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14. ABSTRACT

Our experiments in genetically engineered mice have led in year 2 to several novel findings regarding Kras and Pten-driven tumorigenesis throughout the genital tract (including the ovaries) and in the peritoneal cavity. I summary, our results show ovarian tumors occur when Kras and Pten are induced at extraovarian sites. No phenotype is observed in Kras only and Pten only mice, suggesting that both pathways are needed for in vivo tumorigenesis. The tumor grade varies according to site of injection. The intraductal route of injection leads to high grade endometrioid ductal tumors, in contrast to the low and intermediate grade endometrioid tumors seen in the ovaries and endometrium, respectively. IP injection triggers ovarian tumors phenotypically identical with those occurring post IB injections and the findings (highly consistent with the diagnosis of peritoneal carcinomatosis) displayed similar clinical traits in both female and male mice. Our human studies performed in year 2 show for the first time that distinct plasma miRNA expression patterns may serve as highly specific and sensitive diagnostic biomarkers to discriminate between healthy, endometriosis, and EAOC cases. Corroborated with our ongoing studies on tissue miRNA and mRNA profiling and with preclinical data from our mouse models, we aim to identify new markers of disease that can help with diagnosis and treatment.

15. SUBJECT TERMS

Ovarian cancer, preclinical animal model, Kras, Pten, MUC1

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INTRODUCTION

Ovarian epithelial tumors are highly diverse and the exact tissue of origin is still unclear. Until recently, all five histological subtypes of ovarian epithelial tumors (serous, endometrioid, mucinous, clear cell and transitional adenocarcinomas) were believed to arise in the coelomic epithelium that covers the ovarian surface epithelium (OSE) which then undergoes metaplasia and changes to müllerian -like epithelium before malignant transformation. More recently, however, it has been suggested that they could instead arise directly from extra-ovarian tissues that are embryologically derived from the müllerian ducts. Although scientific evidence in support of both theories exists, further studies on disease pathogenesis are needed.

Our studies use previously described, genetically engineered mice (Cre-loxP) that carry the lox-Stop-loxP-KrasG12D oncoallele and a floxed region within region encoding for the phosphatase domain of the Pten gene (KrasPten mice[1]). We postulate that similarly to intrabursal injections, AdCre injection along various other sites of the genital tract of KrasPten and of our recently described MUC1KrasPten mice [2] will allow us to study *in vivo* tumor initiation and progression and to identify important disease pathogenesis mechanisms in ovarian tumors and other cancers of the genital tract. Furthermore, in conjunction with our clinical studies we aim to identify novel disease biomarkers that may help in the early diagnostic of ovarian cancer and provide new therapeutic/preventive targets.

BODY

We present below our progress in year 2, according to the tasks and milestones described in the original application.

Aim 1 (Months 1-18). To investigate the Müllerian tract versus the OSE as the potential originating sites for ovarian epithelial tumors in KrasPten mice.

Task 1 (Months 0-2). To secure IACUC approval.

This task has been completed, as originally proposed.

Note: Prompted by our novel findings, the complexity of our in vivo work has increased in the past year, demanding implementation of additional surgical procedures (further detailed below) like ovariectomies, ovarian transplantation etc. Consequently, we had applied for and secured approval of an amendment to our animal protocol. Approval letter of the amended protocol is included in the Appendices section.

Task 2 (Months 0-6). To set up large animal breeding protocols and implement genetic screening of littermates.

This task has been completed and the animal breeding protocols have been successfully implemented.

Note: The proposed animal breeding protocols will continue throughout the entire duration of our studies. We are permanently screening large number of litters (more than 240 mice/year), as originally proposed. The breeding protocols are highly complex, as our works requires the separate maintenance of breeders for single Tg (Kras, Pten, MUC1) lines, double Tg (KrasPten) as well as triple Tg (MUC1KrasPten) lines. Tail DNA extraction and PCR protocols for genotyping are performed according to protocols described by us and others[1,3].

Task 3 (Months 6-9). To complete survival surgery and AdCre injection via 4 routes: intrabursal, intraductal, intrauterine and intraperitoneal.

This task has been completed.

We have attempted in year 1 to inject AdCre in the mouse oviducts using the same needles as used for IB injections (26G). However, due to the extremely small diameter of the oviduct, we experienced difficulties targeting the lumen of the duct, as the needle often pierced through it. To address this, we had contacted Hamilton company and ordered custom made needles (34G) and 5 µL syringes.

In year 2, we had designed a modified procedure for intraductal (ID) injections. The new procedure requires first to seal off the communication of the oviduct with the ovary (distally) and uterine horn (proximally) using microsurgical staples and staple applicator. This approach diminishes the chances that AdCre spreads loco-regionally and that remains more confined to the oviduct.

After successfully implementing this new surgical procedure, we were able to trigger intraductal tumors (Fig. 1). The tumors occurred in the injected site and not in the contra-lateral control, and in late stages spread throughout the peritoneal cavity and on the diaphragm (Fig. 1).
Morphologically, the tumors display endometrioid histology, similarly to the tumors triggered in the bursa and endometrium. Surprisingly, however, although the Kras and Pten mutations are driving the tumorigenic process in all mice, the ductal tumors displayed high grade (Fig 2) unlike the ovarian and endometrial tumors that were low or intermediate grade respectively (not shown).
In light of the fact that in humans most of the high grade serous ovarian carcinomas may arise from the fallopian tubes, our findings provide for the first time, preclinical evidence in support of the fact that the oviducts (the fallopian tube equivalent in mice) provide a microenvironment that may be more accommodating for aggressive tumors.

Task 4 (Months 6-15). Disease monitoring, sample acquisition, pathology.
We have completed the experiments originally described in our proposal. However, due to the intriguing findings in year 1, describing both peritoneal and ovarian tumorigenesis in IP injected female mice, we explored new hypotheses in year 2. Testing these new hypotheses required new surgical protocols which we successfully implemented in our work, as described below.

