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Subject Manuscript AJP05-0160 Version 1

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[redacted]

RE: Ma 60 Version 1, [redacted]
laminin

Dear Dr. [redacted]

Thank you for submitting the above-referenced manuscript to The American Journal of Pathology for consideration.

Unfortunately, the manuscript is not acceptable for publication in its present form based on the recommendations of three reviewers (comments below) and an Associate Editor. However, we would be willing to re-evaluate a revised version of the manuscript if the authors feel able to fully respond to the enclosed comments. It is likely that a successful resubmission would need to undergo substantial revision and include additional experimentation. The resubmission would undergo full re-evaluation and no promise can be made as to whether such a revision would be found acceptable for publication.

Resubmitted manuscripts will be considered if they are received within 120 days of this decision. The revised manuscript and a cover letter detailing changes, point-by-point, should be sent (on disk) to the Managing Editor, The American Journal of Pathology, 9650 Rockville Pike, Bethesda, MD 20814-3993. The resubmission package should include the following: one printed copy of the cover letter and full manuscript (including tables and references), one disk containing files of the cover letter and full manuscript (with the appended electronic publishing form), and four sets of figures (publication quality hard copies).

Your interest in The American Journal of Pathology is appreciated.

Sincerely,

[redacted] Ph.D.

Comments:
The manuscript by [redacted] et al. is straightforward and clearly written and presents an aspect of laminin alpha2 deficiency that has not been tackled yet.

Extensive immunostaining data is presented to identify the laminin chains that are expressed in

testis, however the beta3 chain is ignored. Is it because of a technical problem (no specific antibody?). At least, the authors should mention why they do not present beta3 immunostaining in Figure 2.

In the Results section (page 12), it seems to me that the statement "endogenous laminin alpha1 is not expressed in sufficient amounts to compensate for the absence of laminin alpha2" could be answered (at least partly) by presenting both mRNA quantification data and protein immunoblotting for laminin alpha1 chain in dy3K/dy3K mice in addition to the results presented in the transgenic mice (Table 3 and Figure 5). This would be interesting since,in Figure 1, the levels of laminin alpha1 do seem identical in WT and dy3K/dy3K mice (although immunohistochemistry is by no way a quantitative method).

In the Results section (page 13), the authors mention that "loss of laminin alpha2 and gamma3 chains are compensated for by laminin alpha1 and gamma 1 as this chain is upregulated ...". Isn't the beta1 chain also upregulated as presented in Figure 5?

A minor comment regarding to the quantification data presented in the manuscript: the statistical method used to determine the p values is not described and should appear in the methods section.

REVIEWER 2:
Comments:
#AJP05-0160
The manuscript describes a functional role for the laminin ?2 chain in spermatogenesis. dy3K/dy3K mice, lacking the laminin ?2 chain showed less lumen formation of seminiferous tubules and a reduced number of spermatides.

In addition, the authors could show that the basement membrane underlying sertoli cells was irregular, with thinner or ruptured segments. The authors performed a detailed analysis of laminin chain expression in normal and mutant testis and they could show that the laminin ?3 chain is absent in dy3K/dy3K, though it is well expressed in wildtype.

The authors crossed their previously characterized laminin ?1 chain overexpressing mice into the dy3K/dy3K background to analyze a rescuing function of the laminin ?1 chain in spermatogenesis and demonstrated that a two-fold increase of laminin-1 is sufficient to obtain fertile males.

Overall this is a very nice and well-presented study, which provides new insights into laminin ?2 function. I was only left with two main questions: (1) Is basement membrane integrity in double-transgenic mice restored and may the ruptured basement membrane be the primary reason for the testis phenotype in dy3K/dy3K?

(2) dy3K/dy3K die very early due to multiple defects and development in general is likely severely impaired. Could impaired spermatogenesis therefore reflect a delay in development? dy/dy mice with some residual laminin ?2 chain expression have a longer life span and were the perfect model to be analyzed to address this question.

REVIEWER 3:
Comments:
This paper demonstrates that lack of laminin alpha 2 chain in dy3k/d3k mice leads to abnormal testicular development and eventual lack of spermatocytes, a finding that goes along with the fact that these mice are infertile. The authors show that these abnormal testes also lack laminin gamma 3 chain. By studying a transgenic animal that lacks laminin alpha2 chain but overexpress alpha 1 chain, the investigators demonstrate rescue of the abnormal testicular phenotype. The paper is clearly written, contains convincing, high quality figures and has obvious clinical significance. There are few points that this reviewer would like to be addressed:

The characterization of the antibodies used to recognize laminin gamma 1 and 3 chains is unavailable. Reference to "manuscript in preparation" is insufficient to properly review this paper. Information demonstrating that the antibodies do indeed recognize laminin gamma 1 and 3 chains should be provided.

Laminin gamma 3 chain is described as severely reduced, but the figures show absence. Please clarify.

Lack of basement membrane disruption is seen only at EM level but not by immunostaining. Please clarify.

In page 16 of discussion the authors state " our data indicate that male patients with laminin alpha 2 deficient may be infertile". According to the published literature are they infertile or not? This is an important point that cannot be addressed by a may be.

