

Award Number:
W81XWH-09-1-0028

TITLE:
Regulation of Mammary Progenitor Cells by p53 and Parity

PRINCIPAL INVESTIGATOR:
Luwei Tao MD, PhD candidate
D. Joseph Jerry PhD

CONTRACTING ORGANIZATION:
University of Massachusetts-Amherst
Amherst, Massachusetts, 01003

REPORT DATE:
January 2010

TYPE OF REPORT:
Annual Summary report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

✓ Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE: 01-01-2010		2. REPORT TYPE: Annual Summary Report		3. DATES COVERED (From - To) 1 Jan 2009-31 Dec 2009	
4. TITLE AND SUBTITLE Regulation of Mammary Progenitor Cells by p53 and Parity				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0028	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Luwei Tao, D. Joseph Jerry				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Massachusetts-Amherst Amherst, Massachusetts, 01003				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S.Army Medical Research and Material Command Fort Detrick, Maryland, 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Breast cancer is the most frequent cancer among women in the United States. A full term pregnancy early in reproductive life can reduce breast cancer incidence in women by up to 50% and p53, an important tumor suppressor gene, was shown to be a major effector for this protection effect. We hypothesized that p53 may negatively regulate the proliferation and self-renewal of mammary stem/progenitor cells and that the increased p53 in parous gland may limit mammary stem/progenitor cells population and reduce the transformation risk. Mammosphere assays showed that decreased p53 dosage in <i>Trp53</i> ^{+/-} and <i>Trp53</i> ^{-/-} mice led to increased numbers and size of mammospheres. Meanwhile, the number of secondary and tertiary mammospheres was not affected by (IR) regardless of their genotype. The cell cycle analysis also showed that the <i>Trp53</i> ^{-/-} mammospheres have higher proliferation rate than <i>Trp53</i> ^{+/-} spheres. Serial dilution and transplantation experiments also showed that the <i>Trp53</i> ^{-/-} epithelium had significantly increased frequency of long-term regenerative MaSCs compared to <i>Trp53</i> ^{+/+} epithelium. The BrdU labeling experiment showed that <i>Trp53</i> ^{-/-} mammary gland contains less label-retaining epithelial cells (LRECs) than <i>Trp53</i> ^{+/+} epithelium. Our data suggest that p53 negatively regulates the self-renewal of mammary stem cells. The MaSCs pool expands with decreased p53 dosage, which may result in a higher transformation risk. Meanwhile, the p53-mediated apoptosis pathway is compromised in the mammosphere-initiating cells.					
15. SUBJECT TERMS p53, parity, stem cells, progenitor cells, ionizing radiation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

Page

Introduction.....	4
Body.....	5-7
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusion.....	10
References.....	11-12
Appendices.....	13-16

Introduction

Breast cancer is the most frequent cancer among women in the United States¹. Understanding the biological behavior of mammary stem cells (MaSCs) and progenitor cells sheds light on the mechanism of mammary gland malignant transformation^{2,3}. A full term pregnancy early in reproductive life can reduce breast cancer incidence in women by up to 50%⁴. The research in our lab has shown that the p53 tumor suppressor pathway is a crucial downstream effector for this protection mechanism^{5,6}. Parous mammary epithelium showed significantly higher apoptotic response to ionizing radiation (IR) which was p53-dependent^{7,8}. Recent research showed p53 might also participate in the regulation of tissue-specific stem cells^{9,10}. In this project, we proposed that p53 negatively regulates the proliferation and self-renewal of mammary stem/progenitor cells. The increased p53 activity in parous gland may reduce the mammary progenitor/stem cell pool resulting in decreased risk of mammary tumors. For the past year, we focused on the role of p53 in the regulation of mammary stem/progenitor cells. The mammosphere assay showed that basal level p53 negatively regulated the proliferation and self-renewal of mammary stem/progenitor cells upon serial passages. Meanwhile the ionizing radiation (IR) treatment did not change the number of secondary or tertiary spheres, which suggested that the p53-mediated apoptosis pathway may be compromised in the mammary stem/progenitor cells. We also used serial dilution and transplantation experiment, which was not proposed initially, to determine the frequency of real long-term regenerative stem cells in mammary gland. We found that the *Trp53*^{-/-} epithelium had significantly increased frequency of long-term regenerative MaSCs compared to *Trp53*^{+/+} epithelium. The BrdU labeling experiment was used to see if p53 can potentially regulate the cell division and distribution of chromosome during mitosis of mammary stem/progenitor cells. We showed that *Trp53*^{-/-} mammary gland contains less label-retaining epithelial cells (LRECs) than *Trp53*^{+/+} epithelium. In conclusion, our data suggest that p53 negatively regulates the self-renewal of mammary stem cells. The MaSCs pool expands with decreased p53 dosage, which may results in a higher transformation risk. Meanwhile, the p53-mediated apoptosis pathway is compromised in the mammosphere-initiating cells, suggesting different functional status of p53 in cells of different cell differentiation stage.