Hypothesis 1. Peritoneal carcinomatosis seen when AdCre is injected IP in KrasPten or MUC1KrasPten mice is the consequences of a direct mutagenesis of the peritoneal lining and not of the OSE.
The peritoneal lining is made of the mesothelium, a monolayer of flattened squamous-like epithelial cells resting on a thin basement membrane supported by dense irregular connective tissue. To rule out that the peritoneal tumors occurred as a consequence of mutagenesis first triggered in the OSE and to test the direct role the lining mesothelium has in peritoneal tumorigenesis, we first ovariectomized (unilaterally or bilaterally) the KrasPten (or MUC1KrasPten mice) during survival surgery, let the mice recover for 3 weeks and then injected them IP with AdCre 5 x 10e8 plaque forming units (p.f.u.). Our preliminary results show:

- The wild type mice that have been transplanted with one KrasPten ovary remain healthy, even after more than 16 weeks of observation after IP injection AdCre.
- The KP or MKP mice that have been transplanted with ovaries carrying wild-type Kras and Pten develop peritoneal disease with wide-spread dissemination (Fig. 3) 94 and 52 days post IP AdCre, respectively.
- One KP female mice with bilateral ovariectomy developed a massive abdominal tumor more than 100 days post IP AdCre (Fig. 4).

Overall, these preliminary findings suggest that IP injection triggers direct mutagenesis in the peritoneal lining and that peritoneal tumors seen throughout the peritoneal cavity of IP injected female mice are occurring from the peritoneal rather than ovarian mesothelium.

Hypothesis 2. If the lining mesothelium is the direct source of peritoneal carcinomatosis seen in IP injected female mice, tumorigenesis can be similarly triggered in male mice injected IP with AdCre 5 x 10e8 pfu.
To test this, we injected a total of 15 male mice IP with the same dose of AdCre (5 x 10e8 pfu). Of the 15, 7 were MUC1KrasPten, 5 were KrasPten and 2 were Pten only. In confirmation of our hypothesis, the mice carrying both tumor-driving mutations (i.e. the MUC1KrasPten and the KrasPten mice but not Pten) developed tumors. The tumor findings were suggestive of primary peritoneal carcinomatosis highly resembling the peritoneal disease seen in IP injected female mice. The mice developed hemorrhagic ascites and widely disseminated peritoneal spread (Fig. 5). Histologically, the tumors show high proliferation of reactive mesothelial cells and the presence of epithelial cells, confirmed with positive calretinin 8 staining and negative staining for desmin (Fig. 6).
Taken together these experiments demonstrate that similarly to the OSE, the mesothelial cells from the peritoneal lining can give rise to epithelial tumors that show wide-spread loco-regional dissemination and are often accompanied by accumulation of hemorrhagic ascites. The role of the mesothelium is further confirmed in male mice that develop IP tumors with similar characteristics.

Task 5 (months 9-18). Data analysis, manuscript(s) writing and submission
The manuscript on the characterization of MUC1KrasPten and KrasPten mice with ovarian tumors triggered by intrabursal AdCre is currently in press at Oncogene (ref[2] and manuscript pdf attached).
A second manuscript, describing the morphopathogenic characteristics of lesions triggered via IB/IP/IU/ID routes in expanded mouse cohorts is currently in preparation.

Aim 2 (Months 12-36). To profile disease heterogeneity and to identify immune biomarkers of natural and vaccine-induced immune responses in mice with either endometriosis, ovarian cancer or endometriosis progressing to ovarian cancer.
We have made significant progress on this aim. We briefly summarize below our work in this aim, with emphasis on progress made in year 2. Further details can be found in our manuscript, (attached pdf).
We demonstrated in year one that a dendritic cell (DC) vaccine loaded with MUC1 peptide and then matured under type 1 polarizing conditions (DC1), using a maturation cocktail that combines GM-CSF, IL-4, LPS, IFNγ and Poly I:C, is superior to other DC maturation stimuli and can enhance the DC ability to secrete high levels of IL-12. We also showed that type 1 polarized, mature DC1 upregulate all emblematic co-stimulatory and maturation markers (CD40, CD80, CD86 and CD83) and display only slight phenotypic differences with classical DC (IL-4 and LPS matured). Importantly however, only the DC1 (and not the LPS-matured DC) released detectable level of IL-12p70 [2], supporting our rationale for DC1-based vaccination.
In year two, we enlarged our vaccination groups and confirmed the therapeutic potential of DC1-MUC1 vaccine using the triple transgenic MUC1KrasPten mice with MUC1-positive tumors as a preclinical model.
The DC1-MUC1 vaccine was administered subcutaneously (s.c.) in the right flank, at weeks 4, 6 and 8 after AdCre injection (according to the vaccination diagram originally proposed). A total of 3,000 MUC1-loaded DC1 cells were administered in each vaccine. This low number represents the mouse mass-adjusted equivalent of an adult human vaccine of approximately 6.5 million DC. Our results show that the DC1-MUC1 vaccine significantly prolongs survival in vaccinated MUC1KrasPten mice (n=10, p=0.033) compared to MUC1KrasPten non-vaccinated mice (n=13, Fig. 7A). No mice died prior to the scheduled time for the first vaccine dose.

In order to identify biomarkers of vaccination-induced immune changes in the host, we used a combination of ELISA (for MUC1-specific, serum IgM and IgG antibody detection) flow cytometry (for splenic Treg analysis and effector T cell cytokine detection) and real time PCR array (for T cell gene induction).
MUC1-specific antibodies were detectable in the serum and ascites of both vaccinated and non-vaccinated mice although the frequency and amplitude of systemic antibody responses were not increased by DC1-MUC1 vaccination (not shown). These findings confirmed our expectation that DC1 vaccination does not favor humoral responses and are in line with our previous results showing that in order for antibody responses to be triggered, soluble peptide antigen needs to be administered alongside the preloaded DCs [4,5].
Although IFNγ was not significantly upregulated in the spleen of DC1-MUC1 recipients (Fig. 7B), flow cytometry profiling revealed decreases in CD4+CD25+Foxp3+ Treg accumulation (p=0.00001, Fig. 7C) and significant improvement in the CD8 to Treg ratio in the spleen of vaccinated mice (p=0.007, Fig. 7D).
In addition, multiplex profiling with a targeted, quantitative real-time PCR immune array revealed significant up-regulation of CD80, CD40, CD45 and Jak2 genes in the spleens of vaccinated mice (Fig. 7E). Of note, since our experiments had overall survival as the primary endpoint, all our immune measurements (for antibodies, T cell, cytokines etc) were performed at necropsy, which occurred later in mice subjected to vaccination and may account for some of the variability in the observed immune responses.
Altogether, our results demonstrate that type-1 polarized DC1-MUC1 can circumvent tumor-mediated immune suppression in the host, activate multiple immune effector genes and effectively prolong survival. We also identified CD80, CD40, CD45 and Jak 2 as immune biomarkers of response to vaccination, as originally proposed.

