+* MECs ($p<0.001$). Meanwhile, *Trp53−/−* outgrowths occupied significantly higher percentage of fat pad than the *Trp53+/+* outgrowths ($p<0.01$).

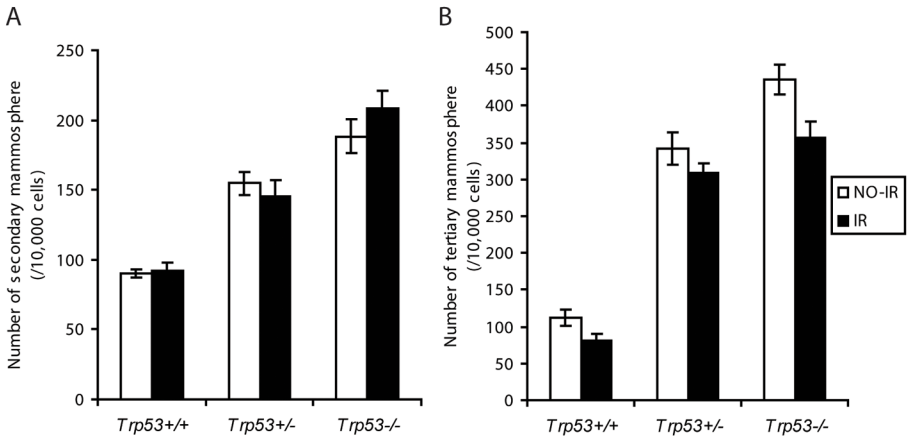

Fig. 2.

p53-mediated apoptosis is compromised in mammary stem/progenitor cells. Single cell suspensions were treated with 0-Gy or 5-Gyγ-irradiation before being plated and the number of secondary (A) and tertiary (B) mammospheres were compared. The mammospheres of different p53 genotypes showed no difference between control (NO-IR) and irradiated (IR) samples ($p>0.05$). The data shown represent 12 replicates for each treatment. The results were reproduced in a second independent experiment.

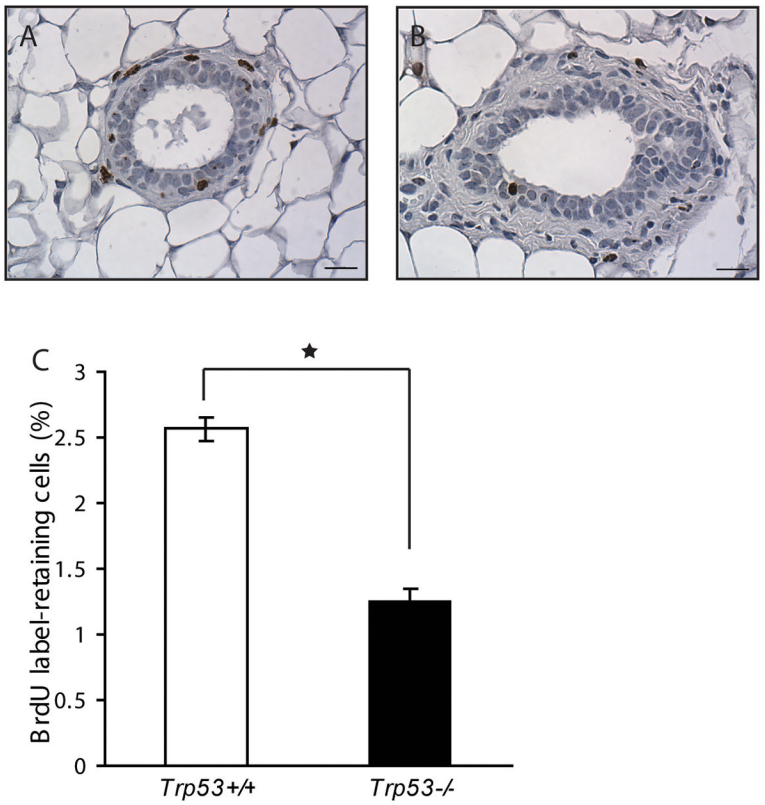

Fig. 3.

Trp53−/− mammary epithelium contained fewer label-retaining epithelial cells (LRECs). LRECs were found in both luminal and basal compartments and the distribution of LRECs were similar among *Trp53+/+* (A) and *Trp53−/−* (B) mammary glands. Quantitative analysis showed that the *Trp53−/−* glands contained significantly lower number of LRECs than the *Trp53+/+* glands (C) ($p<0.01$).

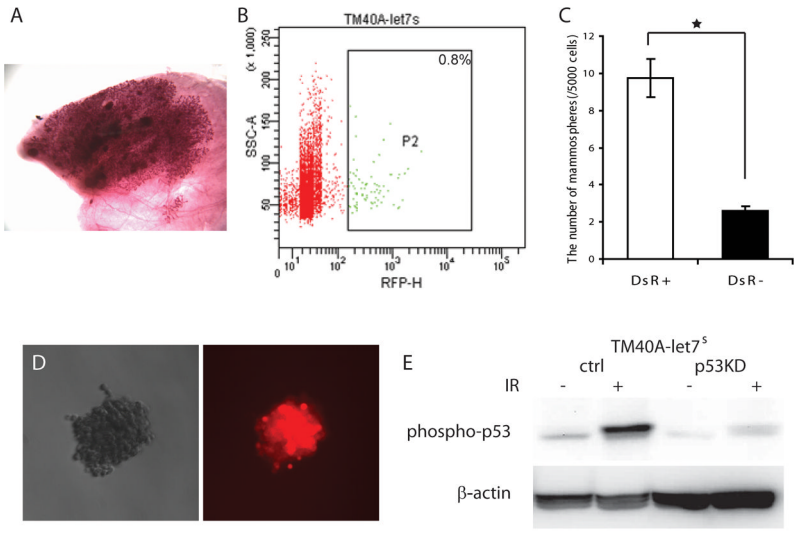

Fig. 4.