Body

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 for 11 generations as described before. Wild type 3 weeks old BALB/cj recipient mice for transplantation were bought 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) supplemented with 5% Fetal Bovine serum (Gibco), 2mg/ml collagenase (Worthington), 100u/ml hyaluronidase (Sigma), 100u/ml pen/strep (Gibco) and 100µg/ml gentamicin (Gibco) for 6 hours. Cell pellet was collected and further dissociated with 1ml pre-warmed 0.05% Trypsin-EDTA (Gibco) and 200µl 1mg/ml DNase I (Roche). Cell suspensions were sieved through a 40µm cell strainer to obtain a single cell suspension.

Mammosphere culture

Primary single cells were seeded into ultralow 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), 4ug/ml heparin (Sigma), 100u/ml Pen/Strep (Gibco), 5µg/ml gentamicin (Gibco)¹¹. 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 10min. 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 ionizing responses of ionizing responses, single cell suspensions received either a 0Gy or 5Gy dose of γ -radiation from a cesium-137 source before being plated.

Limiting dilution and transplantation

Primary mammary epithelial cells 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^{12,13}. *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.

Label retaining cells

3 weeks old BALB/c-*Trp53*^{+/+} and BALB/c-*Trp53*^{-/-} mice were injected with BrdU 300µg/10g body weight for 7 days^{14,15}. 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) and the whole slides were counted for the total epithelial cells (7000 ~ 14000 cells per slides) and LRECs.

Propidium Iodide (PI) staining for cell cycle analysis

Primary mammospheres were passaged 7 days after culture and the secondary mammosphere cells were harvested and trypsinized to get single cell suspension 3 days after culture. The cell suspension was immediately fixed with 7 volume of 100% ethanol and stored in 4°C. For PI staining, cells were pellet down and stained in PBS supplemented with 0.1% glucose, 10µg/ml

RNase A (Sigma) and 50mg/ml PI (Sigma) for 30min at 37°C. Cells were pellet down again to stop the staining process and resuspended in 200µl PBS with 0.1% BSA and 2mM EDTA for analysis. All the cell cycle analysis data were collected with the LSRII flow cytometer facility from BD biosciences and analyzed with ModFit software.

p53 IHC staining

Secondary mammospheres were pellet down 6 hours after 0Gy or 5Gy γ -radiation and fixed with 100% ice-cold methanol for 10 min. Fixed mammospheres were resuspended in 2% melted agarose in PBS and further embedded in paraffin for sectioning and staining. Slides were stained using CM5 anti-mouse p53 antibody (1:800) and DAB staining kit (DAKO). *Trp53*^{-/-} mammospheres were using as negative control.

Results

Effect of p53 genotype on number and size of mammospheres

Both *Trp53*^{+/-} and *Trp53*^{-/-} mammary epithelium gave rise to similar mammospheres as *Trp53*^{+/+} epithelium under ultralow attachment culture condition. The existence of longer-regenerative MaSCs in the mammospheres was confirmed by transplantation of secondary mammospheres into the cleared fat pad. Upon serial passages, *Trp53*^{-/-} and *Trp53*^{+/-} epithelial cells gave rise to significantly higher number of secondary and tertiary mammospheres than wild type epithelium ($P < 0.01$) and the number of *Trp53*^{-/-} mammospheres is higher than *Trp53*^{+/-} ($P < 0.05$) (Figure 1a), suggesting an increased self-renewal rate of mammospheres-initiating cells with decreased p53 dosage. Meanwhile, the decreased p53 also led to increased size of mammospheres, suggesting a higher proliferation rate (Figure 1b), suggesting a higher proliferation rate.