Aim 3 (Months 0-60). To validate in human specimens the disease biomarkers identified (in aim 2) in mice with endometriosis and ovarian tumors

In collaboration with Xin Huang PhD and Robert P Edwards MD, we recently concluded a biomarker discovery study, focusing on plasma miRNA. Using global profiling of more than 1,000 miRNAs via quantitative PCR (qPCR) in a 20-patient discovery cohort, we identified 24 candidate miRNAs, which are differentially expressed between healthy controls (n=6), endometriosis (n-=7), and endometriosis-associated ovarian cancer (EAOC, n=7) patients.
For validation, the 24 miRNAs were further tested in an expanded cohort (n=88) of healthy individuals (n =20), endometriosis (n = 33), EAOC (n = 14), and serous epithelial ovarian cancer cases (SOC, n= 21, included as controls). The clinical demographics of our patient cohort and the experimental approach are shown in Table 1 and Fig. 8, respectively.
We next evaluated the diagnostic power of these miRNAs as potential biomarkers, using receiver operating characteristic (ROC) curves and classification models. We identified three distinct miRNA signatures with reliable differential expression between healthy individuals, endometriosis, and EAOC patients. When profiled against the control SOC category, our results revealed different miRNAs, suggesting that the identified signatures are reflective of disease-specific pathogenic mechanisms.
In line with our proposed studies, we explored next whether some of the most differentially expressed miRNAs between healthy controls and patients with endometriosis-associated ovarian cancer (EAOC) are also differentially expressed between healthy mice and KrasPten mice with endometrioid ovarian tumors. To achieve this we induced tumors in six female mice, sacrificed the mice when moribund, and collected serum at necropsy. Five healthy (non-injected) female mice were sacrificed as controls. Serum miRNAs were extracted and subjected to RT-qPCR analysis to measure expression of miR-15b, 16, 21,191, and 195, because among the top 10 differentially expressed human miRNAs between healthy controls and EAOC patients (Table 2), these five are the only miRNAs that have mouse orthologs. Our results demonstrate that four of the five miRNAs can also delineate the EAOC mice from healthy controls (Fig. 9), suggesting a potential EAOC-specific pathogenesis leading to the dysregulation of miR-15b, 16, 21, and 195, and further validating the biological relevance of the plasma miRNA expression signature we have identified as biomarkers of human EAOC.

We also investigated whether miRNAs in patients' plasma reflect miRNA expression pattern in corresponding diseased tissues. To address this question, we profiled miRNA expression in six pairs of endometriosis tissue or EAOC primary tumors and corresponding plasma samples using the NanoString technology [6], which provides digital counting of miRNA copy numbers without the need for miRNA amplification. Our preliminary results show that although a modest correlation was detected in all paired tissue and plasma samples, distinct miRNA expression profiles, especially among the highly expressed miRNAs, were detected, suggesting that plasma and tissue samples have different miRNA expression profiles.
Having profiled plasma, we are attempting next to perform a multi-platform tissue profiling (miRNA, RNA, DNA) using paraffin embedded tissue specimens from endometriosis and EAOC cases plus normal controls. We are reporting here that in collaboration with Dr Edwards and a team of physicians at Magee Womens Hospital (Drs Esther Elishaev and Rohit Bhargava, pathologists, Drs Ted Lee and Suketu Mansuria, surgeons) and staff scientists (Lindsay Mock, Traci Davis etc) we have completed the accrual of 155 specimens, exceeding the originally proposed target numbers (Table. 3). The molecular profiling using Nanostring is currently ongoing.

Progress on Milestone #1: first round of publication submissions.
This milestone has been completed. The manuscript on tumorigenesis following IB injections is currently in press at Oncogene (manuscript attached).
Using the MUC1KrasPten animal model we studied variations of MUC1 isoforms and its consequences on tumor growth. The provide data for a manuscript D. The manuscript (appendix), generated in collaboration with Olja Finn, is currently in press at Cancer Immunology Immunotherapy.

Progress on Milestone # 2: second round of publication submissions. This milestone is proposed for years 2-3.

In collaboration with Xin Huang we had recently finalized a manuscript on the role of miRNA as disease biomarkers in endometriosis and endometriosis-associated ovarian cancer. The manuscript has been submitted and is currently under review.

Progress on Milestone # 3: first R01 submission. Originally planned for year 3, we report that we secured our first R01 earlier than proposed, in year 2. Funding for the R01 award has been released in February

KEY RESEARCH ACCOMPLISHMENTS

Our experiments in genetically engineered mice have led in year 2 to several novel findings regarding Kras and Pten-driven tumorigenesis throughout the genital tract (including the ovaries) and in the peritoneal cavity. I summary, our results show:

- Ovarian tumors occur when Kras and Pten are induced at extraovarian sites. No phenotype is observed in Kras only and Pten only mice, suggesting that both pathways are needed for in vivo tumorigenesis.
- The tumor grade varies according to site of injection. The intraductal route of injection leads to high grade endometrioid ductal tumors, in contrast to the low and intermediate grade endometrioid tumors seen in the ovaries and endometrium, respectively. This novel finding opens the door for future studies on the role of the environment (ovarian bursa versus the oviduct) in generating tumors with different potentials for cell differentiation and growth.
- IP injection triggers ovarian tumors phenotypically identical with those occurring post IB injections and the findings (highly consistent with the diagnosis of peritoneal carcinomatosis) displayed similar clinical traits in both female and male mice. Ongoing investigations using molecular profiling of peritoneal implants from males and female mice (ovariectomized or not) aim to highlight genetic differences/similarities between primary ovarian tumors and peritoneal disease. Our preclinical studies will provide new insights on the role of ovarian epithelium and the surrounding tissue and potentially provide new drugable targets.

Our studies on plasma miRNAs (Suryawanshi et al, manuscript under review) show for the first time that distinct plasma miRNA expression patterns may serve as highly specific and sensitive diagnostic biomarkers to discriminate between healthy, endometriosis, and EAOC cases. Corroborated with our ongoing studies on tissue miRNA and mRNA profiling and with preclinical data from our mouse models, we aim to identify new markers of disease that can help with diagnosis and treatment.

REPORTABLE OUTCOMES

Manuscripts- In year 2, the PI has co-authored two manuscripts, currently in press.

1. Immunobiology of human mucin 1 in a preclinical ovarian tumor model. RA Budiu, E Elishaev, J Brozick, M Lee, RP Edwards, P Kalinski and AM Vlad. Oncogene. In press.

2. Human mucin MUC1 RNA undergoes different types of alternative splicing resulting in multiple isoforms. Lixin Zhang, Anda Vlad, Christine Milcarek and Olivera J Finn. Cancer Immunology Immunotherapy. In press.

