

Manuscript EMM-2011-01063

p53 suppresses type II endometrial carcinomas in mice and governs endometrial tumour aggressiveness in humans

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Review timeline:

Submission date:	08 November 2011
Editorial Decision:	05 December 2011
Revision received:	20 March 2012
Editorial Decision:	20 April 2012
Revision received:	20 April 2012
Accepted:	23 April 2012

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

05 December 2011

Thank you for submitting your manuscript for consideration to EMBO Molecular Medicine. Three referees have now seen it, whose comments are shown below.

As you will see, all three referees find your study of interest. However, they also raised a certain number of issues that are explicitly reported in the reviews. Nevertheless I would like to highlight few points that are particularly important for our journal:

- Despite Ref. #1 recommendation to remove the human data, we would encourage you not to. Instead please provide additional information regarding intensity of staining and clarify p53 mutation status (ref. #1)
- Clarify the expression pattern of pAKT as associated with survival (ref. #2 major remark)
- Amend nomenclature of tumor type and provide better images of different cancer types as recommended by Ref. #3

I would like to invite you to submit a revised version of the manuscript, fully addressing the comments of all three reviewers, within the time constraints outlined below. Please note that it is EMBO Molecular Medicine policy to allow only a single round of major revision and that, as acceptance or rejection of the manuscript will depend on another round of review, your responses should be as complete as possible.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

All aspects of the mouse model are high, but the human studies require additional studies as my remarks below explain.

Referee #1 (Other Remarks):

This is an interesting manuscript that reports a novel and important genetic mouse model of Type II endometrial carcinoma. The authors created a Trp53 conditional knock-out strain that deletes Trp53 in the epithelium of the genitourinary tract. The deletion of Trp53 resulted in Type II endometrial carcinomas arising in aged female mice. Not only did the mice develop invasive cancers of varied histologic appearances (serous, clear cell, MMMT) they also developed precursor lesions histologically similar to those seen in humans.

In their initial attempts to characterize the mouse model the authors carried out some immunohistochemical analyses, which revealed activation of the Pi3k/Pten pathway in a significant subset of the tumors. To follow up these results, the authors undertook immunohistochemical analyses of a large number of primary human endometrial carcinomas using a TMA. They report that immunostaining with p53 is an independent predictor of outcome for endometrial carcinoma "that should be routinely employed by pathologists to assist with risk stratification of endometrial carcinoma".

The mouse model is novel and should be published. However it is disappointing that there is not more of an attempt to characterize the mouse tumors beyond morphology and immunohistochemistry. It is this reviewer's opinion that it might be best to remove the human studies (for the reasons cited below) and report the mouse model as a brief communication or short report. This could then be followed by future studies further characterizing the mouse model. I have no doubt that this model will be a powerful tool for furthering our understanding of Type II tumors and will generate great interest in the field.

Although the human studies are interesting they do not add much to the existing literature. It has been found in a number of previous studies (on smaller cohorts) that immunohistochemical staining for p53 is an independent prognostic marker for endometrial carcinoma. The authors suggest that what is new is that a number of Type I tumors stain for p53, although the majority show much less staining than serous tumors, and that this correlates with outcome. Given the novelty of this finding, additional supportive data are necessary. P53 immunohistochemistry is for the most part a surrogate marker for the presence of p53 mutations. It is generally accepted that missense mutations result in diffuse, intense staining while nonsense mutations lead to a complete absence of staining. It is not clear what less diffuse staining (i.e., scores of 1-10%, 11-50% and even 51-90%) translate to at the molecular level. Such molecular studies on at least a subset of the tumors are required. Additional studies on scoring the immunostaining needs to be done before such strong clinical recommendations can be made. This is especially important given the known problems with TMAs and their inability to reflect the heterogeneity of tumors.

Finally, I think the authors point out something that is often not communicated well in the existing literature. That is: Grade 3 endometrioid tumors, although they may have arisen through a similar pathogenetic pathway as Grade 1 and 2 tumors, are best considered Type II tumors. This may in fact

be due to the acquisition of p53 mutations as several studies have shown that over half of Grade 3 tumors have p53 mutations.

In sum, the mouse model is novel and will be of interest to a large group of investigators. However, it has not been well characterized in this manuscript. But, since those studies are time and labor intensive, I would suggest publishing the mouse model in a short format. Furthermore, the human studies require additional studies and should be separated from the current mouse studies. They don't add significant novel data and only, in my opinion, dilute the impact of this powerful mouse model.

Referee #2 (Comments on Novelty/Model System):

This paper changes the way we stratify risk for type I endometrial cancer by introducing p53 as a novel negative predictor.
Furthermore, the novel mouse model will help molecular and preclinical studies of type II cancers.

Referee #2 (Other Remarks):

This is a well-written manuscript describing the first mouse model of type II endometrial cancer, based on p53 ablation in the mouse endometrium.