The effect of IR on the number of mammospheres of different p53 genotype

Ionizing radiation (IR) is commonly used to cause double strand break, which induces preferentially p53-dependent cell cycle arrest and apoptosis. Upon serial passages, a portion of mammosphere cell suspensions were treated with 5-Gy γ -radiation and seeded parallelly to the untreated cell suspensions so as to test how the mammosphere-initiating cells respond to IR. Surprisingly, of all the three different *Trp53* genotypes, the number of secondary or tertiary mammospheres was not affected by IR (Figure 2), suggesting that the mammosphere-initiating cells are resistant to IR and that the p53-mediated apoptosis pathway is compromised in these cells.

Limiting dilution and transplantation

The limiting dilution and transplantation experiment was done to estimate the frequency of long-term regenerative stem cells. Total mammary cells were isolated from 8-10 weeks old aged-matched BALB/c-*Trp53*^{+/+} and *Trp53*^{-/-} donor mice (10 for each genotype). Recipients were 3 weeks old wild type BALB/cj mice. Total 74 recipients were done. The outgrowth that occupied $>5\%$ of fat pad was regarded as a successful outgrowth. The ratio of successful events for each concentration group was organized in Figure3a. Both *Trp53*^{+/+} and *Trp53*^{-/-} epithelium gave rise to normal-looking outgrowth (Figure 3c,d). The extent of each fat pad being filled was shown in Figure3b. It is noticeable that, in the regenerated glands, *Trp53*^{-/-} outgrowth showed higher percentage of occupation than the wild type outgrowth.

The number of outgrowth for both genotypes was organized as shown in Table1. The frequency

of long-term regenerative stem cells was estimated and compared by single-hit Poisson distribution model (L-Cal software Stemcell Tech). The frequency of regenerative stem cells in BALB/c-*Trp53*^{+/+} epithelium was 1 per 8,085 with $\pm$ 1S.E. range of 6,508 ~ 10,045. In BALB/c-*Trp53*^{-/-} epithelium, the frequency of long-term regenerative stem cell was 1 in 2,445 with $\pm$ 1S.E. range of 2,033 ~ 2,940. Compared to *Trp53*^{+/+} epithelium, the *Trp53*^{-/-} epithelium contained significantly higher frequency of regenerative stem cells ($P < 0.01$), suggesting that basal level p53 negatively regulates the self-renewal of mammary stem cells and that the insufficient dosage of p53 leads to an expansion of mammary stem cells pool.

Label retaining cells

The non-random segregation of chromatids was reported in both embryonic stem cells and multilineage progenitor cells. It has been postulated that the tissue specific stem cells can maintain their “stemness” and protect themselves from mutation through asymmetric segregation of their template DNA strands. LRECs have been reported in mammary gland by using either ³HTdR or BrdU labeling. It is found that the LRECs can divided asymmetrically and retain their template DNA strands. We labeled our BALB/c-*Trp53*^{+/+} and *Trp53*^{-/-} mice with BrdU at 3 weeks old and chased for 9 weeks. Interestingly, the *Trp53*^{-/-} mammary glands contained significantly less LRECs ($1.26 \pm 0.09\%$) than the *Trp53*^{+/+} mammary glands ($2.56 \pm 0.18\%$) ($P < 0.01$, Figure 4a). The BrdU-retaining cells were found in both luminal and basal compartments and the distribution of LRECs were similar among both genotypes (Figure 4b, c).

