Author’s response to reviews

Title: A new mouse model to study the role of ectopic Nanos3 expression in cancer

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Our point-by-point response letter to the comments of the reviewers follows here (in small caps).

(Reviewer 1): The authors generated a mouse model in which a human NANOS3 gene can be conditionally activated. Combining with the established non-small cell lung cancer (NSCLC) model, the authors found that ectopic expression of Nanos3 significantly shortened the animal survival and enhanced bronchiolar dysplasia. Interestingly, these effects of Nanos3 ectopic expression appeared to be female specific. Furthermore, using a mouse allograft assay, the authors revealed Nanos3 ectopic expression might promote lymph node metastases of NSCLC cells. Overall, the experiments are carefully executed and the phenotypes are potential interesting. This study provides a new mouse model to study Nanos3 associated NSCLC and even other cancers.

WE APPRECIATE THE OVERALL POSITIVE EVALUATION BY REVIEWER 1.

Limitations:
1. In this manuscript, the author mentioned Grelet et al demonstrated that Nanos3 enhances the invasion rate of cultured NSCLC cells and is involved in EMT regulation. Then the author detected whether Nanos3 over expression affects tumor progression in vivo. However, the author only detected the E-cadherin expression by IHC. The expression of EMT markers such as Vimentin, snail should also be detected.

VIMENTIN STAINING OF LUNG SECTIONS OF ADENOCARCINOMAS AND BRONCHIOLES FROM CONTROL AND NANOS3 NSCLC MICE WAS INCLUDED IN THE REVISED VERSION OF THE MANUSCRIPT.

ADDITIONAL FIGURE: FIGURE S13

REVISED RESULTS SECTION: LINES 306-310

STAINING FOR SNAIL SUFFERED FROM TECHNICAL DIFFICULTIES, AND WAS THEREFORE NOT INCLUDED.

2. Figure 3: the authors showed that the ectopic expression of Nanos3 had no significant influences on male NSCLC animal survival, but significantly shortened female animal's survival. Is the sex difference due to NSCLC cell metastases? Or do lymph node metastases occur in male Nanos3 NSCLC mice?

THE SEX DIFFERENCE IS NEITHER DUE TO NSCLC CELL METASTASIS NOR TO LYMPH NODE METASTASIS. IN BOTH MALE AND FEMALE MICE, METASTASES COULD NOT BE OBSERVED. THIS FINDING WAS ADDED TO THE REVISED RESULTS SECTION, AND DISCUSSED TO SOME EXTENT.

REVISED RESULTS SECTION: LINES 279-283

REVISED DISCUSSION: LINES 392-397 AND 401-414

3. The image resolution of figure 5 and figure 7 are not enough.

WE AGREE ON THIS. WE HAD HIGH-RESOLUTION FIGURES 5 AND 7 AVAILABLE, BUT AS A CONSEQUENCE OF THE HIGH CONTENT OF THESE FIGURES (DETAILED HISTOLOGY), THE CORRESPONDING FILE SIZES WERE EXCEEDING THE 10-MB LIMIT LISTED IN THE INSTRUCTIONS TO AUTHORS. MEANWHILE WE FOUND ANOTHER WAY OF COMPRESSING THESE FIGURE FILES WITHOUT MUCH LOSS OF RESOLUTION. THESE FILES ARE NOW SUBMITTED FOR FIGURE 5 (FIGURE 6 IN THE REVISED VERSION OF THE MANUSCRIPT) AND FIGURE 7 (FIGURE 8 IN THE REVISED VERSION OF THE MANUSCRIPT).
4. In this manuscript, most part of references were published five years ago.

WE HAVE ADDED NOW THE FOLLOWING MORE RECENT REFERENCES:

- RECENT REVIEW ON GERM CELL DEVELOPMENT (2019):
  HTTPS://WWW.NCBI.NLM.NIH.GOV/PUBMED/30225862
  REVISED BACKGROUND, LINE 61 (REF. 7)

- RECENT REVIEW ON PUF FAMILY AND RNA-BINDING (2018):
  HTTPS://WWW.NCBI.NLM.NIH.GOV/PUBMED/29385744
  REVISED BACKGROUND, LINES 67 AND 78 (REF. 15)

- RECENT REVIEW ON CCR4-NOT AND NANOS (2019):
  HTTPS://WWW.NCBI.NLM.NIH.GOV/PUBMED/30740123
  REVISED BACKGROUND, LINE 75 (REF. 18)

- RECENT REVIEW ON NON-SMALL CELL LUNG CANCER MOUSE MODELS:
  HTTPS://WWW.NCBI.NLM.NIH.GOV/PUBMED/26331885
  REVISED DISCUSSION, LINE 380 (REF. 47)

(Reviewer 2): In the current study, the authors reported a new mouse model based on the ectopic expression of Nanos3, a protein that has been found upregulated in many cancers. The authors have generated a conditional- tissue-specific mouse model for Nanos3 overexpression. They have examined the role of Nanos3 in lung tumorigenesis by crossing human Nanos3 transgenic mice with a known model of NSLC based on the activation of KRAS and the deficiency in TP53 gene. The authors have found that expression of Nanos3 in the lung, increases bronchiolar dysplasia and has a negative effect on female survival.