The authors convincingly show that, with age, these mice display the pathological and morphological progression observed in human patients, including the presence of the characteristic precursor lesions. In addition, they show rather frequent activation of the PI3K/mTOR pathway, more often in more advanced lesions, suggesting that this pathway cooperates with p53 loss to establish type II cancers, especially of the papillary type.

The second part of the manuscript uses a large number of human samples to convincingly show that PI3K signaling is active not only in type I, but also in type II endometrial cancer. Furthermore, p53 expression is validated as a sensitive marker for poor prognosis, even in type I cancers.

In general, this paper represents an important contribution to our understanding of endometrial cancer pathogenesis, and to the development of better prognostic markers.

MAJOR REMARKS

It is striking that, in Fig. 5, p110alpha, pS6, p4E-BP1, and pGSK3beta all predict poor survival, while high pAKT is associated with better survival. This result represents a major inconsistency.

MINOR REMARKS:

A subset of tumors displays activation of mTOR targets without evidence of AKT activation, suggesting that an alternative pathway to mTOR activation may exist. This should be discussed.
Page 6, second paragraph, third line: "...around 6 months of age (1F)". According to the figure legends, this picture corresponds to 10 weeks old mice. This point should be clarified.

Table S1: The total number of patients/samples is listed as 515. However, adding the number of patients divided by age yields 520, and adding all the patients listed by FIGO stage yields 521.

In the legend to Figure 2, "illustrating" is misspelled.

Page 12, line 14: there is an extra semicolon (;).

Referee #3 (Comments on Novelty/Model System):

Wild et al in this study showed solid evidence of p53 gene function governs the aggressiveness of the endometrial carcinoma. The study design was good and the results are convincing. However, I do have the following comments for the authors to modify when they revise the manuscript.

1. Abstract: Since the precursor lesions of EmGD and EIC are important findings in the study, the authors should cover the findings with a few more sentences in the abstract.
2. Introduction and Discussion: There are several concepts about the endometrial cancer the authors need to understand clearly and modify the text and wording accordingly in the manuscript.
 - a) Carcinosarcoma is currently classified into type II cancer category.
 - b) Type II cancer develops in a stepwise fashion from latent precancer which is p53 signature

endometrial glands (Zhang X et al, 2009 and Jarboe et al., 2009) to precancer (EmGD), to non-invasive cancer (EIC), and to full blown carcinoma (endometrial serous carcinoma). It is fine that your current study may not address the issue of latent precancer, but it should be addressed either in the Introduction or Discussion section. The word "precursor" is vague sometimes, particularly in this setting. For instance, EIC as a precursor for endometrial serous carcinoma can be misread as EIC as a precancer. In reality, EIC is a special form of endometrial serous carcinoma which is associated very high extrauterine disease frequency (Zheng and Schwartz 2005). The authors should use the term carefully in the text.

c) EmGD represents an earliest morphologically identifiable precancerous lesion under microscope. Please make corresponding changes in line 6, second paragraph of page 8.

d) Type II cancers develop mainly in elderly women. That is true. However, it is not necessary the background endometrium is always atrophic. This is mainly because many postmenopausal women are using hormone replacement or estrogen like food supplements, which ultimately change the endometrial proliferative status. In those conditions, Zheng et al called as resting endometrium in their publications (Zheng et al., 2011). Therefore, the first sentence in second paragraph on page 3 should be modified accordingly.

e) Serous carcinoma is the prototype cancer for type II endometrial cancer. Previously, papillary structure was emphasized. These days we know that it is not necessary for serous carcinoma always has papillary structures, while carcinomas with papillary structures are not necessary serous type. Therefore, the term uterine papillary serous carcinoma (UPSC) has been gradually dropped. The current most accepted term is endometrial serous carcinoma (ESC) by emphasizing the serous cellular changes rather than structures.

3. Figures:

a) Figure 1J: This is not a classic picture for a mixed serous and clear cell carcinoma mainly because the nuclear grade is too low.

b) Figure 1K: I understand that this picture in the old days, many pathologists would diagnose it as endometrioid carcinoma. But nowadays it is diagnosed as serous carcinoma (glandular type) without problem since all tumor cells have high-grade nuclei.

c) Figure 1N: This is also equal to a serous carcinoma with solid growth pattern.

d) Figure 2B: This EmGD picture is not representative. It looks like EIC. The degree of nuclear atypia looks worse than EIC in figures 2C and 2G. By definition, the degree of nuclear atypia of EmGD falls short of EIC. The better pictures should be used. Many figures of EmGD in supplement data are good.