- **Abstracts-**We have submitted during the past year three abstracts:

1. American Association for Cancer Research, Chicago –March 31-April 4, 2012;
2. University of Pittsburgh, Cancer Institute Scientific Retreat, University of Pittsburgh at Greensburg –June 21-22, 2012.
3. Ovarian Cancer Symposium. “Ovarian Cancer: Prevention, Detection and Treatment of the Disease and Its Recurrence: Molecular Mechanisms and Personalized Medicine”. Herberman Conference Center, Pittsburgh PA, May 10-11, 2012.

- **Presentations-** The PI has given three invited oral presentations:

1. Seventh International Symposium on Regional Cancer Therapies, February 18-20, 2012 at the South Seas Island Resort, Florida.
2. Ovarian Cancer Symposium. “Ovarian Cancer: Prevention, Detection and Treatment of the Disease and Its Recurrence: Molecular Mechanisms and Personalized Medicine”. Herberman Conference Center, Pittsburgh PA, May 10-11, 2012.
3. Surgical Oncology Faculty Retreat, Seven Springs Mountain Resort, February 3-4, 2012

- **Animal models-** In addition to last year’s findings on KrasPten and MUC1KrasPten female mice with ovarian tumors, endometrial tumors and endometrial hyperplasia, we identified in year 2 two novel preclinical models: one for high grade ductal tumors and the other for peritoneal carcinomatosis in both female and male mice.

- **Funding applied for based on work supported by this award.** This award has helped secure one R01 (starting date of funding: March 2012). It has also supported three other new applications in 2012, all unfunded.

1. Multi-PI R01 application (principal investigators: Vlad, Edwards and Kalinski), submitted to the NIH/NCI on Feb 2012.
2. Letter of intent -the DOD Ovarian Cancer Program Idea Award, (April, 2012)- submitted in collaboration with Xin Huang PhD and Edwards RP, MD .
3. Letter of intent – The Ovarian Cancer research Fund (OCRF) Program Project Development Grant (May 2012) submitted in collaboration with Edwards RP, MD, Lee A, PhD and Oesterreich S, PhD.

CONCLUSION

Our studies in mice describe several new preclinical models of tumorigenesis throughout the genital tract. Using these models we have revealed new roles for the coelomic epithelium in ovarian as well primary peritoneal carcinogenesis in female mice. In addition, we explored the role of the peritoneal lining (mesothelium) in male mice and described for the first time peritoneal tumors similar to those in females. We are further exploring the molecular profile of these tumors and our findings will likely advance our understanding of basic mechanisms of tumorigenesis in the ovaries, oviducts and the surrounding peritoneum and may reveal novel diagnostic markers and/or therapeutic targets.

Our studies in humans revealed a combination of plasma miRNAs that can serve as highly specific and sensitive diagnostic biomarkers in endometriosis and EAOC. In combination with other multiplex platforms and with preclinical data from our mouse models, we aim to identify new markers of disease that can help with diagnosis and treatment.

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SUPPORTING DATA

Fig. 1

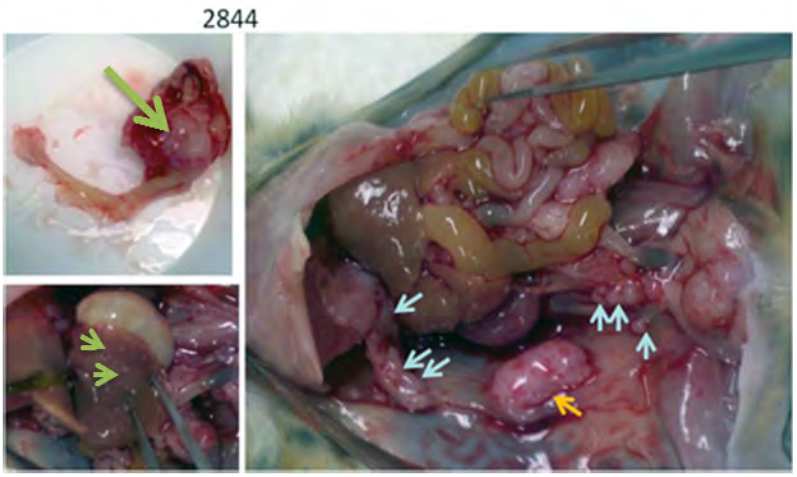

Fig. 1. Intraductal (ID) AdCre triggers tubal tumors with secondary ovarian involvement, peritoneal dissemination and loco-regional invasion. One example of a female mouse (tag ID #2844) injected with 2.5 x 10e5 AdCre pfu in the oviduct. The oviduct was first clipped at both ends (adjacent to the ovary and uterus, respectively) and the AdCre was subsequently injected in the middle of the oviduct. The mouse was sacrificed when moribund. The images were collected at necropsy. Upper left panel shows the ductal tumor (arrow) occurring in the injected (left) oviduct. Lower left panel shows small peritoneal implants on the diaphragmatic side of the liver (arrows). The right panel shows multiple peritoneal implants (arrows) on the diaphragm and mesenterum, and one larger tumor formation on the right peritoneal wall (yellow arrow).