The PI staining for the cell cycle analysis

PI staining and cell cycle analysis were used to compare the proliferation rate of *Trp53*^{+/+} and *Trp53*^{-/-} mammosphere cells. The *Trp53*^{-/-} mammospheres contained significant higher number of S phase cells ($11.395 \pm 2.04\%$) than *Trp53*^{+/+} mammospheres ($8.26 \pm 1.65\%$, $p < 0.05$), suggesting a higher proliferation rate with the absence of p53. The *Trp53*^{+/+} mammospheres showed greater variation of G2 phase cells ($5.8 \pm 4.75\%$) than *Trp53*^{-/-} mammospheres (8.24 ± 1.78), which showed no statistical difference. (Figure 5)

p53 IHC staining

Trp53^{+/+} and *Trp53*^{-/-} mammospheres treated with 5-Gy of γ -irradiation were stained with CM5 antibody for total p53. This is the method that our lab has been using for p53 staining. However, we did not see typical nucleus p53 staining. Most of the staining stays in the cytosole for the *Trp53*^{+/+} mammospheres. The *Trp53*^{-/-} mammospheres staining were much lighter, which may suggest that the staining we saw in the wild type spheres could be specific. (Figure 6) But we definitely still need to optimize the staining conditions and try different antibodies.

Key Research Accomplishments

- Dec 2008- Apr 2009 Mammospheres of different p53 phenotype were cultured for counting number and measuring size. (Finished)
- Apr 2009- Jun 2009 Mammospheres of different p53 phenotype were irradiated to compare the IR response of mammospheres. (Finished)
- May 2009- Oct 2009 PI staining and cell cycle analysis were used to compare the proliferation rate of *Trp53*^{+/+} and *Trp53*^{-/-} mammospheres. (Finished)
- Sept 2009- Nov 2009 Mammospheres of different p53 genotype were irradiated and fixed for p53 staining using CM5 antibody. (Finished)
- Jun 2009- Dec 2009 Mammary epithelial cells of *Trp53*^{+/+} or *Trp53*^{-/-} were injected into wild type host mice cleared fat pad for limiting dilution and transplantation to compare the frequency of real mammary stem cell. (Finished)
- Jun 2009- Jan 2010 Label-retaining cells experiments and BrdU staining were done on *Trp53*^{+/+} and *Trp53*^{-/-} mice to see if loss of p53 changes the segregation of chromosome. (Finished)

Reportable outcomes:

1. Mammosphere assay to compare self-renewal and proliferation of *Trp53*^{+/+}, *Trp53*^{+/-} and *Trp53*^{-/-} mammospheres.
2. The effect of irradiation on mammospheres of different p53 genotype.
3. Limiting dilution and transplantation experiment of *Trp53*^{+/+} and *Trp53*^{-/-} mammary epithelial cells to determine the frequency of long-term regenerative mammary stem cells.
4. BrdU labeling to compare the frequency of label-retaining cells in *Trp53*^{+/+} and *Trp53*^{-/-} mammary glands.

Conclusion

The importance of p53 in breast cancer is highlighted by the dramatic increase of breast cancer risk among women with Li-Fraumeni syndrome. Although the function of activated p53 in mammary epithelium has been extensively studied, its role at basal level under normal conditions is not fully understood, largely due to the extreme low protein level. The comparison of transgenic mice with different *Trp53* genotype allows us to study the function of basal level p53 in mammary epithelium.

Both the mammosphere assay and the limiting transplantation assay data showed that there is increased amount of mammary stem/progenitor cell in the *Trp53*^{-/-} mice mammary gland. Cell cycle analysis also showed that the *Trp53*^{-/-} mammospheres have a higher proliferation rate than the wild type mammospheres. These data suggested that basal level of p53 negatively regulates the self-renewal and proliferation of mammary stem cell. A significant portion of mammary tumors originated from different *Trp53* transgenic mice tumor models showed similar expression pattern as human basal-like tumors, a subtype that is hypothesized to originate from mammary stem/progenitor cells¹⁶⁻¹⁸. It is possible that the expanded MaSCs pool resulted from dysfunctional p53 may become a group of cells of high transformation risk, especially considering their long life span and the ability to give rise to multiple lineages of differentiated cells¹⁸. The increased number of *Trp53*^{+/-} mammospheres also suggested a potential haploinsufficiency of basal p53 dosage for the regulation of mammary stem/progenitor cells, although the difference may be more subtle and require more mice to confirm.