4. Some specific points:

a) Page 4, line 12, the reference Jarboe et al., 2009 does not belong to the text.

b) Page 9, line 7 and others: "papillary EIC" is not a good term. The authors may use "EIC with papillary structures".

c) Page 9, line 9: fibrogenic should be fibrovascular.

d) Page 9, line 20 to 22: continuous transitions from normal looking endometrium to EmGD, to EIC, and to ESC was originally observed and described by Zheng et al. (2004). Therefore, it is not your proposal. Authors should give the credit to previous publication and state supported the previously proposed...

e) Page 16, last sentence: It is too early to recommend that p53 staining is used in every endometrial cancer case. This will not be cost-effective. Therefore, authors should modify the sentence or remove it since authors have developed risk score strategy in this study, which is certainly superior than p53 staining alone in clinical setting.

5. Some relevant questions:

a) Since the authors generated genital-urinary tract specific deletion of Trp53, cancer or cancer like lesions are expected to be developed in other organs such as kidney and bladder. What were the general findings in addition to endometrial type II cancers? Authors should briefly mention in the text.

b) In this mouse model, can authors describe the time (how many weeks) of EmGD starts prior to the observation of full brown type II cancer or EIC?

**University of
Zurich** ^{UZH}**Institute of Physiology****Referee # 1**

Although the human studies are interesting they do not add much to the existing literature. It has been found in a number of previous studies (on smaller cohorts) that immunohistochemical staining for p53 is an independent prognostic marker for endometrial carcinoma. The authors suggest that what is new is that a number of Type I tumors stain for p53, although the majority show much less staining than serous tumors, and that this correlates with outcome. Given the novelty of this finding, additional supportive data are necessary. P53 immunohistochemistry is for the most part a surrogate marker for the presence of p53 mutations. It is generally accepted that missense mutations result in diffuse, intense staining while nonsense mutations lead to a complete absence of staining. It is not clear what less diffuse staining (i.e., scores of 1-10%, 11-50% and even 51-90%) translate to at the molecular level. Such molecular studies on at least a subset of the tumors are required. Additional studies on scoring the immunostaining needs to be done before such strong clinical recommendations can be made. This is especially important given the known problems with TMAs and their inability to reflect the heterogeneity of tumors.

We disagree that our human cancer studies do not add much to the existing literature. We would like to emphasise that we have systematically analysed in a single cohort almost all of the markers that have been previously proposed to be involved in endometrial carcinoma pathogenesis. We identify that the mTOR pathway is frequently activated in type II tumours and that this is predictive of a poor outcome, highlighting a molecular similarity with type I tumours. Using this dataset we have also developed a novel statistical method to assess whether combinations of the 14 tested markers may yield more prognostic information than individual markers alone. Interestingly, this unbiased approach revealed that the immunohistochemical (IHC) status of p53 alone is almost an equally good predictive marker as the 4 protein signature, implying a fundamentally important role of p53 in the pathogenesis of both type I and type II tumours.

We appreciate the reviewer's comment that our IHC findings would be strengthened by molecular analyses of *TP53* gene mutation status. To attempt to allow a molecular interpretation of IHC scores where only a fraction of the tumour cells stain strongly for p53 we undertook deep sequencing analyses of exons 5-8 of the *TP53* gene in 63 endometrial carcinoma cases from endometrioid or serous carcinoma subtypes that displayed different frequencies of IHC staining. DNA was isolated from punches taken directly adjacent to the punches used for the TMA analysis to attempt to minimize heterogeneity between different regions of the tumour. The obtained depth of sequencing coverage (an average of over 2000 reads per amplicon) allowed insight into the heterogeneity of *TP53* mutations present in each individual tumour. This data has been included as a new Figure 7 and new Supporting Information Table 2 in the manuscript and is described in the Results and Discussion sections. Briefly, this data shows that there is an excellent correlation between the frequency of *TP53* mutations in the tumour and the IHC score, particularly for those cases with a strong IHC score. Moreover, we could show that there is an excellent correlation between mutations that are predicted to have a strong detrimental effect on protein function and the frequency of occurrence of these mutations within the tumour population, implying that these mutations provide a selective advantage to the tumour cells that leads to their enrichment in the tumour. Finally, tumours in which the most abundant mutation was present in more than 33% of the sequences had a much worse prognosis than those where the most abundant mutation was present at frequencies less than 33%.



Yemelyanova et al. (Modern Pathology, 2011, 24:1248-1253) similarly identified a correlation between a high p53 IHC score and the occurrence of *TP53* mutations in ovarian carcinomas. These authors additionally identified that 31% of ovarian carcinomas that display completely negative p53 IHC staining are in fact associated with *TP53* mutations (that presumably truncate or render the p53 protein unstable or alter the epitope detected by the antibody). In our endometrial carcinoma cases we also found that some negative p53 IHC score tumours contained *TP53* mutations. However, the frequencies of these mutations were not highly abundant (compared to the frequencies found in tumours that displayed a high p53 IHC score) suggesting that i) these mutations either do not lead to aberrant stabilisation of the p53 protein or render the p53 protein unrecognisable by the p53 antibody and ii) that these mutations do not provide a selective advantage to the tumour cells that harbour them. This latter point is consistent with the good prognosis that p53 IHC negative tumours display in our survival analyses.