Fig. 2

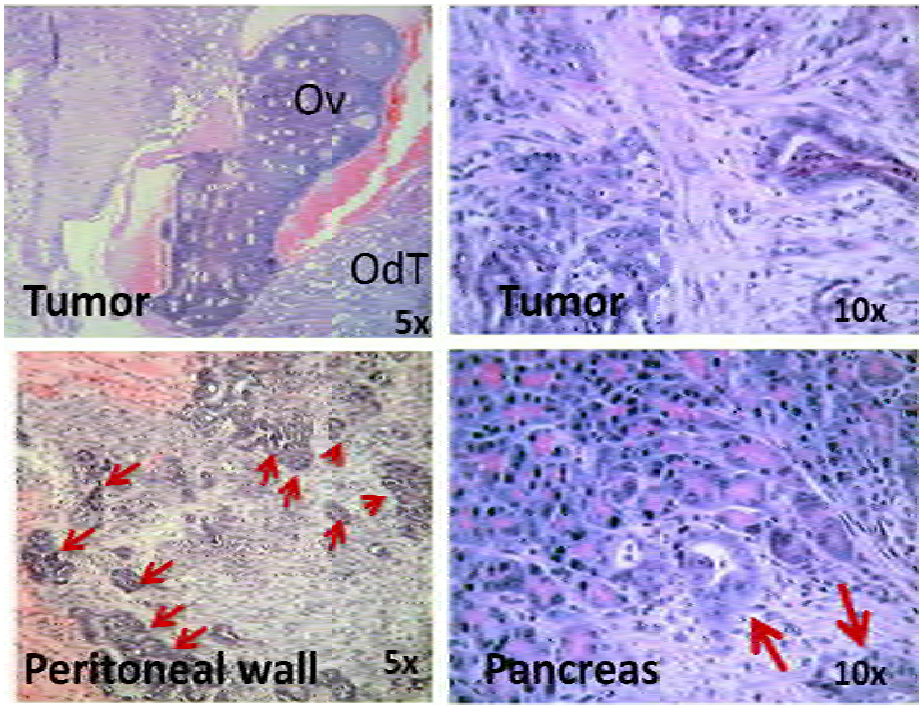

Fig. 2. Intraductal (ID) AdCre triggers high grade endometrioid tubal tumors with peritoneal dissemination and loco-regional invasion. HE staining. The primary tumor (upper panels) and of disseminated tumors in the peritoneal wall and infiltrating the pancreas (lower panels, left and right, respectively). Histologically, the tumors comprise of mostly poorly differentiated tumor cells and display glandular formations (arrows) consistent with the diagnosis of endometrioid high grade ovarian tumors. The gland-like formations are also visible in the peritoneal and pancreatic implants. Ov, ovary; OdT, oviduct.

Fig. 3

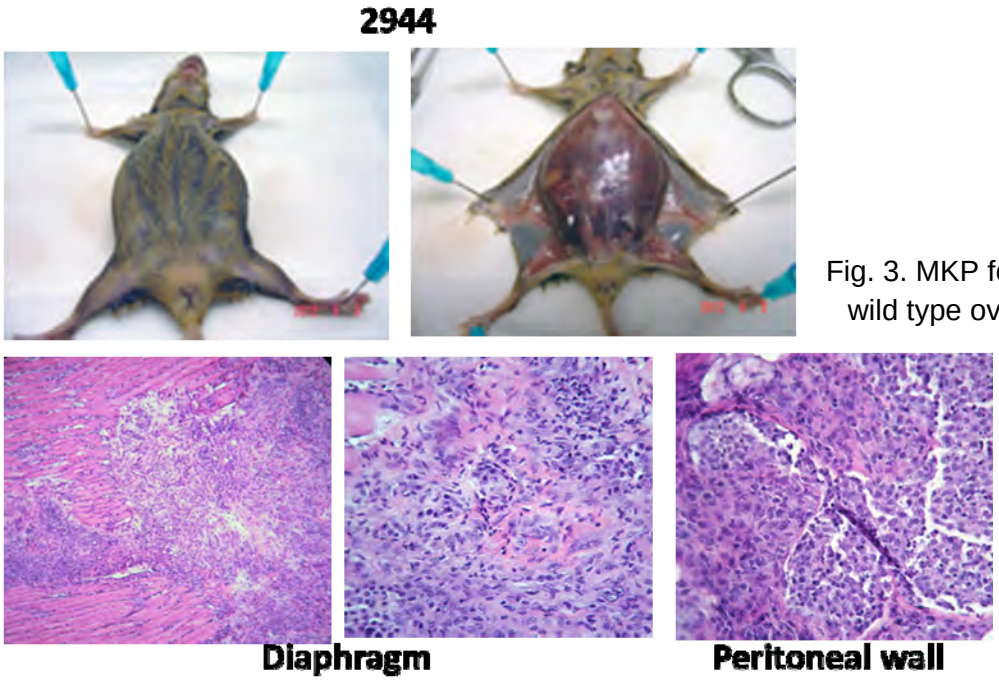

Fig. 3. MKP female mouse transplanted with a wild type ovary and then injected IP with AdCre (5x10e8 pfu) . The mouse developed massive ascites (upper panels) and numerous widespread tumors throughout the abdomen, including the diaphragm (lower left and middle) and peritoneal wall (lower right).

Fig. 4

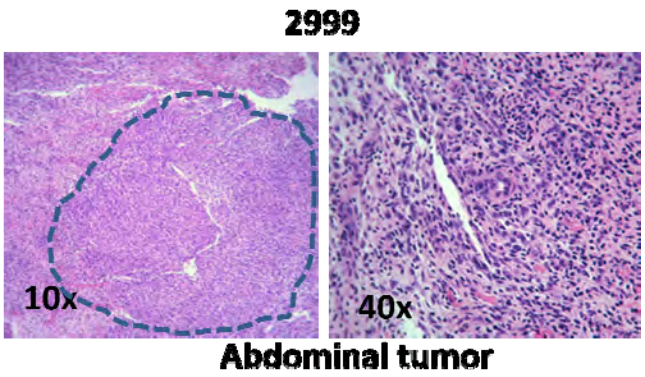

Fig. 4. KrasPten female mouse with bilateral ovariectomy, MKP female mouse transplanted with a wild type ovary and then injected IP with AdCre (5x10e8 pfu) . The mouse developed a massive tumors inside the peritoneal cavity. Left panel shows a tumor area (circled), at 10x. The right panel is a higher magnification of the tumor at left. HE staining.

Fig. 5

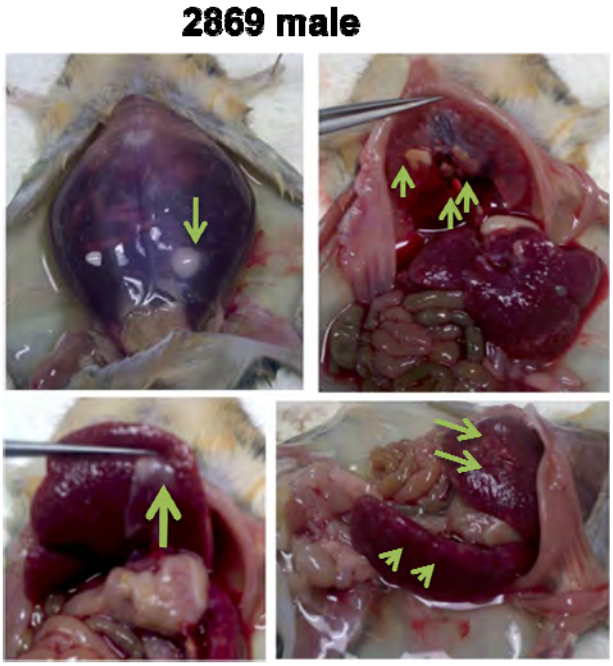

Fig. 5. Intraperitoneal administration of 5 x 10e8 pfu AdCre in male mice triggers primary peritoneal carcinomatosis which resembles the peritoneal disease seen in IP injected female mice. The mice develop hemorrhagic ascites (upper left) and widely disseminated peritoneal tumors. The arrows show tumor implants on the peritoneal wall (upper left), diaphragm (upper right), liver surface (lower left, right) and on the splenic surface (lower right).