The existence of LRECs in mammary epithelium could be due to either quiescent cells that stop proliferating or the asymmetric segregation of chromatids. Smith reported that by using ³HTdR as the first label for LRECs and BrdU as secondary label for recently proliferating cells, most LRECs were actively synthesizing DNA yet retained their ³HTdR labeled strands, suggesting that asymmetrically dividing cells contribute to most of LRECs^{14,15}. However, the contribution of quiescent cells still can not be ruled out. In our experiment, the frequency of LRECs in wild type mice after 9 weeks chasing was 2.56±0.18%, which is close to Smith reported by using ³HTdR. Interestingly, we showed that *Trp53*^{-/-} epithelium contained less LRECs than wild type epithelium. One simple explanation is that the *Trp53*^{-/-} epithelium contains less quiescent cells than wild type, which is possible considering the important role of p53 in regulation of cell cycle checkpoint and senescence. Another rather tempting explanation is that p53 may regulate the partition of sister chromatids during mitosis, which could be decisive for the fate of daughter cells¹⁹. We showed that p53 negatively regulated MaSCs self-renewal and that *Trp53*^{-/-} epithelium contained more long-term regenerative MaSCs than wild type epithelium. It will be interesting to discuss whether p53 is also involved in the non-random segregation of chromatides during mitosis.

Woodward et al reported that the mammary progenitor cells are more resistant to IR than differentiated cells²⁰. We also showed that the mammosphere-initiating cells are resistant to IR and the number of secondary or tertiary mammospheres were not changed after radiation. We also used the p53 staining protocol that worked in regular tissue to stain irradiated mammospheres and we did not get typical nucleus staining. It is reported that ES cells could not activate p53-dependent stress response and that the p53 protein in ES cells were cytoplasmic and translocated ineffectively into nucleus^{21,22}. It is possible that similar mechanism may exist in

the mammary stem/progenitor cells to protect the body from losing the cells to DNA damage.