Not surprisingly, we also identified tumours that display a strong p53 IHC score but which do not display an obvious dominant *TP53* mutation. It is possible that these tumours may harbor mutations in *TP53* exons that we have not sequenced, or that p53 may be aberrantly stabilized in these tumours due to alterations in one of the many regulatory pathways that control p53 protein stability and function (eg. loss or mutation of Mdm2). In this respect, we believe that p53 IHC has the advantage of being able to identify either *TP53* mutations or other aberrations in the p53 pathway in endometrial tumours. In summary, we believe that these new gene mutation studies provide molecular evidence to support our proposal that p53 IHC status is an excellent predictive marker of endometrial carcinoma prognosis that could be employed routinely in the clinic. We also now provide new pictures in Supporting Information Figure 8 that show representative p53 IHC staining patterns for each IHC score to aid other investigators who may wish to perform similar studies on independent cohorts of tumours.

Referee #2**MAJOR REMARKS**

It is striking that, in Fig. 5, p110alpha, pS6, p4E-BP1, and pGSK3beta all predict poor survival, while high pAKT is associated with better survival. This result represents a major inconsistency.

We thank the reviewer for raising this pertinent question. An important point to clarify is that the PI3K-AKT-mTORC1 signalling pathway should not be considered as a strictly linear cascade. There are numerous inputs into this pathway (amino acids, growth factors, cellular energy status, hypoxia) at different levels, as well as several points of negative feedback regulation, most prominently several mechanisms downstream of mTORC1 activation that involve S6K-mediated inactivation of IRS proteins or PDGF receptors. This feedback normally serves to balance the input and output signals to/from mTORC1. In the context of tumours, genetic alterations that lead to constitutive activation of mTORC1 (such as mutations or silencing of TSC1 or TSC2) cause constitutive feedback inhibition of AKT signaling (see Manning, 2004, JCB 3: 399-403). Thus, a lowered activation status of AKT can in some instances be a consequence of overactive mTORC1 signalling and AKT must be considered as being both upstream and downstream of mTORC1.

We attempted to further clarify the activation status of the AKT kinases in our cohort of human tumour samples by IHC staining using antibodies against phospho-Thr-308 AKT (phosphorylation at this site results from activity of the PKD1 kinase and is necessary for AKT activation) and against phospho-AKT substrate (this antibody recognizes phosphorylated moieties in substrates that are targeted by AKT, thus serving as a global readout of AKT kinase activity). Unfortunately, despite several attempts in our own staining facilities and using those of another diagnostic pathology unit in a hospital in Germany, we were unable to obtain reliable stainings for these antibodies. This is a recurring problem that we have observed when attempting to monitor AKT activity in a variety of different tumour types. The staining for all AKT activation markers is almost always very weak. We speculate that this may be partly due to the quality and sensitivity of the available antibodies but also in large part due to the aforementioned feedback inhibition mechanisms that act as a cellular “rheostat” to keep AKT activation at a low and appropriate level.

Given that the AKT data are therefore less “concrete” than the mTORC1 activation status data (for which we have multiple downstream markers that show the same trend) we have altered the text in numerous places in the manuscript to remove the emphasis on the “PI3K-AKT-mTORC1” pathway and instead describe this either as the “mTORC1” pathway or to describe “PI3K and/or mTORC1 activation” depending on the relevant data set. This focus emphasizes that mTORC1 is the common downstream effector of several different genetic alterations that are known to occur in endometrial tumours (see also below).

MINOR REMARKS:

A subset of tumors displays activation of mTOR targets without evidence of AKT activation, suggesting that an alternative pathway to mTOR activation may exist. This should be discussed.

We agree that this interpretation is one possibility (but see above for discussion regarding interpretation of AKT activation data). Indeed, as referenced in the manuscript, it has been reported that loss of TSC2 or LKB1 expression occurs in 13% and 21% of type I endometrial tumours, respectively (Lu et al. 2008, Human Cancer Biology, 14:2543-2550). Genetic mutations in the LKB1-TSC1/2-Rheb signaling axis

can cause mTORC1 activation independently of AKT activation. We have now conducted IHC staining for TSC1 and TSC2 to test this possibility in our cohort of human endometrial tumours. This data has been included as new panels in Figures 4 and 5. While these studies show that some tumours have lower levels of expression than others, there are no major significant differences between subtypes of endometrial tumours, nor do these expression differences provide prognostic information.

We believe that ultimately it will be necessary to perform studies in which comprehensive sequencing analyses are performed for each individual tumour on all of the genes that are known to be positive and negative regulators of the PI3K-AKT-mTORC1 signalling pathway or of other mTORC1 regulatory pathways. It is highly likely that there will be many different combinations of mutations or genetic alterations that lead to activation of some or all components of these signalling cascades in each individual tumour. Our IHC data obtained using P-S6 and P-4E-BP1 as the most downstream readouts of activation of mTORC1 should provide further impetus to undertake such ambitious analyses in future studies.

