Author's response to reviews

Title: Characterisation of prostate cancer lesions in heterozygous Men1 mutant mice

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Author's response to reviews: see over
Dear Editors,

Please find enclosed our revised manuscript entitled "Characterization of the prostate cancer lesions developed in heterozygous Men1 mutant mice" (MS: 2117687835334112). Following your decision of April 14th, 2010, we have studied seriously the reviewers' comments and requests. We are very grateful to two reviewers’ comments that we found well constructive and thoughtful. We consider that all the concerns raised by the reviewers are indeed important issues. More importantly, we found that all these issues can be appropriately addressed by more careful data interpretation and balanced discussion. We have done, therefore, all we could to clarify and discuss all the raised issues, and to revise the manuscript accordingly. As suggested by the Reviewer 1, we also submitted the manuscript to an extensive English edition as recommended. I believe, therefore, that the revised manuscript is improved, and that all the concerns raised by the reviewers are appropriately addressed. The manuscript is now we feel of significant interest to your readership.

Please find in the following our detailed response to the reviewers’ comments.

**Point by point answer**

**Reviewer 1:**

Major compulsory revisions:
It is missing in this article whether in human prostate cancers cells the menin-encoding gene is expressed at reduced levels (and also whether the AR, p63, CDKN1B and Nkx3.1 genes are expressed at lower levels) as compared to normal prostate tissue.

The MEN1 gene may have an oncogenic role in prostate cancer. (Paris PL et al. in Prostate Cancer Prostatic Dis. 2009;12(2):184-91.

Reply:
As requested, we have added the information about the expression of the above mentioned genes in human prostate cancers. We have not included the work by Paris et al in our previous manuscript, because our analyses were only carried out in the mouse model, so that the two works may not be comparable. Following the Reviewer 1’s suggestion, we referred to this work in the revised manuscript and discussed the possibility where menin may play a dual role (oncogenic or tumour suppressor) in prostate glands (page 16). The authors did point out the complex nature of Men1-related tumorigenesis and the limited number of samples in their study. We share, therefore, completely their cautious attitude in interpreting their data and will be looking forward to following their and others’ further reports on this issue.

As for the other genes, AR is largely expressed in most prostate cancers (please see page 13); the reduction or absence of p63 staining is a characteristic of carcinoma lesions (see page 12). Please see page 13 for CDKN1B expression and page 16 for menin expression.
The expression of Nkx3.1 is downregulated or completely inactivated in human prostate cancer, in correlation with tumor progression (page 16).

Results:
Page 12; what about Men1 gene LOH in mPIN lesions which have developed in wildtype mice?

Reply:
We have checked menin expression by immunohistochemistry in mPIN lesions developed in Men1 wild-type mice, and found the level was normal, which would not be the case if both Men1 wild-type alleles were lost. This is why we did not seek to detect LOH in these lesions.

Page 13: what about doublestaining for menin and AR to see whether AR is downregulated in the same cells which have downregulated menin expression?

Reply:
We have shown in figure 2B-I that in two out of four mPIN lesions and in all six prostate carcinoma lesions observed in our mutant mice, only a very few cells remained menin-positive. We demonstrated further in figure 3A-H that these few cells were remaining basal cells, but not cancer cells. As the vast majority of cells constituting the lesions were menin-negative, a double immunostaining for menin and AR will not, unfortunately, bring any additional information concerning the potential link between menin inactivation and AR downregulation. We hope that the Reviewer 1 will forgive us for not doing this analysis because of the above technical reason.

Discussion:
Page 16: which common partners are there between menin and AR (list them or provide reference)

Reply:
AR and menin share not only common partners but also common target genes. The term “wide range” was probably not accurate. We revised accordingly the sentence and named some of these common partners and target genes in the discussion of the manuscript, namely Smad3, β-catenin, p27 and cyclins D (page 16-17).

The question arises whether in man, the AR is present in all prostate cancers. The clinical prognosis may be influenced by activation of the AR. A more favourable outcome may depend on the repression of the AR. Androgens may promote growth and malignant transformation of prostate tissue. This observation provides the rationale for approaches that either prevent the production of androgens or blocks their action. Androgen deprivation therapy (ADT) is the standard initial approach for all patients with metastatic prostate cancer. ADT utilizes either bilateral orchidectomy or medical castration (i.e. a gonadotropin releasing hormone agonist with or without an anti-androgen). This approach may not be very effective, and even highly undesirable, for patients whose prostate tumor is AR-negative anyhow!

Reply:
AR is indeed expressed in the majority of human prostate cancers, resistant or not to ADT. Most prostate cancers with metastasis respond to androgen deprivation therapy (ADT), at least in the beginning. But after 1-2 years of treatment, it has almost always been observed that they became androgen-independent, probably through the reactivation of AR.
independently from androgens (via AKT activation or AR amplification for example). The reviewer's comment is indeed thoughtful. Our present data do not allow us, however, to give any further comment on ADT.

