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TITLE: The Role of SnoN and Ski in Mammary Epithelial Cell Transformation

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14. ABSTRACT

Ski and SnoN are members of Ski oncoprotein family. Overexpression of Ski or SnoN can induce transformation of chicken embryonic fibroblast. Ski/SnoN is recently identified as a repressor of TGF signaling pathway which is an important tumor suppression pathway at the early stage of tumorigenesis. Higher level of Ski/SnoN is found in transformed mammary epithelial cells. Ski/SnoN might play a role in regulation of the transformation of mammary epithelial cell by antagonizing TGF signaling pathway. In my project, I try to explore the role of Ski/SnoN in the transformation of mammary epithelial cells.

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Introduction:

SnoN, a member of Ski oncoprotein family, is an important repressor of TGFβ signaling pathway (1). SnoN antagonize TGFβ signaling pathway by direct binding to the Smad complex which leads to the repression of TGFβ responsive gene transcription (1, 2). The binding of SnoN to Smad complex can disrupt the interaction of R-Smad and Co-Smad. Furthermore, the binding of SnoN can recruit transcription repressors and release transcription activators. By these ways, the transcription of TGFβ responsive genes is efficiently blocked. However, the role of SnoN in vivo has not been clearly elucidated. In my project, I try to explore the role of SnoN in vivo and determine whether it is TGFβ dependent or not. In order to answer these questions, knock-in mutant SnoN (mSnoN) mice in which the interaction of SnoN and TGFβ pathway is cut off by introducing the mutations in Smad binding sites, have been constructed. These mice allow us to explore the role of SnoN and Smad interaction in vivo and the role of SnoN which is independent of TGFβ pathway.

Body:

In our lab, knock-in mice with mutant SnoN which can't bind to the Smad complex have been constructed. Mutant SnoN can't antagonize TGFβ signaling pathway and becomes more stable due to the defect in Smad dependent degradation of SnoN (3, 4, 5). These mice will allow us to study the role of SnoN in vivo and distinguish whether it is TGFβ dependent or not. MEFs (mouse embryonic fibroblast cells) from mutant mice show a different response in senescence. I hypothesize that SnoN can play a role in the regulation of senescence. Now, I have found that high levels of SnoN can induce premature senescence in cells. The level of SnoN is critical for the regulation of senescence. High level of SnoN can accelerate the senescence response in cells. The regulation of SnoN in senescence seems to be independent of its repression function in TGFβ signaling pathway. Furthermore, SnoN has been found to interact with PML protein and is localized in PML nuclear body in senescent cells. The interaction of SnoN and PML protein is required for activation of p53 pathway which leads to premature senescence in MEFs. Based on the data in MEFs, we have generated a model for SnoN induced senescence. Moreover, more senescent cells are also observed in tissue from mutant mice. These mice are resistant to carcinogen induced tumorigenesis. It further support SnoN can function as a tumor suppressor in vivo by induction of senescence.

Accomplishments in 2006:

1. MEFs from mutant mice show an accelerated or premature senescence (Fig 1).

2. The level of SnoN is critical for the regulation of senescence in MEFs (Fig 2).

3. SnoN induced senescence is TGFβ signaling pathway independent (Fig 2 and 3).

4. p53 pathway is responsible for the premature senescence in mutant MEFs (Fig 4)

5. SnoN can form senescence associated nuclear speckles which are colocalized with PML bodies in senescent cells (Fig 5a).

6. SnoN can directly bind with PML protein and PML protein is required for SnoN induced senescence (Fig 5b).

Accomplishments in 2007

1. Both p53 and PML are required for SnoN induced senescence. PML is critical for the induction of p53 in SnoN induced senescence. A higher level of p53 is found in mutant MEFs when they start to enter senescence. Knocking down p53 in mutant MEFs by shRNA can rescue premature senescence. It suggests p53 is the major contributor for the premature senescence in mutant MEFs. We already know PML is required for premature senescence in mutant MEFs. More interestingly, knock-down PML by shRNA can significantly eliminate the high level of p53 in MEFs. (Fig 5c).