TM40A cells as an *in vitro* model to test the function of basal level p53. (A) Whole mount of a TM40A outgrowth 20 weeks after transplantation. (B) The let7c-sensor plasmid was introduced into TM40A cells (TM40A-let7^{s}). The TM40A-let7^{s} cells contained 0.8% of DsRed positive (DsR^{+}) progenitor cells. Background levels of fluorescence were determined using control cells. (C) The DsR^{+} cells gave rise to significantly more mammospheres than DsR^{-} cells ($p<0.01$). (D) Most cells in the DsR^{+} mammospheres remained DsRed positive. (E) Western blot showed that phosphorylated p53 (phospho-p53) was reduced in TM40A-let7^{s}-p53KD cells compared to the control cells (TM40A-let7^{s}-ctrl).

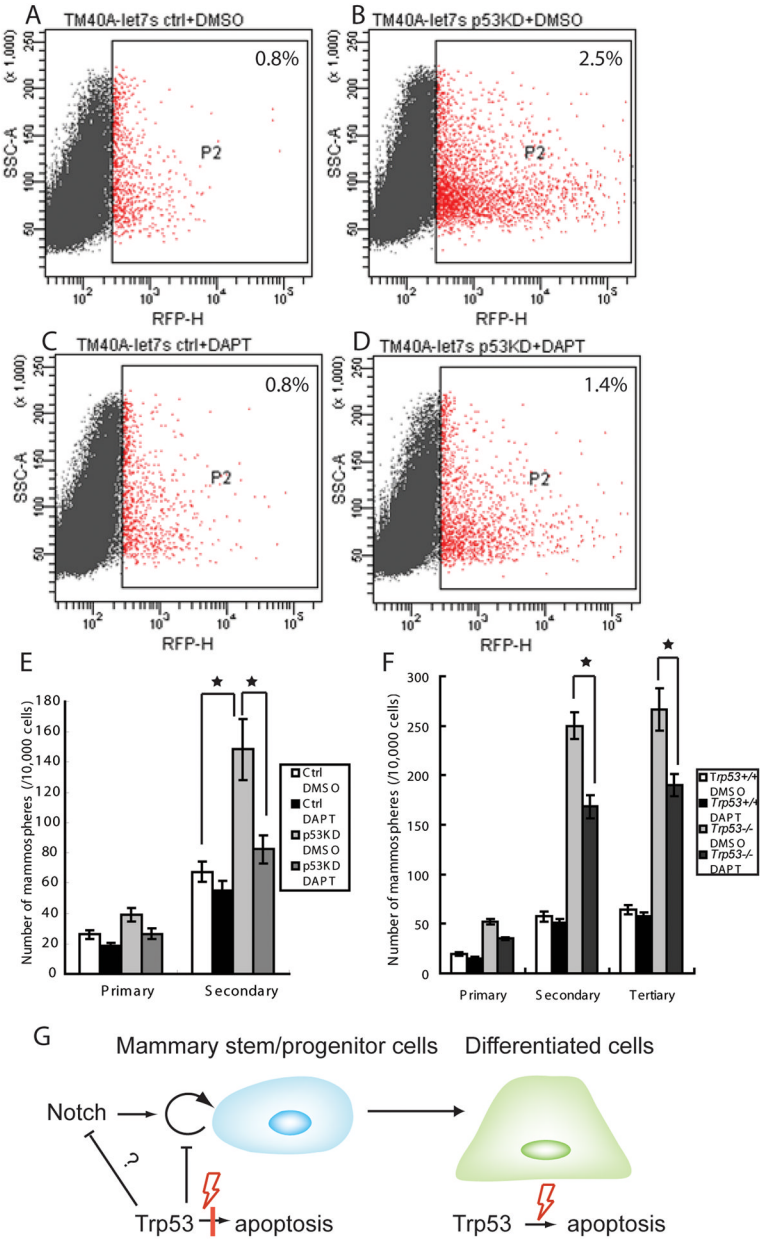

Fig. 5.

Inhibition of mammary stem/progenitor cells with γ-secretase inhibitor. (A-D) TM40A-let7^{S}-p53KD cells contained more DsR^{+} progenitor cells compared to the TM40A-let7^{S}-ctrl cells. The expansion of DsR^{+} progenitors in TM40A-let7^{S}-p53KD cells can be inhibited by the treatment of DAPT, while the DsR^{+} cells in TM40A-let7^{S}-ctrl cells were not affected. (E) TM40A-let7^{S}-p53KD cells gave rise to significantly more secondary mammospheres than control cells ($p<0.01$). The number of p53KD mammospheres decreased significantly with the treatment of 5μM DAPT ($p<0.01$), while the number of control mammospheres were not affected ($p>0.05$). (F) Mammospheres were treated with either 5μM DAPT or DMSO control during serial passages. The number of *Trp53−/−* mammospheres decreased significantly after the treatment of DAPT ($p<0.01$). The number of *Trp53+/+* mammospheres was not changed with DAPT treatment. (G) Model of p53 tumor suppression function in different cell types. p53 restricts the self-renewal of mammary stem/

progenitor cells; however the p53-mediated apoptosis response is compromised in these cells. In the differentiated cells, the p53-mediated apoptosis pathway becomes functional. Notch may be inhibited by basal levels of p53 in mammary stem/progenitor cells. Insufficient p53 can result in increased Notch activity which leads to the expansion of mammary stem/progenitor cells.