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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

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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 \(Trp53^{-/-}\) and \(Trp53^{-/-}\) 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 \(Trp53^{-/-}\) mammospheres have higher proliferation rate than \(Trp53^{+/+}\) spheres. Serial dilution and transplantation experiments also showed that the \(Trp53^{-/-}\) epithelium had significantly increased frequency of long-term regenerative MaSCs compared to \(Trp53^{+/+}\) epithelium. The BrdU labeling experiment showed that \(Trp53^{-/-}\) mammary gland contains less label-retaining epithelial cells (LRECs) than \(Trp53^{+/+}\) 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.
Introduction

Breast cancer is the most frequent cancer among women in the United States\(^1\). 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) and100ug/ml gentamicin (Gibco) for 6 hours. Cell pellet was collected and further dissociated with 1ml pre-warmed 0.05% Trpsin-EDTA (Gibco) and 200\(\mu\)l 1mg/ml DNase I (Roche). Cell suspensions were sieved through a 40\(\mu\)m 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), 5\(\mu\)g/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 60\(\mu\)l 1mg/ml DNase I for 10min. Cell suspensions obtained from dissociation were sieved through 40\(\mu\)m 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 \(\gamma\)-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: 50,000/10\(\mu\)l, 10,000/10\(\mu\)l, 5,000/10\(\mu\)l, 2,500/10\(\mu\)l, 1,000/10\(\mu\)l, 100/10\(\mu\)l\(^{12,13}\). \(Trp53^{++}\) 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 300\(\mu\)g/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 mammosospheres 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, 10\(\mu\)g/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 ± 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 ± 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±0.09%) than the Trp53+/+ mammary glands (2.56±0.18%)(P<0.01, Figure4a). 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±2.04%) than Trp53+/+ mammospheres (8.26±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±4.75%) than Trp53/-/ mammospheres (8.24±1.78), which showed no statistical difference. (Figure5)

**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 lost 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 cells. 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 cells. 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. 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 cells. 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 cells. 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. 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


Appendices and Supporting data

Figure 1.

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

Figure 2.

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.
Figure 3.

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 extent of each fat pad filled by the outgrowth. (c) HE staining of a Trp53+/+ outgrowth. (d) HE staining of a Trp53-/- outgrowth.

Figure 4.

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.

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| 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.