**Peer Review File**

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**Review Comments**

1. One major weakness is that there does not seem to be any defined objective for the study. Rather, it seems to simply be the results of an investigation of unexpected tumors that developed while attempting to replicate an experimental model system developed previously.

R: Genetically engineered mouse is an ideal model to advance our understanding of biological behaviors of cancer cells. Establishing an ovarian cancer model induced by *Kras* activation and *Pten* deletion to further study the pathogenesis of ovarian cancer is our original intention. However, these important finding in this study demonstrates that it's crucial to avoid accidental exposure of adenovirus to successfully establish the mouse model of ovarian cancer, otherwise, non-specific genetic manipulations can induce the formation of RMS. Besides, the study points to a potential role of *Kras*/*Pten* mutation combinations in RMS pathogenesis and provides useful clues for its origins. (see Page 2, line 3-5)

Changes in the text: Genetically engineered mice are ideal models to advance our understanding the tumorigenesis of ovarian cancer. Our original objective was to establish an ovarian cancer model induced by *Kras* activation and *Pten* deletion.

2. It should be noted whether the transgenic alleles in the final mice used in the study were homozygous or heterozygous.

R: The transgenic alleles after genetic manipulation are as follows: 1) *Kras* gene is heterozygous, expressing mutant $kras^{G12D}$ and wild-type *Kras* respectively, 2) *Pten* gene is homozygous with complete deletion. (see Page 5, line 23-25)

Changes in the text: Transgenic alleles after genetic manipulation were as follows: 1)

The *Kras* gene was heterozygous, expressing mutant $Kras^{G12D}$ and wild-type *Kras* respectively, 2) The *Pten* gene was homozygous; hence, showed complete deletion.

3. Page 3, line 23: the location should read the University of Iowa (not Lowa).

R: Thank you for your reminder, and we have corrected this mistake in the manuscript. (see Page 4, line 26)
Changes in the text: University of Iowa.

4. Page 3, line 26: The time of adenoviral injection after the hCG injection should be stated.

R: In the revised manuscript, we described the time and order of drug use in detail. After using 5 U of pregnant mare serum gonadotropin for 2 days, 5 U of human chorionic gonadotropin were used to promote synchronized ovulation, and 1.5 days later the animals were anesthetized and a dorsal incision was made to expose the ovaries. (see Page 4, line 27-30)
Changes in the text: After using 5 units (U) of pregnant mare serum gonadotropin (Sigma) for 2 days, 5 U of human chorionic gonadotropin (Sigma) were used to promote synchronized ovulation, and 1.5 days later the animals were anesthetized and a dorsal incision was made to expose the ovaries.

5. The primers used for testing recombination efficiency should be identified or, if used previously, the reference should be provided.

R: We provided the primer sets designed for testing recombination efficiency in the revised manuscript. The primer sets used were as follows: $Kras^{G12D}$ (Forward: GTCTGGAATTCCGCAAGCTA; Reverse: GCACGCAGACTGTAGAGCAG), Recombined $Kras^{G12D}$ (Forward: GTCTTTCCCCAGCACAGTGC; Reverse: CTCTTGCCTACGCCACCAGCTC), *Pten* (Forward:

CAAGCACTCTGCGAACTGAG; Reverse: AAGTTTTTGAAGGCAAGATGC), Recombined *Pten* (Forward: ACTCAAGGCAGGGATGAGC; Reverse: GCTTGATATCGAATTCCTGCAGC). (see Page 5, line 16-22)

Changes in the text: The primer sets used were as follows: $Kras^{G12D}$ (Forward: GTCTGGAATTCCGCAAGCTA; Reverse: GCACGCAGACTGTAGAGCAG), Recombined $Kras^{G12D}$ (Forward: GTCTTTCCCCAGCACAGTGC; Reverse: CTCTTGCCTACGCCACCAGCTC), Pten (Forward: CAAGCACTCTGCGAACTGAG; Reverse: AAGTTTTTGAAGGCAAGATGC), and recombined Pten (Forward: ACTCAAGGCAGGGATGAGC; Reverse: GCTTGATATCGAATTCCTGCAGC).

6. Figure 2: It is unclear how many "subcutaneous lumps" were found. Much of the text, including the Abstract and Introduction indicates one, but the Results section and Figure 2 indicate that there were more (i.e. 8/10 mice). The wording of the text needs to be modified to remove the text that indicates that "a subcutaneous lump accidentally developed". Also, what is the nature of the disease progression associated with the subcutaneous lumps that lead to the mice becoming moribund?