2. Determine the interaction of SnoN and PML protein and mapping the binding site of PML in SnoN.

In transiently transfection experiment, overexpression of SnoN and PML in 293T cells can cause them to interact together. In senescence cells, the endogenous interaction of SnoN and PML is shown as well (Fig 6a and 6b). Co-transfection of different truncation of SnoN with PML allows us to determine whether the binding site of PML in SnoN. The partly deletion of SAND domain in SnoN (SnoN d 322-366) could completely abolish the interaction between SnoN and PML (Fig 6c).

3. Elevated SnoN level in cells causes its colocalization to PML NB but not SAND domain deleted SnoN

Overexpression of SnoN in the early passages of MEFs could cause the formation of SnoN speckles in nuclei which are colocalized with PML nuclear bodies. However, overexperssion of SAND domain deleted SnoN which no longer binds to PML, leads to the localization of SnoN in whole nuclei (Fig 7a). So it supports the interaction of SnoN and PML is necessary for the formation of SnoN speckles in nuclei and colocalization of SnoN with PML nuclear bodies.

4. The interaction of SnoN with PML is required for the induction of p53 and premature senescence in cells.

Overexpression of either SnoN or mSnoN in the early passages also causes the increased level of p53 and premature senescence in MEFs. However, overexpression of SAND domain deleted SnoN fails to premature senescence in MEFs (Fig 7b). It confirms that the interaction between SnoN and PML is essential for the premature senescence.

5. SnoN induced senescence decreases Ras and c-Myc induced transformation in MEFs.

In soft agar assay, the combination of Ras and c-Myc can cause the anchorage independent growth in MEFs. However, less and smaller colonies are formed in mutant MEFs. Accordingly, Ras induced senescence is enhanced in mutant MEFs. So it suggests that premature senescence in mutant MEFs might repress the transformation induced by oncogenes (Fig 8).

6. SnoN induced senescence could also represses the process of skin carcinogenesis induced by the treatment of DMBA and TPA.

In order to confirm the tumor suppression function of premature senescence in mutant mice, carcinogen induced skin tumorigenesis is performed in mice. By treatment of DMBA and TPA, more and bigger papillomas are observed in wild type mice than in mutant mice (Figure is in preparation). It supports that the premature senescence in mutant mice indeed repress the tumorigenesis in vivo.

REPORTABLE OUTCOMES:

1. Manuscript for this project is in preparation since the tumor suppression function of SnoN through senescence is clear.
2. This part of work has been present in Department Retreat in Oct, 2007 and the thesis committee meeting in Dec, 2007.

Summary:

In MEFs with knock-in mSnoN, it has been shown that SnoN itself can induce a premature senescence when highly expressed. The high level of SnoN is able to interact with PML and localized to PML NB. The interaction of SnoN with PML can induce the high level of p53 which is responsible for premature senescence. The premature senescence blocks the transformation of MEFs induced by oncogenic Ras and seems to repress the carcinogen induced tumorigenesis in mice.

Next steps:

1. Confirm that less incidence of tumor in mutant mice treated by DMBA and TPA is due to the enhanced senescence response.
2. Determine whether Sno induced senescence is due to it oncogenic function or tumor suppressor function.
3. Look for the ageing phenotypes in mutant mice which might be correlated with the premature senescence in cells.

Supporting data:

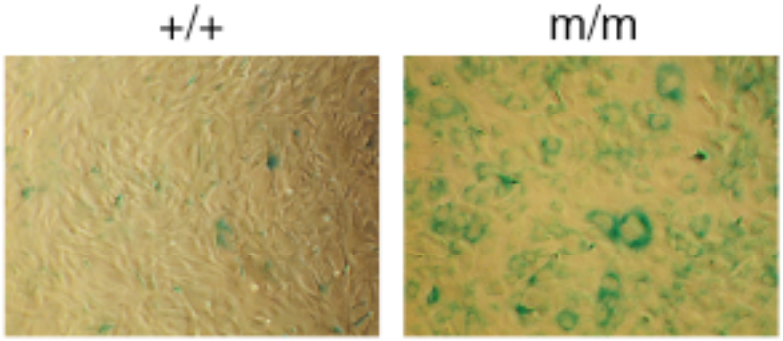

Fig 1: A premature senescence was found by SA β-gal staining (senesecen associated β-gal staining) in MEFs with knock-in mSnoN at passage 6.