Fig. 6

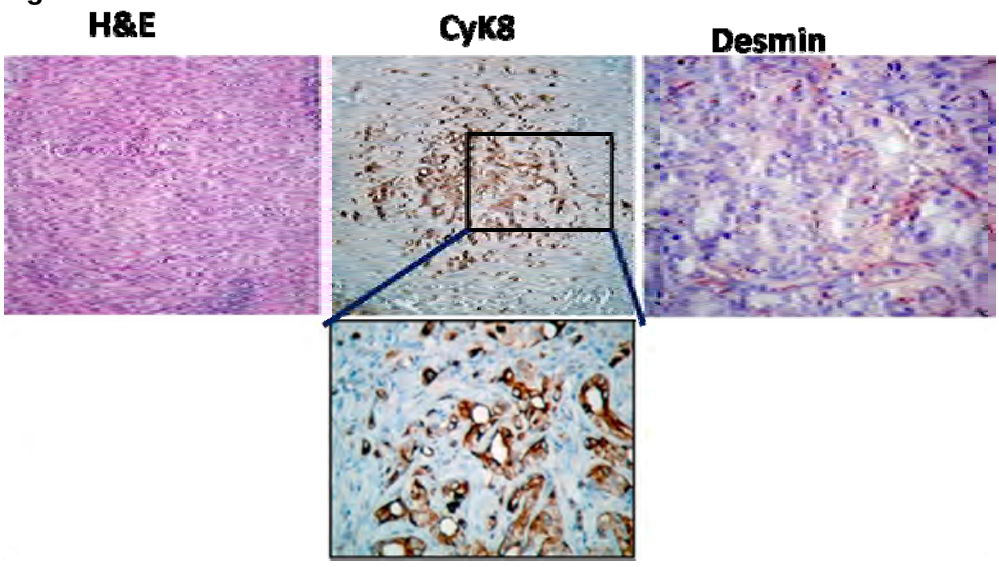

Fig. 6. Histological appearance of a diaphragmatic implant via HE (upper left) and IHC (middle and right panels).The tumor cells show glandular morphology, and can be visualized with cytokeratin 8 staining (middle panel; lower panel shows a magnification of boxed tissue in the upper panel). Stroma is visualized with an antibody to desmin (upper right).

Fig. 7

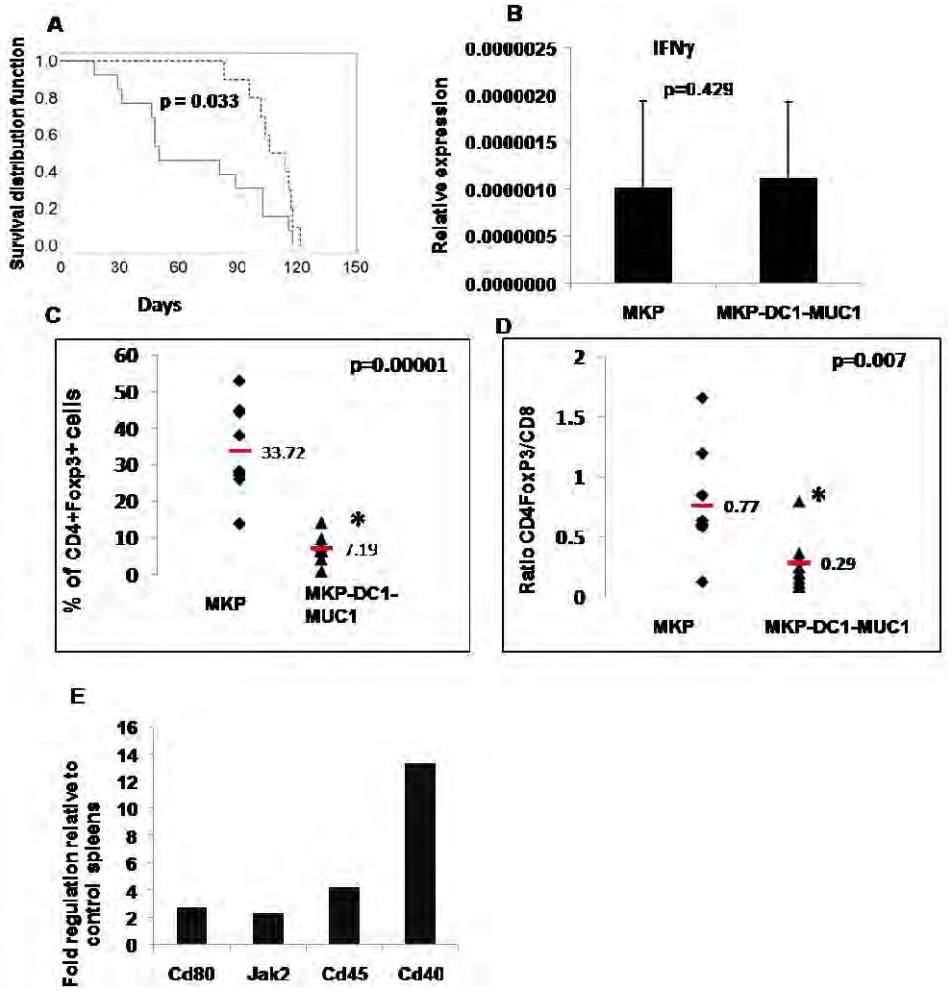

Fig. 7. Effects of MUC1 vaccination on survival, loco-regional immune suppression and overall immunity. (A) Kaplan–Meier curve of overall survival comparing MKP (solid line, n=13) and MKP vaccinated mice (dotted line, n=10), using log-rank test (p=0.033). Analyses were performed using SAS 9.0, assuming statistical significance at $p<0.05$.