R: We are sorry that the expression has confused you. As stated in the results section, large subcutaneous lumps developed in most of the compound mutant mice carrying LSL-$Kras^{G12D}$ and $Pten^{Fl/Fl}$ (8/10). According to your suggestion, we have modified the texts with a more rigorous expression. (see Page 2, line 14; Page 3, line 24-25; Page 6, line 15; Page 6, line 22; Page 16, line 3)

The animals with the subcutaneous lumps were moribund about five weeks post AdCre administration probably due to the end-stage cachexia and multiple organ dysfunction sydrome under high tumor burden. (see Page 6, line 30 and Page 1, line 1)

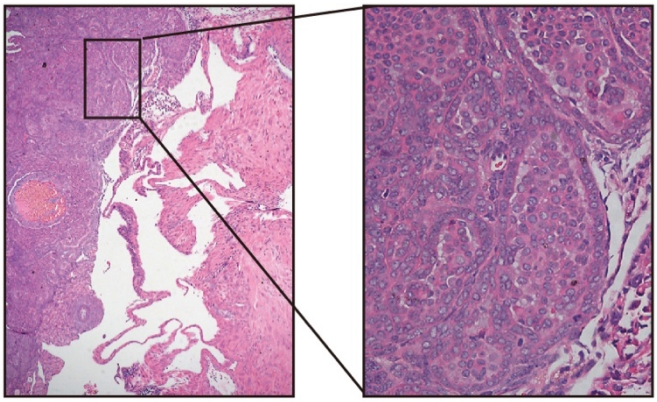
Changes in the text: subcutaneous lumps;

The animals with the subcutaneous lumps were moribund approximately five weeks post AdCre-eGFP administration (range, 4.5-6.5 weeks), probably because of the end-stage cachexia and multiple organ dysfunction syndrome under high tumor burden.

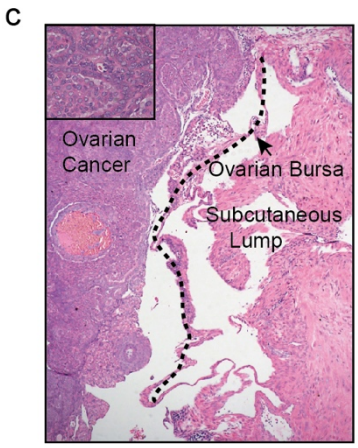
7. In the caption to Figure 2, the “normal” tissues shown in 2a need to be identified. Also, the “typical glandular epithelium of OEAs” described in the text (page 5, line 8) is not apparent in Fig. 1c. Further, the IHC images for Pten and KRas targets shown in 2b and 2d need to include positive and negative controls.

R: All cells of the transgenic mice in this study carried conditional alleles, and we used liver tissue as a normal tissue control. (see Page 16, line 17)

We are sorry that the morphology of glandular ovarian cancer is not clear enough because of the low magnification. In the revised Figure 1c, we provided a partially enlarged picture of ovarian cancer in the upper left corner of the original picture. (see Page 16, line 10) And, here we provided the picture of figure 1c with more details for reference.

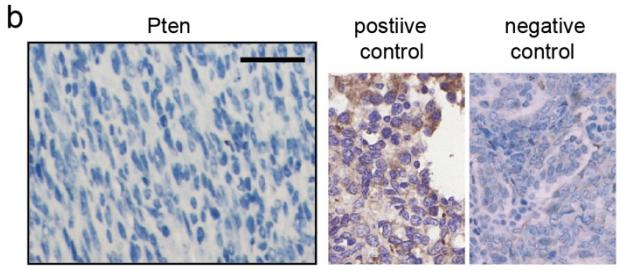


(revised figure 1c)