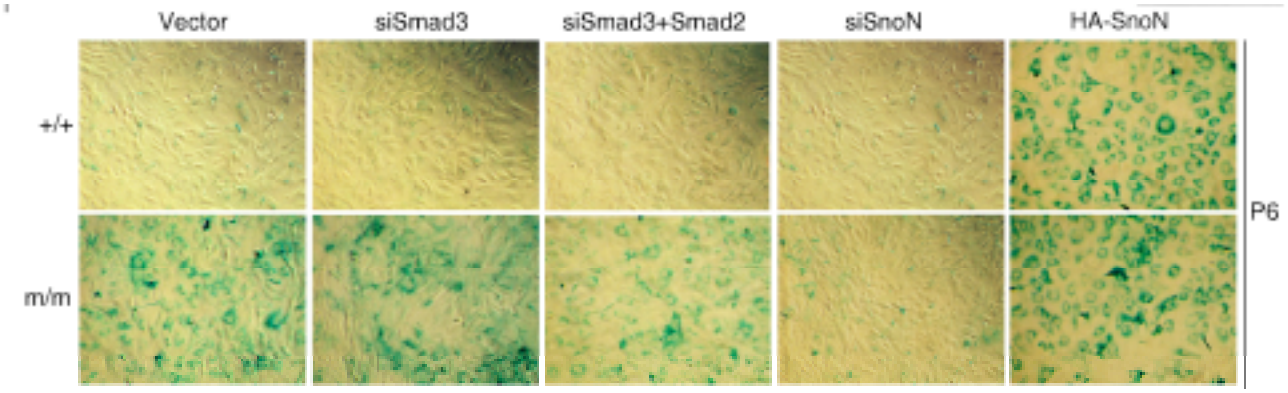

Fig 2: The premature senescence in mutant MEFs can be rescued by knocking down SnoN but not knocking down Smads. Moreover, the premature senescence can be induced in wt MEFs when SnoN is overexpressed. The senescence was shown by SA β-gal staining in wt and mutant MEFs after infection by either vector or shRNA constructs.

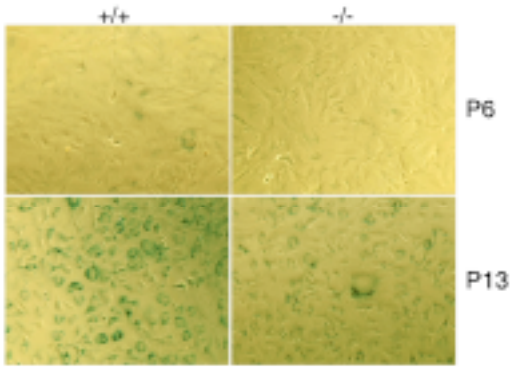

Fig 3: The premature senescence in MEFs with knockout SnoN is delayed. The senescence was shown by SA β-gal staining in wt and knockout SnoN MEFs at passage p6 and p13. It further supports that the role of SnoN in senescence is TGFβ pathway independent.