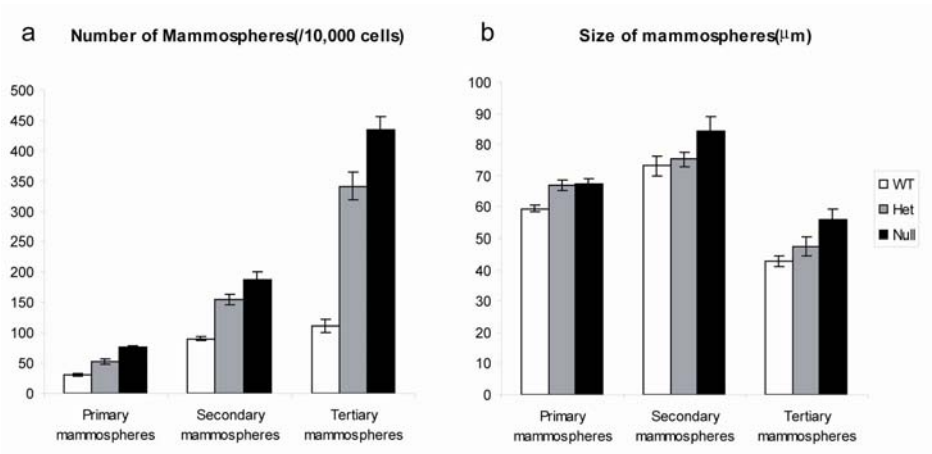
References

1. Jemal, A. *et al.* Cancer statistics, 2007. *CA Cancer J. Clin.* **57**, 43-66 (2007).
2. Shackleton, M. *et al.* Generation of a functional mammary gland from a single stem cell. *Nature* **439**, 84-88 (2006).
3. Stingl, J. *et al.* Purification and unique properties of mammary epithelial stem cells. *Nature* **439**, 993-997 (2006).
4. Rosner, B., Colditz, G. A. & Willett, W. C. Reproductive risk factors in a prospective study of breast cancer: the Nurses' Health Study. *Am. J. Epidemiol.* **139**, 819-835 (1994).
5. Jerry, D. J. *et al.* A mammary-specific model demonstrates the role of the p53 tumor suppressor gene in tumor development. *Oncogene* **19**, 1052-1058 (2000).
6. Jerry, D. J. Roles for estrogen and progesterone in breast cancer prevention. *Breast Cancer Res.* **9**, 102 (2007).
7. Becker, K. A. *et al.* Estrogen and progesterone regulate radiation-induced p53 activity in mammary epithelium through TGF-beta-dependent pathways. *Oncogene* **24**, 6345-6353 (2005).
8. Dunphy, K. A., Blackburn, A. C., Yan, H., O'Connell, L. R. & Jerry, D. J. Estrogen and progesterone induce persistent increases in p53-dependent apoptosis and suppress mammary tumors in BALB/c-Trp53^{+/-} mice. *Breast Cancer Res.* **10**, R43 (2008).
9. Meletis, K. *et al.* p53 suppresses the self-renewal of adult neural stem cells. *Development* **133**, 363-369 (2006).
10. Dumble, M. *et al.* The impact of altered p53 dosage on hematopoietic stem cell dynamics during aging. *Blood* **109**, 1736-1742 (2007).
11. Dontu, G. *et al.* In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev.* **17**, 1253-1270 (2003).
12. Stingl, J., Eaves, C. J., Kuusk, U. & Emerman, J. T. Phenotypic and functional characterization in vitro of a multipotent epithelial cell present in the normal adult human breast. *Differentiation* **63**, 201-213 (1998).
13. Siwko, S. K. *et al.* Evidence that an early pregnancy causes a persistent decrease in the number of functional mammary epithelial stem cells--implications for pregnancy-induced protection against breast cancer. *Stem Cells* **26**, 3205-3209 (2008).
14. Booth, B. W., Boulanger, C. A. & Smith, G. H. Selective segregation of DNA strands persists in long label retaining mammary cells during pregnancy. *Breast Cancer Res.* **10**, R90 (2008).
15. Smith, G. H. Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. *Development* **132**, 681-687 (2005).
16. Herschkowitz, J. I. *et al.* Identification of conserved gene expression features between murine mammary carcinoma models and human breast tumors. *Genome Biol.* **8**, R76 (2007).

17. Sorlie, T. *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc. Natl. Acad. Sci. U. S. A* **98**, 10869-10874 (2001).
18. Stingl, J. & Caldas, C. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nat. Rev. Cancer* **7**, 791-799 (2007).
19. Armakolas, A. & Klar, A. J. Cell type regulates selective segregation of mouse chromosome 7 DNA strands in mitosis. *Science* **311**, 1146-1149 (2006).
20. Woodward, W. A. *et al.* WNT/beta-catenin mediates radiation resistance of mouse mammary progenitor cells. *Proc. Natl. Acad. Sci. U. S. A* **104**, 618-623 (2007).
21. Lin, T. *et al.* p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. *Nat. Cell Biol.* **7**, 165-171 (2005).
22. Qin, H. *et al.* Regulation of apoptosis and differentiation by p53 in human embryonic stem cells. *J. Biol. Chem.* **282**, 5842-5852 (2007).

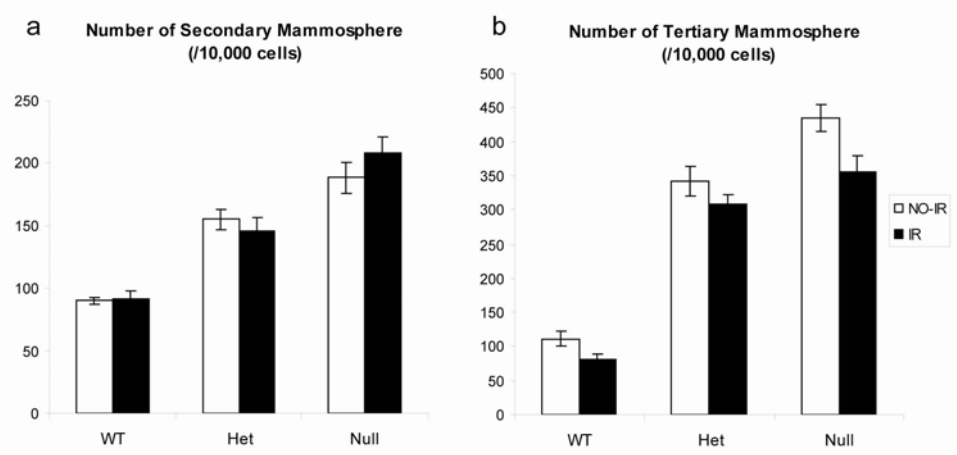
Appendices and Supporting data

Figure1.