Page 6, second paragraph, third line: "...around 6 months of age (1F)". According to the figure legends, this picture corresponds to 10 weeks old mice. This point should be clarified.

The original figure legend was incorrect. The pictures are representative of 10 week old kidneys and uteri but of 6 month old epididymides. We have adapted the figure legend to clarify this.

Table S1: The total number of patients/samples is listed as 515. However, adding the number of patients divided by age yields 520, and adding all the patients listed by FIGO stage yields 521.

We apologise for this inconsistency. Supplementary Information Table 1 has been corrected ($N_{\text{total}}=521$).

In the legend to Figure 2, "illustrating" is misspelled.

This has been corrected.

Page 12, line 14: there is an extra semicolon (;).

This sentence has been corrected.

Referee #3

However, I do have the following comments for the authors to modify when they revise the manuscript.

1. Abstract: Since the precursor lesions of EmGD and EIC are important findings in the study, the authors should cover the findings with a few more sentences in the abstract.

In principle we agree that this would be a good idea, but unfortunately this is not possible due to the strict word limit of the abstract. Defining “endometrial glandular dysplasia” and “endometrial intra-epithelial carcinoma” in the abstract requires too many words and would take away from the other messages of the paper. We therefore chose to use the general term “precursor lesions” in the abstract and these have been well defined and discussed in the manuscript in several places.

2. Introduction and Discussion: There are several concepts about the endometrial cancer the authors need to understand clearly and modify the text and wording accordingly in the manuscript.

a) Carcinosarcoma is currently classified into type II cancer category.

We thank the reviewer for raising this issue. In our study, three mice developed carcinosarcomas (Table 1). This strongly confirms one of our major findings; i.e. the generation of a mouse model of type II endometrial cancer. Immunohistochemical and molecular studies strongly support the inclusion of uterine carcinosarcomas in the epithelial group, especially in the type II category of endometrial carcinomas. However, classification of carcinosarcomas as type II endometrial carcinoma is still a matter of debate. According to the WHO classification of tumours of the female genital tract (Tavassoli & Devilee (Eds.): World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. IARC Press: Lyon 2003) uterine carcinosarcomas are separately classified as a mixed epithelial and mesenchymal neoplasm whose prognosis is worse than that of members of the epithelial category. This separate classification is also the case in a couple of current textbooks for gynecologic pathology (e.g.: Nucci & Oliva: Gynecologic Pathology. A Volume in Foundations in Diagnostic Pathology Series. Goldblum (Ed). Churchill Livingstone: 2009). Others include carcinosarcomas within the type II category (Dedes et al., Nat Rev Clin Oncol 2011). Nonetheless, we decided to keep carcinosarcomas as a separate tumour entity for our statistical analyses to be as precise and descriptive as possible.

b) Type II cancer develops in a stepwise fashion from latent precancer which is p53 signature endometrial glands (Zhang X et al, 2009 and Jarboe et al., 2009) to precancer (EmGD), to non-invasive cancer (EIC), and to full blown carcinoma (endometrial serous carcinoma). It is fine that your current study may not address the issue of latent precancer, but it should be addressed either in the Introduction or Discussion section. The word "precursor" is vague sometimes, particularly in this setting. For instance, EIC as a precursor for endometrial serous carcinoma can be misread as EIC as a precancer. In reality, EIC is a special form of endometrial serous carcinoma which is associated very high extrauterine disease frequency (Zheng and Schwartz 2005). The authors should use the term carefully in the text.

We appreciate this helpful clarification and have altered the relevant section of the Introduction accordingly as follows. “Endometrial serous adenocarcinomas are believed to arise from precursor lesions termed endometrial glandular dysplasia (EmGD) that progress to a non-invasive cancer termed endometrial intraepithelial carcinoma (EIC) (Fadare & Zheng, 2009; Zheng et al, 2007; Zheng et al,

2004; Zheng et al, 2011). Approximately 50-75% of these lesions display *TP53* mutations (Jia et al, 2008; Liang et al, 2004). p53 immunohistochemical signatures, termed latent precursors, can also be observed in benign endometrial glands, suggesting that *TP53* mutation occurs as the very first step of serous adenocarcinoma formation {Jarboe, 2009 #358; Zhang, 2009 #357}.”

c) EmGD represents an earliest morphologically identifiable precancerous lesion under microscope. Please make corresponding changes in line 6, second paragraph of page 8.

This sentence has been changed as suggested. “EmGD represents the earliest morphologically identifiable serous carcinoma precancerous lesion in humans (Yi & Zheng, 2008; Zheng et al, 2004)”

d) Type II cancers develop mainly in elderly women. That is true. However, it is not necessary the background endometrium is always atrophic. This is mainly because many postmenopausal women are using hormone replacement or estrogen like food supplements, which ultimately change the endometrial proliferative status. In those conditions, Zheng et al called as resting endometrium in their publications (Zheng et al., 2011). Therefore, the first sentence in second paragraph on page 3 should be modified accordingly.