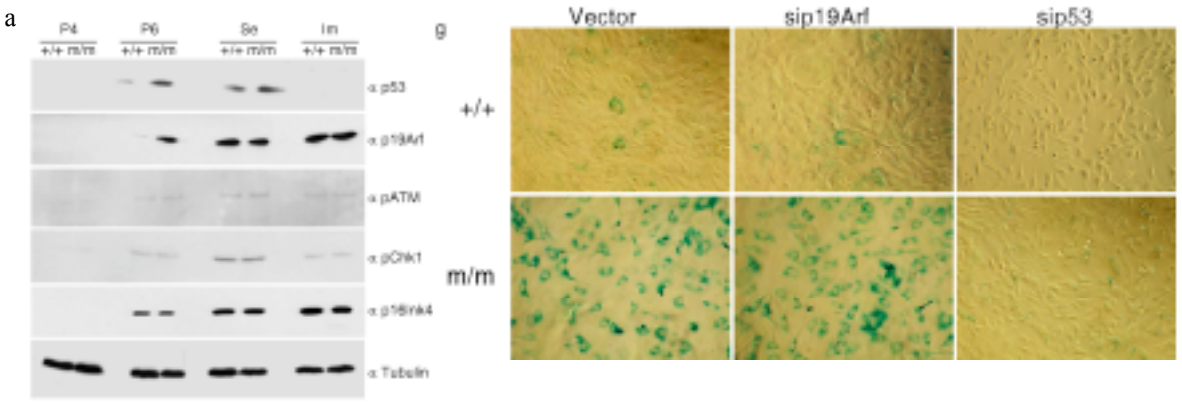

Fig 4: p53 is required for the premature senescence in mutant MEFs. a. The level of p53 and its regulators included p19Arf, phosphor-ATM, phosphor-Chk1 are checked by western blotting in wt and mutant MEFs at different passages. The level of p16Ink4a is checked as well. b. Knocking down p53 by infection of shRNA for p53 in mutant MEFs can rescue the premature senescence but not knocking down p19Arf. The senescence was shown by SA β-gal staining.

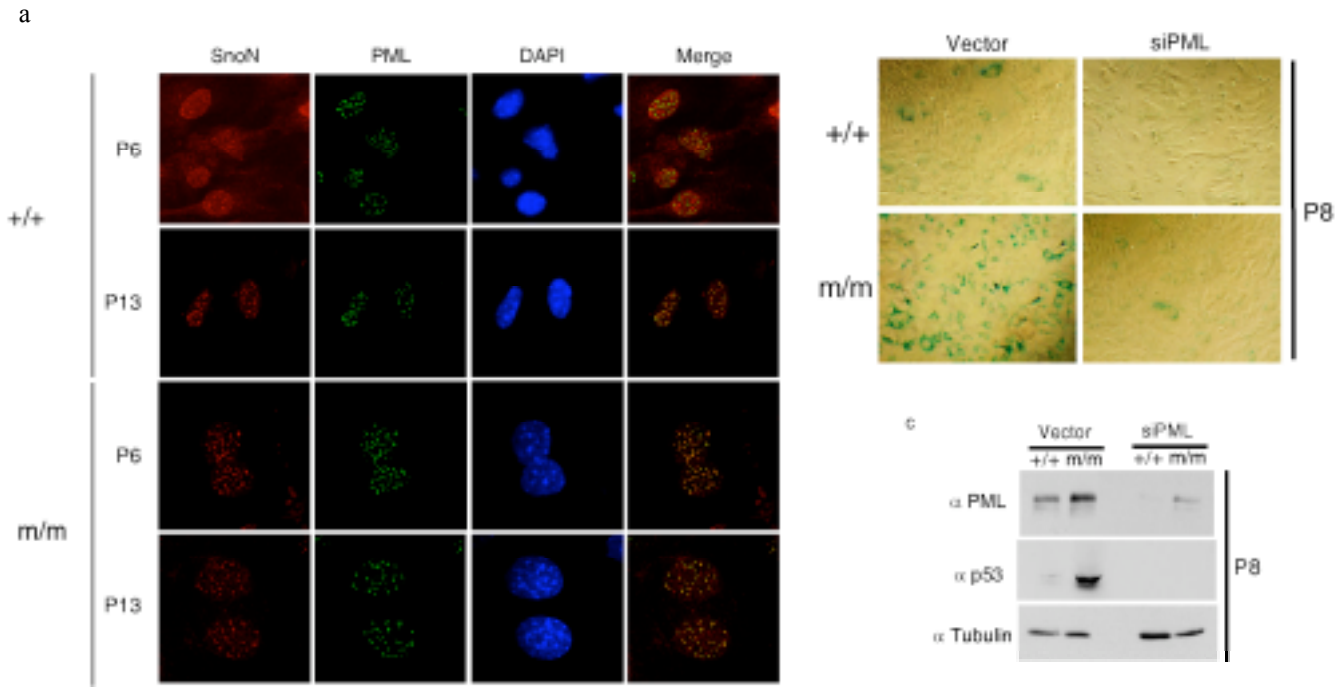

Fig 5: SnoN is colocalized with PML nuclear bodies in senescent cells and PML is required for the premature senescence in mutant MEFs. a. SnoN (Red) and PML (Green) were stained by anti-SnoN or anti-PML antibody in wt and mutant MEFs at different passages. Nuclei were stained by DAPI staining (Blue). b. Knocking down PML by infection of shRNA for PML in mutant MEFs can rescue the premature. The senescence was shown by SA β-gal staining.). c. Knocking down PML by infection of shRNA for PML in mutant MEFs can eliminate the high level of p53 as well.