Dear Dr

Thank you for considering our manuscript entitled '[redacted]' for publication in *American Journal of Pathology*. We have revised the manuscript to incorporate the reviewers' suggestions. Specifically, we have included the following experimentation: 1) immunostainings of laminin β3 chain in wild-type and dy^{3K}/dy^{3K} testis, 2) quantification of laminin α1 chain expression in dy^{3K}/dy^{3K} and dy^{3K}LNα1TG testis, 3) electron microscopy studies to show restoration of basement membranes in dy^{3K}LNα1TG testis, 4) characterization of laminin γ1 and γ3 chain antibodies by ELISA. In addition, we have rewritten the manuscript according to the reviewers' proposals. We have outlined each of the changes to the manuscript on the attached pages.

Please find the enclosed paper copy of the revised manuscript as well as an electronic copy of the manuscript and cover letter. Enclosed are also four sets of figures and the electronic publishing form.

Thank you again for your interest in our work and the quick response to our first submission. We feel that the changes made in response to the reviewers' comments have improved the manuscript, and hope that it is now acceptable for publication.

Yours Sincerely,

Response to reviewers' comments on manuscript #AJP05-0160 Version 1, Laminin α1 chain corrects infertility caused by absence of laminin α2 chain.

Reviewer 1:

1) The reviewer wanted us to present laminin β3 chain immuno-staining. We have now stained sections of wild-type and dy^{3K}/dy^{3K} testis with laminin β3 chain antibodies. In the results section we report (as data not shown) that laminin β3 chain was not detected in testis. The antibody against laminin β3 chain is described in the methods section and stained positive control sections well.

2) The reviewer wanted us to examine the levels of laminin α1 chain in dy^{3K}/dy^{3K} testis. This has now been dealt with and we have also analyzed the levels of laminin α1 chain in dy^{3K}LNα1TG testis. mRNA quantification data revealed that laminin α1 chain mRNA is upregulated ~2.4-fold in dy^{3K}/dy^{3K} testis and ~4-fold in dy^{3K}LNα1TG testis. This new set of data has been incorporated into Table 3. The previous table showed a ~5-fold increase in the levels of laminin α1 chain mRNA in LNα1TG mice compared to wild-type. Now, we show a ~4-fold increase. We have now analyzed more samples and obtained this new value. However, this minor difference does not affect the conclusion of our manuscript.

3) We agree with the reviewer that it should be stated (now on page 14) that also laminin β1 chain is upregulated. This is now described.

4) In the methods section we have now included that the statistical significance was examined by using Student's t-test. We thank reviewer 1 for bringing this to our attention.

Reviewer 2:

1) The reviewer wanted to know whether the basement membrane integrity was restored in dy^{3K}LNα1TG testis. In a new Figure 8 we show by transmission electron microscopy that the basement membrane is definitely restored in dy^{3K}LNα1TG testis. Thus, it may well be that the primary reason for the testis phenotype in dy^{3K}/dy^{3K} mice is the ruptured basement membrane.

2) Since dy^{3K}/dy^{3K} mice die very early and it could be that development in general is impaired, the reviewer asked whether the impaired spermatogenesis reflects a delay in development. We can not entirely exclude this possibility. However, many organs in dy^{3K}/dy^{3K} mice do not display impaired development. For example, kidney, bladder,

heart and eye look normal. Thus, we do not think that development in general is impaired. This has been stated more strongly in the manuscript on page 11. The reviewer further suggested that *dy/dy* mice were the perfect model to address this question. The *dy/dy* mice do not reproduce and are sterile according to The Jackson Laboratory. Still, we agree with the reviewer that it would be of some interest to analyze testis of *dy/dy* mice. However, these studies are hampered by the fact that at present we are not allowed to import *dy/dy* mice from Jax Lab to our animal facilities due to the health status of the mice. Please see: **http://jaxmice.jax.org/health/d1.pdf** for further information. Yet, these are studies we intend to do in the future but we hope that the manuscript now can be published without this piece of information.

Reviewer 3:

1) The reviewer asked us to provide information that the previously uncharacterized antibodies against laminin γ1 and γ3 chains do indeed recognize laminin γ1 and γ3 chains, respectively. In a new Figure 1 we show by ELISA titration curves that the antibodies react with laminin γ1 and γ3 chains, respectively. Although the laminin γ3 antibody in ELISA shows some minor binding to laminin γ1 chain fragment LN/LEa, it is also clear from immunofluorescence stainings that the laminin γ3 chain antibody does not react with laminin γ1 chain. In laminin α2 chain deficient testis, laminin γ3 chain is absent. If it would react with laminin γ1 chain, it would stain the laminin α2 chain deficient testis (or dy^{3K}LNα1TG testis), which is rich in laminin γ1 chain.

2) We had previously cautiously described the expression of laminin γ3 chain in dy^{3K}/dy^{3K} testis as severely reduced, but as the reviewer points out the figures show an apparent absence. Thus, we now describe laminin γ3 chain expression as absent in dy^{3K}/dy^{3K} testis.

3) The reviewer wanted us to clarify why lack of basement membrane is seen only at the EM level but not by immunostaining. As we now discuss in the discussion section, the presence of basement membrane components does not necessarily reflect the presence of a basement membrane defined by electron microscopy, an important point that is gradually becoming clear also from other studies. Other basement membrane components (such as other laminin chains, collagen type IV and perlecan) can be present but not assembled into a basement membrane. For example, it has been shown that the basement membrane in laminin α2 chain deficient muscle is disrupted despite the presence of other basement membrane components.

4) The reviewer asked whether male patients with laminin α2 chain deficiency are fertile or not according to the published literature. To our knowledge, no literature is available that deals with the question about MDC1A and male fertility. Nevertheless, in the discussion we have included the following sentence: "Finally, to our knowledge there is no published literature that deals with the issue of MDC1A and fertility".