It is useful to refer to the article of Imachi et al.:
Menin, a product of the MENI gene, binds to estrogen receptor to enhance its activity in breast cancer cells: possibility of a novel predictive factor for tamoxifen resistance.
Menin positive breast tumours had a worse outcome than menin-negative ones. Menin functions as a transcriptional regulator of ER# and is a possible predictive factor for tamoxifen resistance. Menin-negative patients had a more favourable disease-free survival than menin-positive group by Log-rank-test. On the basis of the results of this study, it can be hypothesized that expression of menin in breast cancer cells might be associated with resistance to anti-estrogens.
In analogy with the presence of ER# receptor in breast cancer, the presence of the androgen receptor in prostate cancer may be predictive of the outcome and survival and direct medical treatment.

Reply:
We totally agree with the point raised by these comments and added the article by Imachi et al in the revised manuscript. Indeed, menin may possess an "oncogenic" potential, through its role of transcriptional coactivator of ER in certain circumstances or in certain stages of cancer development. The data obtained by Paris et al. (that you mentioned above) and Imachi et al. do suggest such an oncogenic role for menin in prostate and breast cancer, particularly in cancer recurrence. This possibility is now discussed in the revised manuscript (page 16).

Conclusion:
Page 18:
Do the genetic loci found in reference 18 include the genes studied in the present paper (menin, AR, p63, CDKN1B, Nkx3.1)?

Reply:
The genetic loci found in reference 40 (page 18) do not include the above mentioned genes, except 11q13 that contains the MEN1 gene, but none of the other genes analyzed in the present paper. The locus 11q13 was found to be associated with prostate cancer susceptibility. But the use of SNPs in this study is not discriminating enough to evaluate the oncogenic or tumor suppressor potential of this locus regarding to prostate cancer.

Figure legends:
Page 27 (Figure 3) How was "reduced intensity of AR expression, relative to the average AR staining intensity" defined? How much reduced?? What was average?

Reply:
In fact, we counted cells showing no, or very low, AR expression in normal prostate glands and prostate carcinomas. It seems to us that the wording "average" could be confusing. The corresponding text was revised to better explain how we counted these cells (page 13 and 28).

For p63 I see hardly a difference between Fig 3B (wildtype mouse) and 3F (adenocarcinoma in Men1 mutant mouse).

Reply:
This is because we chose to focus the image in 3F on a part of prostate carcinoma which contained residual basal cells, in order to better show that the menin-positive cells which remained in the prostate lesions were actually p63-positive basal cells. Indeed, the total number of cells on this image (please see blue Dapi staining) is much higher compared with that in 3B. So, the proportion of p63-positive basal cells over total cells observed in prostate carcinomas is substantially lower than that seen in normal prostate glands from wild-type mice.

Minor essential revisions:
Specific, but general remark:
Many sentences have to be formulated more carefully, throughout the entire paper!
In addition, the English language has to be reviewed
For example:
Abstract:
Background:
Intriguingly, other MEN1-specific lesions in hormone-dependent organs like breast and prostate were observed in the Men1 mutant mice.
Results:
.., while none of the control mice developed cancerous lesions 'in the prostate'.
Background:
'Inactivating' mutations of the MEN1 gene predispose patients to multiple ... high frequency (which frequency?) sex-cord stromal cell tumours
Intriguingly, we have found three cases of breast cancer in heterozygous MEN1 mice. (3 out of how many mice?)
Dreijerink et al. 'have' in stead of 'has'

Reply:
We have sent our manuscript for extensive edition and added all the missing information in the background part.

Reviewer 2:
The article entitled « Characterization of the prostate cancer lesions developed in heterozygous Men1 mutant mice » by C Seigne et al. reports the results of the study of the prostate in male heterozygous Men1 mutant mice. The Authors show that incidence of in situ carcinoma and prostate cancer was increased compared with wild type mice. The expression of menin was reduced in carcinomas and partial loss of Men1 allele was detected in some lesions. Menin-negative prostate cancer cells did not display p63 expression and androgen receptor was expressed but heterogeneously. The expression of CDKN1B was decreased in the prostate cancer cells of mutant mice. The paper is clear well written and discussion is fair.

Minor essential revisions
1-The main concern with that study is that while the number of lesions was given in details for the description of the lesions and the menin expression, there is no detail about the number of the lesions involved in the studies of p63 expression and AR expression. The Authors describe “cancerous cells” but we don’t know which number of prostate cancers have been studied and if this result is homogeneous in all cancers studied. The same is true for expression of AR and CDK1B

Reply:
The reviewer is correct. We have added the number of mice used for each expression analysis in the figure legends of the revised manuscript.
2-Some sentences in the Results are too speculative, particularly concerning AR expression (p13, "They also suggest that menin inactivation may lead to the deregulation of AR expression"). This is only an association between menin activation and AR expression not a causal relationship!

Reply:
Indeed, we have no intention to claim that the AR downregulation has a causal relationship with menin inactivation. The sentence has been accordingly revised (page 13).

I hope that you and reviewers will find our replies and arguments reasonable, scientific and convincing.

Thanking you very much for your consideration of our revised manuscript, and looking forward to hearing from you soon,

Yours sincerely,

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