Thank you for this clarification. We have altered the sentence about the atrophic endometrium/estrogenic effect as this information is not directly relevant to the topic of the current paper.

e) Serous carcinoma is the prototype cancer for type II endometrial cancer. Previously, papillary structure was emphasized. These days we know that it is not necessary for serous carcinoma always has papillary structures, while carcinomas with papillary structures are not necessary serous type. Therefore, the term uterine papillary serous carcinoma (UPSC) has been gradually dropped. The current most accepted term is endometrial serous carcinoma (ESC) by emphasizing the serous cellular changes rather than structures.

We are aware of the nomenclature changes. According to the reviewer’s comment, we have changed the diagnoses in Figures 1 and 2 as well as in the manuscript. Growth patterns are now provided in brackets only.

3. Figures:

a) Figure 1J: This is not a classic picture for a mixed serous and clear cell carcinoma mainly because the nuclear grade is too low.

We have replaced the picture in Figure 1J with a new image that shows the more typical high nuclear grade that we observe in these mouse tumours.

b) Figure 1K: I understand that this picture in the old days, many pathologists would diagnose it as endometrioid carcinoma. But nowadays it is diagnosed as serous carcinoma (glandular type) without problem since all tumor cells have high-grade nuclei.

We have changed the diagnosis in Figure 1K accordingly. The list of diagnoses in Table I has also been adjusted.

c) Figure 1N: This is also equal to a serous carcinoma with solid growth pattern.

The neoplasia in Figure 1N is a non-endometrioid (type II) endometrial cancer. Per definition, undifferentiated carcinomas are those lacking any evidence of differentiation (Tavassoli, Devilee (Eds.):

World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. IARC Press: Lyon 2003) which is the case for the tumour in Figure 1N. Therefore, we have designated the lesion as undifferentiated carcinoma rather than serous adenocarcinoma with solid growth pattern. Both entities in humans show a very poor clinical outcome and a distinction is in some respects an academic discussion. For the present type of study, however, it is important to be as precise as possible and to emphasize that some tumour lesions of our mouse model did not show any differentiation.

d) Figure 2B: This EmGD picture is not representative. It looks like EIC. The degree of nuclear atypia looks worse than EIC in figures 2C and 2G. By definition, the degree of nuclear atypia of EmGD falls short of EIC. The better pictures should be used. Many figures of EmGD in supplement data are good.

Thank you for this comment. We have replaced the images in 2B and 2C to better reflect the representative nuclear atypia that are characteristic of these types of lesions. We have attempted in the text of the manuscript to better emphasise that what we observe in the mouse are a spectrum of lesions that are consistent with increasing degree of malignancy in terms of nuclear morphology and structure of the epithelium. We have generally classified these precursor lesions based on the EmGD/EIC descriptors previously defined for human tissues. One benefit of the mouse model is that there are many, many lesions that may be observed. We therefore also observe some lesions within the malignant spectrum that exhibit overlapping features of both EmGD and EIC.

4. Some specific points:

a) Page 4, line 12, the reference Jarboe et al., 2009 does not belong to the text.

Thank you. This mis-citation has been moved to the relevant position in the rewritten section of the Introduction (see point 2b for details).

b) Page 9, line 7 and others: "papillary EIC" is not a good term. The authors may use "EIC with papillary structures".

We have altered this term as suggested.

c) Page 9, line 9: fibrogenic should be fibrovascular.

This has been corrected.

d) Page 9, line 20 to 22: continuous transitions from normal looking endometrium to EmGD, to EIC, and to ESC was originally observed and described by Zheng et al. (2004). Therefore, it is not your proposal. Authors should give the credit to previous publication and state "supported the previously proposed..."

We agree completely with this point and it was not our intention to give the impression that this was a new idea but rather to illustrate that the mouse model is completely consistent with the proposed model in human disease. We have altered the relevant section of the results to better illustrate this point.

"Based on their morphologies and relative frequencies of occurrence, it appears that these lesions represent a spectrum of histological changes that occur in the progression from a normal epithelium to a carcinoma, consistent with the model proposed for human endometrial serous adenocarcinoma {Zheng, 2004 #354}. Papillary adenocarcinomas in our model appear to form exclusively from the surface epithelium of the lumen (Fig 2I) whereas adenocarcinomas with an acinar growth pattern arise from endometrial glands (Fig 2E). Supporting the progression model, and consistent with observations in

human disease {Zheng, 2004 #354}, the endometrial epithelium directly surrounding several papillary adenocarcinomas (Fig 3G shows an example) displays a continuous transition from normal epithelium (Fig 3J) to EmGD (Fig 3M) to EIC (Fig 3P) to a papillary adenocarcinoma growth pattern (Fig 3P).”

e) Page 16, last sentence: It is too early to recommend that p53 staining is used in every endometrial cancer case. This will not be cost-effective. Therefore, authors should modify the sentence or remove it since authors have developed risk score strategy in this study, which is certainly superior than p53 staining alone in clinical setting.