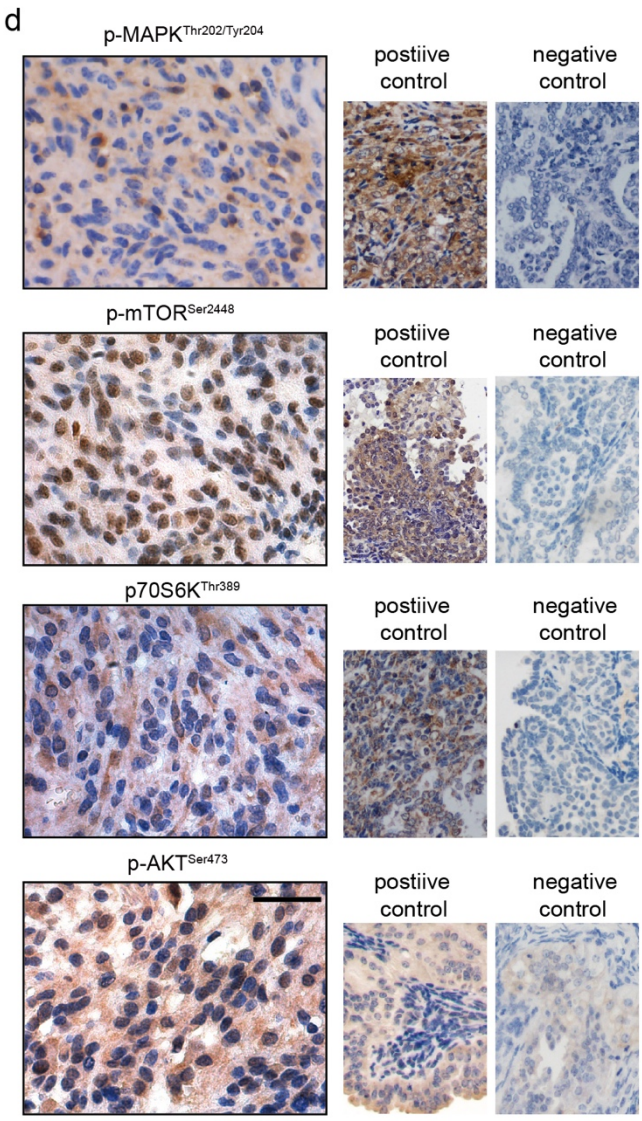



We provided postive and negative controls of *Pten* and *Kras* targets shown in 2b and 2d in the revised Figure 2. (see Page 16, line 19-25)

(revised Figure 2b)




(revised Figure 2d)




Changes in the text:

Figure 2a. T1 and T2 are tumor tissues and N1 and N2 are normal tissues (liver tissue) from the indicated mice (lane 2, 4).

Figure 1c. Upper left: a partially enlarged picture of ovarian cancer.

Figure 2b. Immunohistochemical staining for Pten in the tumor section (left), positive control (ID8 cells allograft), and negative control (recombined *Kras*$^{G12D}$/*Pten* ovarian cancer) (right).

Figure 2d. Immunohistochemical staining for p-MAPK$^{Thr202/Tyr204}$, p-mTOR$^{Ser2448}$, p-S6K$^{Thr389}$, and p-AKT$^{Ser473}$ (left), positive control (recombined *Kras*$^{G12D}$/*Pten* ovarian cancer), and negative control (recombined *Kras*$^{G12D}$/*Pten* ovarian cancer with INK128 treatment for p-mTOR$^{Ser2448}$, p-S6K$^{Thr389}$ and p-AKT$^{Ser473}$, and PD0325901 treatment

for p-MAPK$^{Thr202/Tyr204}$ (right).

8. There is no description of the source of the published data that was used to generate results shown in Figure 4b and 4d, the nature of those datasets, nor any description of how that data was extracted and analyzed.

R: Thank you for your suggestion, and we have added a more detailed description of the data in the revised manuscript. We used the Oncomine database (http://www.oncomine.org) to analyze published transcriptional data of diverse kind of human sarcomas and found that the up-regulation of myoD1 and myogenin were specific in RMS (Fig. 4b). (see Page 8, line 23-24) To reveal genomic alterations of *Kras* and *Pten* in human RMS, we then analyzed their alterations at genomic levels with cBioPortal database (https://www.cbioportal.org/). (see Page 8, line 30 and Page 9, line 1)

Changes in the text: We analyze published transcriptional data of diverse kind of human sarcomas from the Oncomine database (http://www.oncomine.org) and found that the up-regulation of myoD1 and myogenin were specific to RMS (Fig. 4b).

To reveal genomic alterations of *Kras* and *Pten* in human RMS, we then analyzed their alterations at genomic levels using cBioPortal database (https://www.cbioportal.org/) (Fig. 4d).

9. Page 6, line 31: The phrase "the top volume recommended in the previous reports" needs to be referenced.

R: The phrase "the top volume recommended in the previous reports" was referenced in the revised manuscript. (see Page 9, line 14)

Changes in the text: First, the syringes we initially used were too large to easily control the volume injected, which frequently resulted in injecting > 5 μl of the Adcre-eGFP solution, which is the maximum volume recommended in the previous reports (19,22,23).

10. Page 7, line 1: The results associated with the conclusion that “the incidence rate of RMS was significantly decreased” need to be shown. A vague statement is not sufficient to demonstrate that adopting the additional measures was sufficient to reduce the incidence of subcutaneous tumors.

R: The incidence rate of RMS was significantly decreased, showing that 10% (1/10) of mice developed subcutaneous lumps. (see Page 9, line 23)

Changes in the text: With the above improvements to our genetic manipulation protocols, the incidence of RMS was significantly decreased (1/10, 10%).