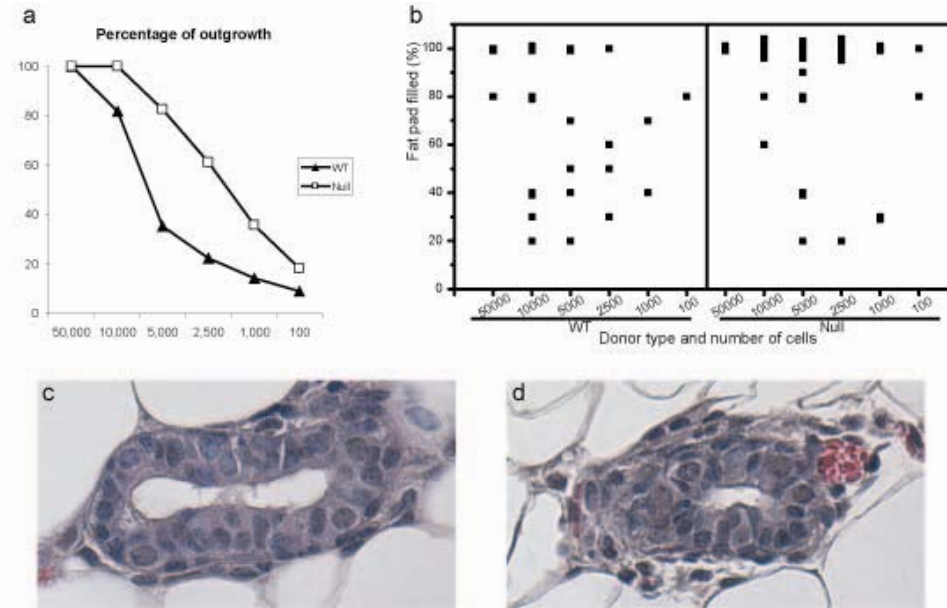
Mammosphere assay of BALB/c-*Trp53*^{+/+}, *Trp53*^{+/-} and *Trp53*^{-/-} mice. (a) Number of mammospheres upon serial passage. (b) Size of mammospheres upon serial passage.

Figure2.



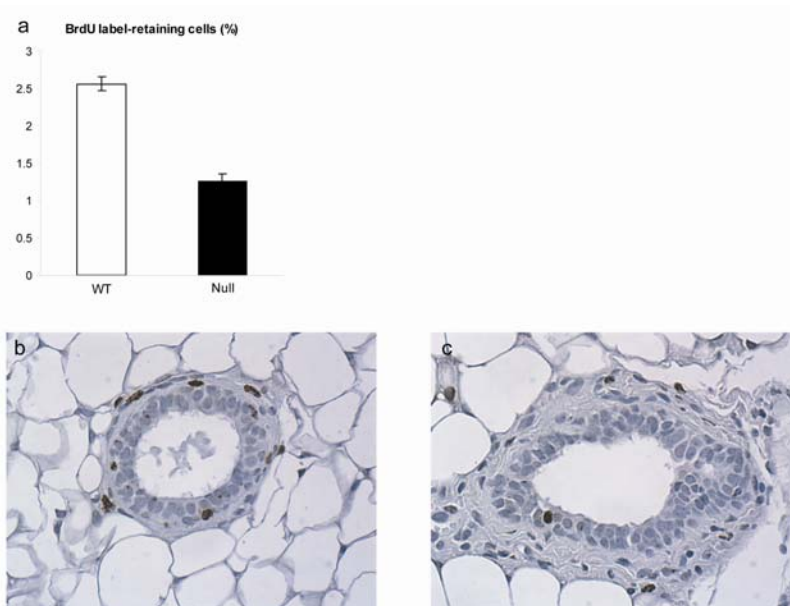
Effect of Ionizing Radiation (IR) on number of mammospheres. (a,b) Number of secondary and tertiary mammospheres of different *Trp53* genotype was not changed with IR treatment.