(B) Quantitation of Ifng gene expression by real time PCR (qRT-PCR) in the spleen of vaccinated and non-vaccinated mice. (C, D) Percentages of CD4+Foxp3+ cells (C) and ratios of CD8 to Tregs (E) in vaccinated (n=10) versus non-vaccinated (n=10) MUC1KrasPten mice (D). The plotted values were obtained from flow cytometry dot plots of spleen cells. p value, Student t test. (E) Immune genes that were significantly upregulated in the spleens of vaccinated (n=4) and non-vaccinated controls (n=4) with similar tumor load at cull time. The qRT-PCR experiments were run using the 84 gene immune profiler (Th1-Th2-Th3 immune profiler, SA Biosciences). A cut-off of p=0.05 was used to select significant changes in expression. (CD80, p=0.02; Jak2, p=0.02; CD45, p=0.03; CD40, p=0.02). Jak2 (Janus kinase 2); CD (cluster of differentiation).

Table 1. Demographic and clinical information of the study cohort.

	EAOC (n=14)	Endometriosis (n = 33)	SOC (n=21)	Healthy (n = 20)
Age <40 years	2 (14.2%)	21 (63.6%)	0 (0%)	9 (45%)
Age >40 years	12 (85.7%)	12 (36.3%)	21 (100%)	11 (55%)
Smoking				
Yes	3 (21.4%)	9 (27.2%)	3 (14.2%)	NA
No	11 (78.6%)	21 (63.6%)	13 (61.9%)	20 (100%)
Unknown	0 (0%)	3 (9%)	5 (23.8%)	NA
Alcohol consumption				
Yes	6 (42.8%)	17 (51.5%)	7 (33.3%)	0
No	7 (50%)	12 (36.3%)	13 (61.9%)	20 (100%)
Unknown	1 (7.4%)	4 (12.1%)	1 (4.7%)	NA
Family History of Cancer Yes	6 (42.85%)	11 (33.3%)	11 (52.3%)	0
No	6 (42.8%)	15 (45.4%)	9 (42.8%)	20 (100%)
Unknown	2 (14.2%)	7 (21.2%)	1 (4.7%)	0
Stage of disease				
I	5 (35.7%)	NA	3 (14.2%)	NA
II	4 (28.5%)	NA	0	NA
III	5 (35.7%)	3 (9%)	16 (76.1%)	NA
IV	0	NA	2 (9.5%)	NA
Race				
Caucasian	14 (100%)	29 (87.8%)	21 (100%)	9 (45%)
African- American	0	4 (12.1%)	0	7 (35%)
Hispanic	0	0	0	4 (20%)

Fig. 8

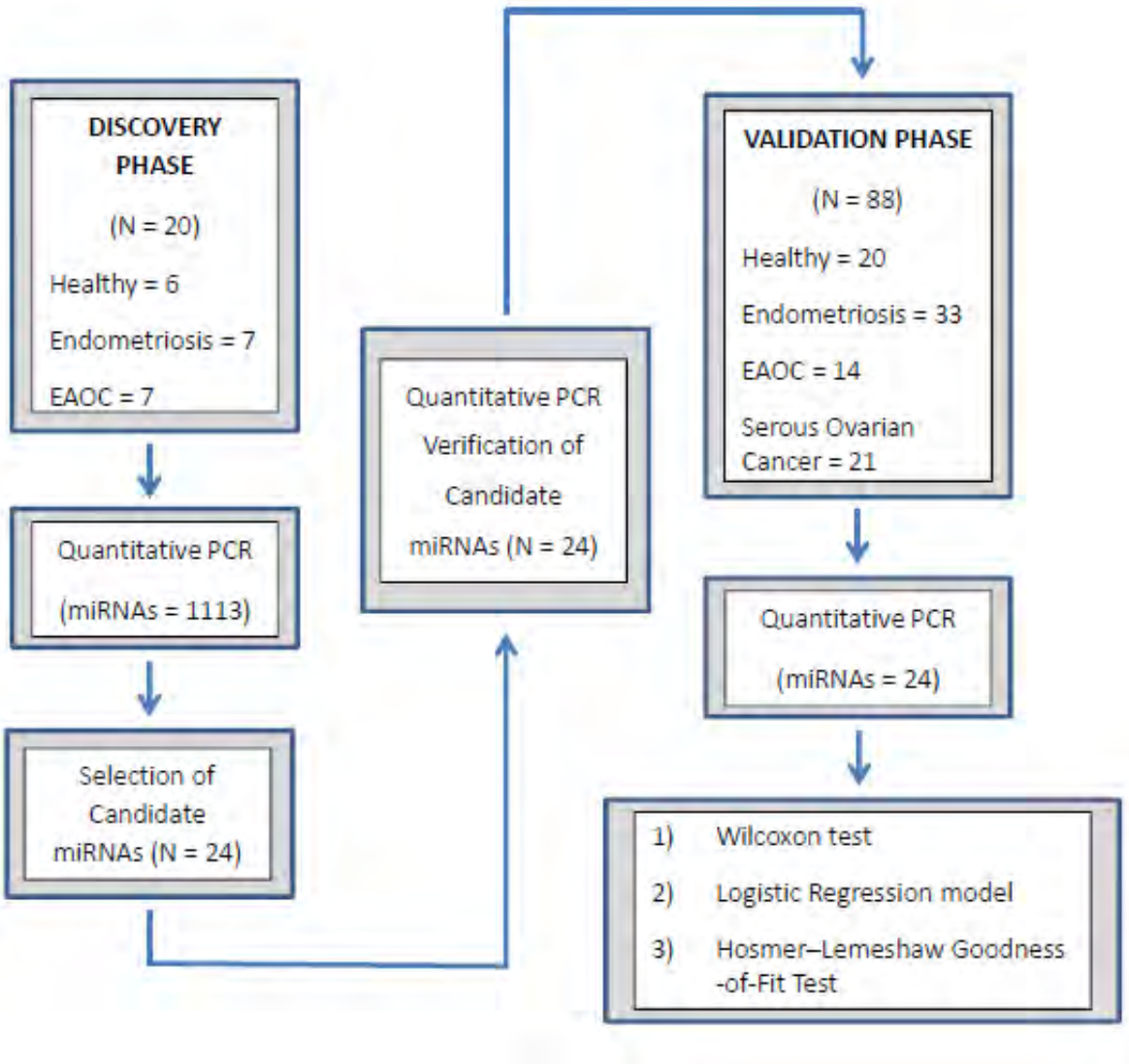

Fig. 8. Experimental design

Table 2. The 10 most differentially expressed miRNAs in pair- wise comparisons among healthy controls, endometriosis, and EAOC samples.