We disagree with this suggestion that we should exclude the recommendation to perform p53 immunohistochemistry. p53 IHC stainings are routine procedures in virtually all pathology laboratories worldwide. The cost-effectiveness is best determined by the prognostic value of the procedure. In our case we present convincing evidence that the prognostic value is extremely high. We would like to emphasise that we have systematically analysed in a single cohort almost all of the markers that have been previously proposed to be involved in endometrial carcinoma pathogenesis. We identify that the mTOR pathway is frequently activated in type II tumours and that this is predictive of a poor outcome, highlighting a molecular similarity with type I tumours. Using this dataset we have also developed a novel statistical method to assess whether combinations of the 14 tested markers may yield more prognostic information than individual markers alone. Interestingly, this unbiased approach revealed that the immunohistochemical (IHC) status of p53 alone is almost an equally good predictive marker as the 4 protein signature, implying a fundamentally important role of p53 in the pathogenesis of both type I and type II tumours.

We appreciate the reviewer’s comment that our IHC findings would be strengthened by molecular analyses of *TP53* gene mutation status. To attempt to allow a molecular interpretation of IHC scores where only a fraction of the tumour cells stain strongly for p53 we undertook deep sequencing analyses of exons 5-8 of the *TP53* gene in 63 endometrial carcinoma cases from endometrioid or serous carcinoma subtypes that displayed different frequencies of IHC staining. DNA was isolated from punches taken directly adjacent to the punches used for the TMA analysis to attempt to minimize heterogeneity between different regions of the tumour. The obtained depth of sequencing coverage (an average of over 2000 reads per amplicon) allowed insight into the heterogeneity of *TP53* mutations present in each individual tumour. This data has been included as a new Figure 7 and new Supporting Information Table S2 in the manuscript and is described in the Results and Discussion sections. Briefly, this data shows that there is an excellent correlation between the frequency of *TP53* mutations in the tumour and the IHC score, particularly for those cases with a strong IHC score. Moreover, we could show that there is an excellent correlation between mutations that are predicted to have a strong detrimental effect on protein function and the frequency of occurrence of these mutations within the tumour population, implying that these mutations provide a selective advantage to the tumour cells that leads to their enrichment in the tumour. Finally, tumours in which the most abundant mutation was present in more than 33% of the sequences had a much worse prognosis than those where the most abundant mutation was present at frequencies less than 33%.

Yemelyanova et al. (Modern Pathology, 2011, 24:1248-1253) similarly identified a correlation between a high p53 IHC score and the occurrence of *TP53* mutations in ovarian carcinomas. These authors additionally identified that 31% of ovarian carcinomas that display completely negative p53 IHC staining are in fact associated with *TP53* mutations (that presumably truncate or render the p53 protein

unstable or alter the epitope detected by the antibody). In our endometrial carcinoma cases we also found that some negative p53 IHC score tumours contained *TP53* mutations. However, the frequencies of these mutations were not highly abundant (compared to the frequencies found in tumours that displayed a high p53 IHC score) suggesting that i) these mutations either do not lead to aberrant stabilisation of the p53 protein or render the p53 protein unrecognisable by the p53 antibody and ii) that these mutations do not provide a selective advantage to the tumour cells that harbour them. This latter point is consistent with the good prognosis that p53 IHC negative tumours display in our survival analyses.

Not surprisingly, we also identified tumours that display a strong p53 IHC score but which do not display an obvious dominant *TP53* mutation. It is possible that these tumours may harbor mutations in *TP53* exons that we have not sequenced, or that p53 may be aberrantly stabilized in these tumours due to alterations in one of the many regulatory pathways that control p53 protein stability and function (eg. loss or mutation of Mdm2). In this respect, we believe that p53 IHC has the advantage of being able to identify either *TP53* mutations or other aberrations in the p53 pathway in endometrial tumours. In summary, we believe that these new gene mutation studies provide molecular evidence to support our proposal that p53 IHC status is an excellent predictive marker of endometrial carcinoma prognosis that could be employed routinely in the clinic. We also now provide new pictures in Supporting Information Figure 8 that show representative p53 IHC staining patterns for each IHC score to aid other investigators who may wish to perform similar studies on independent cohorts of tumours.

5. Some relevant questions:

a) Since the authors generated genital-urinary tract specific deletion of *Trp53*, cancer or cancer like lesions are expected to be developed in other organs such as kidney and bladder. What were the general findings in addition to endometrial type II cancers? Authors should briefly mention in the text.