Figure3.



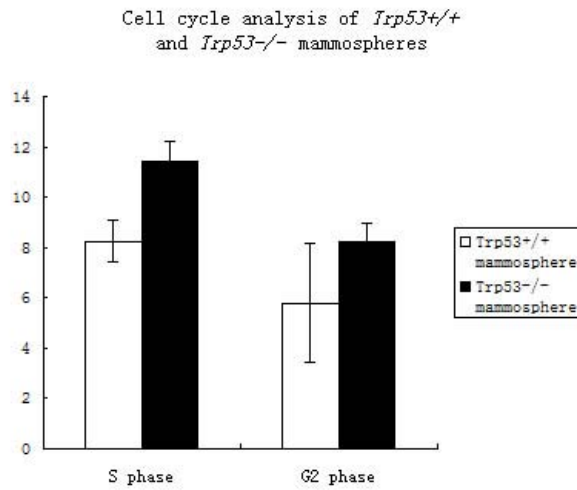
Limiting dilution and transplantation of epithelium from BALB/c-*Trp53*^{+/+} and *Trp53*^{-/-} mice. (a) The percentage of successful outgrowth events in each concentration group. (b) The extend of each fat pad filled by the outgrowth. (c) HE staining of a *Trp53*^{+/+} outgrowth. (d) HE staining of a *Trp53*^{-/-} outgrowth.

Figure4.



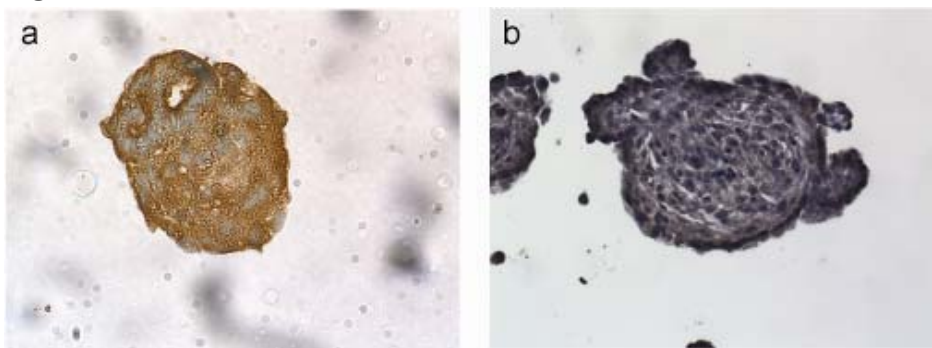
LRECs in *Trp53*^{+/+} and *Trp53*^{-/-} mice mammary glands. (a) Frequency of LRECs in BALB/c-*Trp53*^{+/+} and *Trp53*^{-/-} mice mammary glands. (b) BrdU staining of a *Trp53*^{+/+} mammary gland. (c) BrdU staining of a *Trp53*^{-/-} mammary gland.

Figure 5.



Cell cycle analysis of *Trp53*^{+/+} and *Trp53*^{-/-} mammosphere cells.

Figure 6.



p53 staining of irradiated mammospheres. (a) p53 staining of *Trp53*^{+/+} mammosphere. (b) p53 staining of *Trp53*^{-/-} mammosphere.

Table 1.

Cell number	Mice number	<i>Trp53</i> ^{+/+}	<i>Trp53</i> ^{-/-}
50,000	3	3	3
10,000	11	9	11
5,000	17	6	14
2,500	18	4	11
1,000	14	2	5
100	11	1	2
Frequency		1 in 8,8085	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	

Mammary cells isolated from age-matched *Trp53*^{+/+} and *Trp53*^{-/-} mice were injected into wild type mice cleared fat pad. The number of tested mice and successful events were indicated in the table. The frequency of long-term regenerative mammary stem cells was estimated using L-cal software from Stemcell Tech.