Major comments:

- In general, this work basically describes the phenotype observed in NSCLC mouse model that express Nanos3 in the lung, so the data shown in Supplementary figure 4 demonstrating the expression of Nanos3 in the NSCLC lung should be shown in the main figures.
FOLLOWING THE SUGGESTION OF REVIEWER 2, FIGURE S4 WAS CHANGED NOW TO A MAIN FIGURE (FIGURE 3 IN THE REVISED VERSION OF THE MANUSCRIPT). LINES 245 AND 262.

- The effect the authors observed in female mice survival upon Nanos3 expression in the lung is very interesting and the paper should benefit from more experiments or discussion that explain the gender bias. Is the expression of Nanos3 interacting partners increased in NSCLC in females? Is Nanos3 function suppressed by testosterone or androgen receptor signaling? Since there is increased bronchiolar dysplasia in Nanos3 NSCLC females, does it correlate with enhanced expression of myofibroblast activation?

WE DISCUSSED THESE COMMENTS OF THE REVIEWER MORE THOROUGHLY IN THE REVISED DISCUSSION SECTION.

REVISED DISCUSSION, LINES 401-414.

UNFORTUNATELY, WE ARE UNABLE TO EXECUTE ADDITIONAL ANIMAL EXPERIMENTS (AS THE CORRESPONDING AUTHOR IS CURRENTLY RETIRED AND HAS NO MORE RESEARCH FUNDS AVAILABLE, WHILE ALL HIS CLOSE COLLEAGUES ARE WORKING NOW IN VARIOUS OTHER SETTINGS).

- It is confusing that in vivo there is not sign of metastasis in Nanos3 NSCLC mice but the Nanos3 tumor derived cells are more metastatic. Does Nanos3 have a role in migration or invasion? The authors should include a better characterization of the cell lines in the manuscript. Do these cells exhibit enhanced expression of migration or invasion markers? Do the levels of Nanos3 correlate with higher invasive potential?

WE HAVE INCLUDED A BETTER CHARACTERIZATION OF THE LUNG TUMOR DERIVED CELL LINES LUTDCO AND LUTDNA3 IN THE REVISED VERSION OF THE MANUSCRIPT.

ADDITIONAL FIGURE FILE 18: FIGURE S18

ADDITIONAL FIGURE FILE 19: FIGURE S19

REVISED RESULTS, LINES 341-347.

- There are some graphs that lack units or do not show standard deviation or statistical analysis, which should be included in the figures and figure legends.

WE HAVE ADDED NOW THE FOLLOWING MISSING FEATURES:

- STANDARD ERROR IN FIGURE 2C

- UNIT OF THE Y-AXIS OF FIGURE 6A (FIGURE 7A IN THE REVISED MANUSCRIPT)
- STANDARD ERRORS IN FIGURE 6B (FIGURE 7B IN THE REVISED MANUSCRIPT)

- UNIT OF Y-AXIS IN SUPPLEMENTARY FIGURE S4C (MAIN FIGURE 3 IN THE REVISED MANUSCRIPT)

- STATISTICAL ANALYSIS OF SUPPLEMENTARY FIGURE S5 (FIGURE S8 IN THE REVISED MANUSCRIPT).

- Histology sections in figure 3 is too small to appreciate differences, I recommend to include panels with bigger magnification or accompany with quantitation (same in figure 5 and 7).

PANELS WITH INCREASING MAGNIFICATIONS OF THE TISSUE SECTIONS SHOWN IN FIGURES 3, 5, 7 (CORRESPONDING TO FIGURES 4, 6 AND 8 IN THE REVISED MANUSCRIPT) ARE NOW INCLUDED AS SUPPLEMENTARY FIGURES.

FOR REVISED FIGURE 4: FIGURES S6 AND S7 HAVE BEEN ADDED; REVISED RESULTS, LINES 287-289.

FOR REVISED FIGURE 6: FIGURES S9, S10 AND S11 HAVE BEEN ADDED; REVISED RESULTS, LINES 296-301.

FOR REVISED FIGURE 8: FIGURES S16 AND S17 HAVE BEEN ADDED; REVISED RESULTS, LINES 338-339.

Minor comments:

- Background section shouldn't exhibit references to figures, I recommend to include references form the literature.