To address this useful comment we have added the following sentence to the results section (page 6) “*Trp53^{ΔA}* mice displayed no pathological alterations in the kidney, ureter, bladder, vas deferens or vesicular glands up until at least 79 weeks of age.”

The following sentences were added to the Discussion section “*Trp53* gene deletion under the control of the *Ksp1.3-Cre* transgene occurs during embryogenesis and induces gene deletion widely in the kidney and genitourinary tract, yet tumours arise only in the endometrium in old mice, suggesting that additional genetic alterations must accumulate and cooperate with *Trp53* deficiency to initiate cancer. The absence of tumours in tissues other than the endometrium likely reflects the very low rate of cellular turnover in these tissues in comparison to the cyclic nature of cellular proliferation in the endometrium, which presumably allows mutations to frequently accumulate in the absence of p53 function.”

b) In this mouse model, can authors describe the time (how many weeks) of EmGD starts prior to the observation of full blown type II cancer or EIC?

Thank you for this excellent question. We have added a new Supporting Information Figure S1 that depicts representative lesions that are observed in mice at different ages. These observations are now described in the results section (page 9/10) by the following sentences. “Furthermore, all mice in the 24-29 week-old cohort displayed some regions of EmGD (Supporting Information Fig 1E,H,N,Q) and



all mice in the 47-58 week-old cohort frequently displayed widespread regions of EmGD and/or EIC (Supporting Information Fig 1F,I,O,R) but did not display any tumours. Thus, as they age, *Trp53*-deficient mice progressively display a spectrum of endometrial histological alterations ranging from low nuclear grade dysplasia through to invasive high grade carcinomas. These mice therefore represent the first model that accurately reproduces the progression and hallmark morphological features of human type II endometrial carcinomas.”

2nd Editorial Decision

20 April 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now globally supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

As you will see below, two referees are not fully satisfied with statements regarding the novelty and recommendation of systematically use p53 staining on endometrial carcinomas. We would appreciate if you could tune down these statements in the main text of the manuscript.

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

I maintain my opinion about the human data. It is not novel and their recommendation of staining every endometrial carcinoma with p53 is not convincing.

Referee #1 (Other Remarks):

Despite the authors rebuttal of the data on the human tumors what they have done is not novel. All of these markers have been previously looked at in primary human tumors. The association of positive p53 immunohistochemical staining with poor prognosis has been known for almost 2 decades, including the relationship of IHC staining and mutations (it has been shown in endometrial carcinoma that negative IHC staining correlates with nonsense mutations, you don't need to cite recent ovarian cancer data). Furthermore, many of the PTEN/AKT/PI3K downstream effectors have been studied at the protein and gene expression level. Therefore, I maintain that mouse model is very powerful and novel and should be characterized and correlated with human tumors. That is what makes this manuscript important and valuable.

Referee #2 (Comments on Novelty/Model System):

The authors have addressed the points raised by the reviewers.

Referee #2 (Other Remarks):

The points raised by the three reviewers have been adequately addressed, and the manuscript is now acceptable for publication.

Referee #3 (Other Remarks):

After extensive revision, the manuscript reaches to the publication level. However, I still insist that one statement on page 15, last sentence of the first paragraph, should be modified. It is too early to recommend that p53 staining is used in every endometrial cancer case in the clinic. This kind of recommendation typically derives from large scale clinical trials, which is certainly not applied to current study.

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Regarding the comments of reviewer 1 and reviewer 3 we have modified the relevant sections of the text according to the suggestions to remove the emphasis on the novelty and strength of the clinical recommendations. The relevant sections now read as follows:

Page 15: In this study, p53 immunoreactivity therefore represents a highly sensitive and predictive marker that assists with risk stratification of endometrial carcinomas.

Page 19: In summary, irrespective of endometrial carcinoma subtype, grade or FIGO stage, we find that strong p53 nuclear immunoreactivity represents an excellent marker to stratify patients into poor outcome groups, even in low grade and low FIGO stage tumours. We suggest that these patients, who would otherwise be stratified into a relatively low-risk group, should actually be considered as high-risk patients who would benefit from additional monitoring and therapy, possibly including the use of drugs to inhibit the PI3K pathway and/or strategies to kill p53 deficient tumour cells (Lane et al, 2010). We suggest that further clinical studies should investigate the possible value of performing p53 immunohistochemistry as a routine procedure to assist with risk stratification of endometrial carcinomas.

The Paper Explained: We have removed the clinical recommendation from the IMPACT section.

Regarding the additional comments of reviewer 1 we believe that our manuscript already does highlight the points that the reviewer makes. We cite the relevant literature in which others have

previously studied similar markers in these tumour types. We agree that the major novelty of our paper is that the mouse model closely correlates with the molecular and histological features of human tumours and have already highlighted these issues in numerous places in the text. We do not believe that any further manuscript changes are necessary to address this opinion of the reviewer.