Endometriosis (n=33) vs. Healthy Controls (n=20)	W.pvalue[1]	a.W.pvalue[2]	FC[3]
hsa.miR.16	0.00000	0.00001	396.62
hsa.miR.195	0.00001	0.00011	62.02
hsa.miR.191	0.00004	0.00028	79.05
hsa.miR.1274b	0.00005	0.00031	24.55
hsa.miR.1974	0.00007	0.00034	15.43
hsa.miR.4284	0.00025	0.00101	16.79
hsa.miR.15b	0.00041	0.00124	26.27
hsa.miR.1978	0.00041	0.00124	51.29
hsa.miR.1979	0.00051	0.00135	30.54
hsa.miR.362.5p	0.00062	0.00149	3.41

EAOC (n =14) vs. Healthy Controls (n=20)	W.pvalue[1]	a.W.pvalue[2]	FC[3]
hsa.miR.21	0.00001	0.00014	147.40
hsa.miR.191	0.00001	0.00014	380.01
hsa.miR.16	0.00002	0.00015	1323.18
hsa.miR.15b	0.00005	0.00032	80.50
hsa.miR.1977	0.00029	0.00117	11.48
hsa.miR.1979	0.00029	0.00117	99.11
hsa.miR.1973	0.00038	0.00131	58.96
hsa.miR.1974	0.00057	0.00170	20.75
hsa.miR.4284	0.00073	0.00196	26.58
hsa.miR.195	0.00137	0.00273	48.02

Endometriosis (n =33) vs. EAOC (n=14)	W.pvalue[1]	a.W.pvalue[2]	FC[3]
hsa.miR.362.5p	0.00014	0.00343	0.14
hsa.miR.1274b	0.00071	0.00855	0.04
hsa.miR.1274a	0.01323	0.09634	0.13
hsa.miR.21	0.01606	0.09634	13.84
hsa.miR.766	0.02195	0.10534	15.11
hsa.miR.1975	0.03142	0.11204	7.75
hsa.miR.1308	0.03527	0.11204	0.13
hsa.miR.191	0.03735	0.11204	4.81
hsa.miR.744	0.05209	0.13192	4.89
hsa.miR.376a	0.05497	0.13192	6.38

Fig. 9

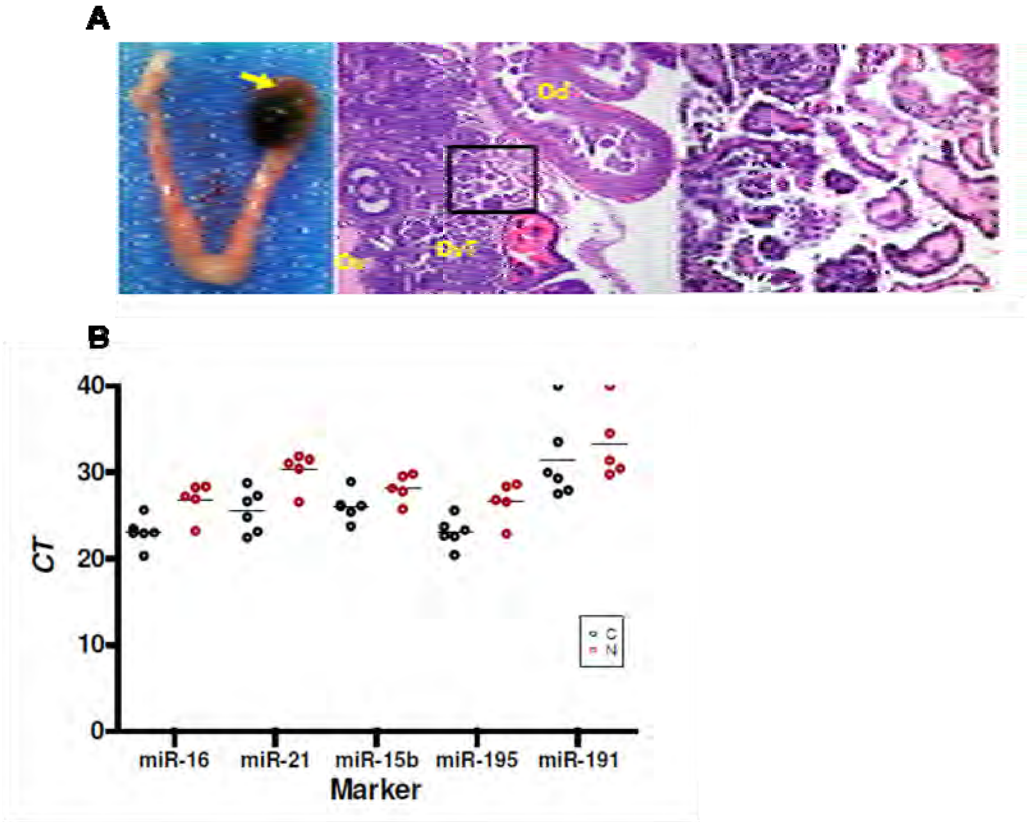

Fig. 9. The plasma miRNA expression signature that differentiates healthy controls from EAOC patients can be detected in a mouse endometrioid ovarian cancer model. A) Left panel shows ovarian tumor at the Ad-Cre injected site (arrow), but no tumor formation seen on non-injected left ovary. Middle panel is 10X HE staining of a cross section of mouse ovarian tumor with ovary (Ov), oviduct (Od), and ovarian tumor (OvT). Right panel shows magnified image of ovarian tumor with endometrioid histology (40X). B) Expression of orthologous miRNAs in healthy mice (n=5) and mice with EAOC (n=6). Four out of the five miRNAs are significantly upregulated in most of the mice with EAOC as compared to normal (p = 0.00009, 0.000433, 0.000209, and 0.00014 for miR-16, 21, 15b, and 195, respectively; student's t-test), similar to the profiles in human EAOC samples. miR-191 did not reach statistical significance, although there is also a trend that expression of plasma miR-191 is elevated in EAOC mice compared to that of in normal mice. Equal amount of RNA extracted from mouse serum was used for RT and qPCR. The raw *CT* values were plotted because currently there is no consensus on endogenous plasma miRNA in mouse that can be used for normalization. C, mice with EAOC tumors; N, normal control mice.

Table 3. Summary of accrued tissue specimens

Benign Endometriosis (N=33)	Abnormal Endometriosis (N=43)	Ovarian Cancer (N=45)	Normal Endometrium (n=34)
▪ Ovarian (n=18) ▪ Non-Ovarian (N=15)	▪ Atypia (n=15) ▪ Concurrent cancer (n=14) subdivided into ▪ Concurrent Endometriosis (n= 14) ▪ Concurrent cancer (n=14)	▪ Endometrioid (n=15) ▪ Clear cell (n=15) ▪ Serous (n=15)	Total (N = 155)