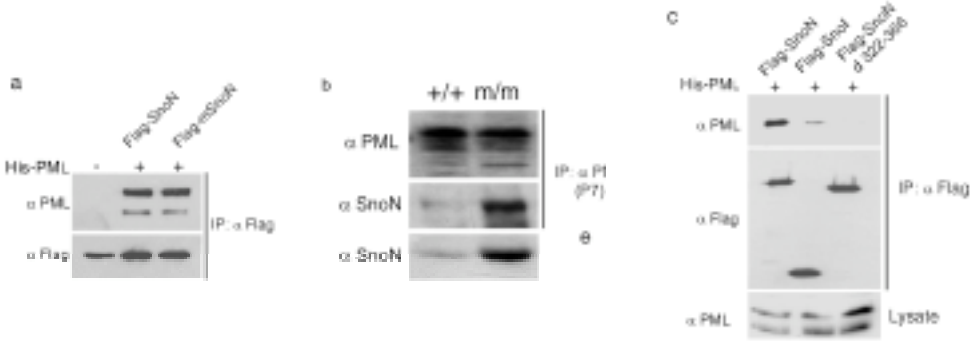

Fig 6: SnoN can directly interact with PML protein in senescent cells. a. Flag tagged SnoN or mSnoN was transiently co-transfected with His tagged mouse PML to 293T cells. Immunoprecipitation (IP) by anti-Flag antibody was used to pull down SnoN or mSnoN. Then, the IP product was checked by anti-Flag and anti-PML antibody. b. IP by anti-PML antibody was performed in +/+ and m/m MEFs at P7. The IP product was checked by anti-PML and anti-SnoN. SnoN level in the lysate was checked as well. c. Flag tagged SnoN, SnoN (1-366), SnoN (366-684) or SnoN d (322-366) was co-transfected with His tagged PML into 293T. IP by anti-Flag antibody was used to pull down SnoN and mutants. The IP products were checked by anti-Flag and anti-PML antibody.