WE DELETED THE REFERENCE TO FIGURE 1A IN THE BACKGROUND SECTION AND REPLACED IT BY THE FOLLOWING REFERENCE FROM THE LITERATURE: DE KEUCKELAERE ET AL., 2018 (REF. 12).

REVISED BACKGROUND, LINES 69 AND 72.

- Define what is exactly the meaning of "ectopic" expression, and discuss whether the expression of Nanos3 in the tumors is found in cancer cells or also in tumor stroma.

THE MEANING OF “ECTOPIC” EXPRESSION WAS DEFINED IN MORE DETAIL IN THE REVISED RESULTS SECTION, LINES 251-255.

THE EXPRESSION OF NANOS3 IN CANCER CELLS AND TUMOR STROMA OF OUR NSCLC TUMOR MODEL IS MORE ELABORATED ON IN THE REVISED RESULTS.
SECTION (LINES 262-268, NEW ADDITIONAL FIGURE S5) AND IN THE REVISED DISCUSSION (LINES 421-427).

THE MEANING OF “ECTOPIC” EXPRESSION AND THE EXPRESSION OF NANOS3 IN CANCER CELLS AND TUMOR STROMA IN GENERAL IS ALSO MORE ELABORATED ON IN THE REVISED DISCUSSION SECTION, LINES 371-376.

- The authors should change the blot shown in figure 2A with a stronger exposure of Nanos3, and include a loading control in the same panel.

THE TOP BAND IN THE BLOT WITH NANOS3 DETECTION (FIGURE 2A, BOTTOM) REPRESENTS THE ACTIN LOADING CONTROL OF THAT BLOT, BUT WAS NOT CORRECTLY ANNOTATED IN THE ORIGINAL MANUSCRIPT.

TO CLARIFY THIS, AND TO MEET THE OTHER CONCERN OF THE REVIEWER, WE HAVE DIVIDED NOW FIGURE 2A (BLOT) INTO A TOP PANEL WITH LOADING CONTROL, A MIDDLE PANEL WITH NANOS 3 DETECTION (MORE STRONGLY EXPOSED) AND A BOTTOM PANEL WITH EGFP DETECTION (INCLUDING ALSO AN ASPECIFIC BAND, EQUALLY SERVING AS LOADING CONTROL). UNFORTUNATELY, NEITHER LIVER SAMPLES NOR LIVER-SPECIFIC TRANSGENIC NANOS3 MICE ARE AVAILABLE AT THIS MOMENT (AND CANNOT BE GENERATED ANYMORE IN OUR FORMER LABORATORY), AND THEREFORE, WE COULD NOT REPEAT THE WESTERN BLOT FOR NANOS3.

(Reviewer 3): In this manuscript (BCAN-D-18-02739), the authors reported a new mouse model allowing examination of Nanos3-associated pathways and investigation of the influence of ectopic Nanos3 expression in various cancer types, particularly in lung cancer. Although without the insight of mechanism, this study provided in vivo evidence regarding the role of Nanos3 in the progression of lung cancer.

WE APPRECIATE THE OVERALL POSITIVE EVALUATION BY REVIEWER 1.

Several points need to be addressed before publication.

1). Since this mouse model is an inducible ectopic overexpression, is the level comparable to that in human lung cancer? I understand it is difficult to compare, but some western blots with those lung tumor-derived cells and established human lung cancer cell lines may help.

WE PERFORMED A WESTERN BLOT WITH PROTEIN LYSATES FROM LUTDCO AND LUTDN3A3 CELL CULTURES IN COMPARISON WITH PROTEIN LYSATES FROM OUR ESTABLISHED NANOS3 OVEREXPRESSING LUNG CANCER CELL LINES, CALU-1 AND SK-LU-1.

ADDITIONAL FIGURE FILE: FIGURE S19.
REVISED RESULTS SECTION, LINES 348-357.

REVISED DISCUSSION, LINES 445-458.

2) Please include some zoom-in pictures for IHC and H/E images.

PANELS WITH INCREASING MAGNIFICATIONS OF THE TISSUE SECTIONS SHOWN IN FIGURES 2, 3, 5, 7 (CORRESPONDING TO FIGURES 2, 4, 6 AND 8 IN THE REVISED MANUSCRIPT) ARE NOW INCLUDED AS SUPPLEMENTARY FIGURES.

FOR REVISED FIGURE 2: FIGURE S4 HAS BEEN ADDED; REVISED RESULTS, LINES 233-234.

FOR REVISED FIGURE 4: FIGURES S6 AND S7 HAVE BEEN ADDED; REVISED RESULTS, LINES 287-289.

FOR REVISED FIGURE 6: FIGURES S9, S10 AND S11 HAVE BEEN ADDED; REVISED RESULTS, LINES 296-301.

FOR REVISED FIGURE 8: FIGURES S16 AND S17 HAVE BEEN ADDED; REVISED RESULTS, LINES 338-339.