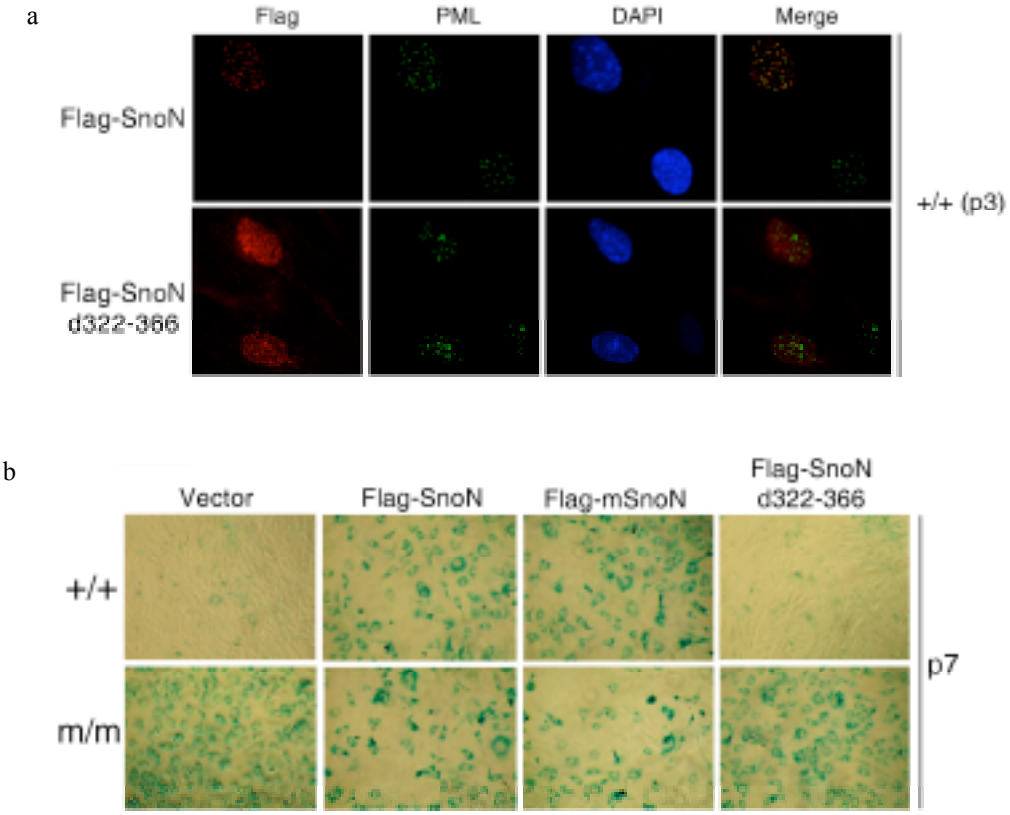

Fig 7: The interaction of SnoN and PML is necessary for the function of SnoN in senescence. a. Immunoflurescent staining by anti-Flag and anti-PML was used to check the localization of SnoN and PML in the infected cells with Flag tagged SnoN or SAND domain deleted SnoN. b. Senescence was analyzed by SA-b-gal staining in the infected cells with Flag tagged SnoN or SAND domain deleted SnoN.

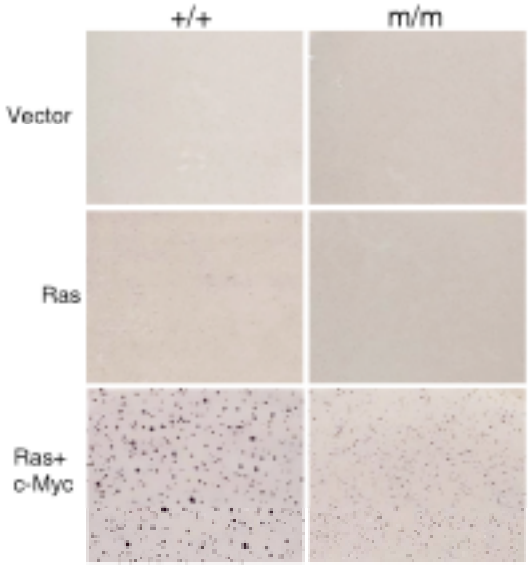

Fig 8: The transformation induced by the overexpression of oncogenes is blocked in mtant MEFs. Either retrovirus based construct with Puromycin selection gene for constitutively activated H-Ras (Q61L) alone or retrovirus based constructs with Neomycin selection gene for c-Myc alone or both were used to infect MEFs at P3. After 24hrs of infection, MEFs were selected by Puromycin or Neomycin or both. The survival cells were cultured in soft agar.

Reference:

1. Luo K. Ski and SnoN: negative regulators of TGF-beta signaling. Curr Opin Genet Dev. 2004;14(1):65-70
2. Wu JW, Krawitz AR, Chai J, Li W, Zhang F, Luo K, Shi Y. Structural mechanism of Smad4 recognition by the nuclear oncoprotein Ski: insights on Ski-mediated repression of TGF-beta signaling. Cell. 2002 Nov 1;111(3):357-67
3. Wan Y, Liu X, Kirschner MW. The anaphase-promoting complex mediates TGF-beta signaling by targeting SnoN for destruction. Mol Cell. 2001;8(5):1027-39
4. Stroschein SL, Bonni S, Wrana JL, Luo K., Smad3 recruits the anaphase-promoting complex for ubiquitination and degradation of SnoN. Genes Dev. 2001 15(21):2822-36
5. Bonni S, Wang HR, Causing CG, Kavsak P, Stroschein SL, Luo K, Wrana JL. TGF-beta induces assembly of a Smad2-Smurf2 ubiquitin ligase complex that targets SnoN for degradation. Nat Cell Biol. 2001 3(6):587-95