A mutation affecting laminin alpha 5 polymerisation gives rise to a syndromic developmental disorder.

Lynelle K. Jones, Rachel Lam, Karen K. McKee, Maya Aleksandrova, John Dowling, Stephen I. Alexander, Amali Mallawaarachchi, Denny L. Cottle, Kieran M. Short, Lynn Pais, Jeffery H. Miner, Andrew J. Mallett, Cas Simons, Hugh McCarthy, Peter D. Yurchenco and Ian M. Smyth
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Original submission

First decision letter

MS ID#: DEVELOP/2020/189183

MS TITLE: A mutation affecting laminin alpha 5 polymerisation gives rise to a syndromic developmental disorder.

AUTHORS: Lynelle Jones, Rachel Lam, Karen McKee, Maya Aleksandrova, John Dowling, Stephen Alexander, Amali Mallawaarachchi, Denny Cottle, Kieran M Short, Lynn Pais, Jeff Miner, Andrew Mallett, Cas Simons, Hugh McCarthy, Peter Yurchenco, and Ian Smyth

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper describes the identification by whole-genomic sequencing of a patient with a missense mutation in the LAMA5-gene encoding the alpha5-chain of laminins. A well conserved region of the polypeptide is affected which is said to be responsible for the assembly of laminins into networks deposited into many types of basement membranes (BM). The authors show that the mutation causes a syndromic condition affecting many organ systems in the patient. Further, they have

created a transgenic mouse harbouring the same amino acid substitution and show that the animals exhibit a hypomorphic phenotype closely resembling that of the patient. Most animals die before or at birth, but a few individuals survive to adolescence. In addition, the authors have performed aggregation studies with a system of a recombinant, experimental variant laminin 111 displaying the same amino acid substitution in the affected LN-domain of alpha5-chains. They show that the mutation disrupts the capability of the recombinant protein system to form aggregates. They conclude that the syndromic condition of the patient is a consequence of a mutated laminin alpha 5-chain that has lost its competence to aggregate and to be incorporated into BMs in a fully functional form.
The manuscript constitutes a major achievement. It represents a major advance in our knowledge, not only by identifying a patient mutation but also by a conclusive elucidation of a pathogenetic mechanism. The paper is generally well written, and publication should be considered. Nevertheless, a number of text modifications are suggested here that may improve the paper.

Comments for the author

Specific suggestions:
Although the artificial recombinant LN111-aggregating system is entirely unfunctional after introduction of the patient mutation, the deposition of the mutated laminin into BMs seems to occur in an appropriate way, both, in mice in situ as well as in cell cultures. This potentially confusing fact is not commented in the present form of the manuscript, but it should be, especially at the end of the first paragraph of the discussion (last two sentences) where the contradiction is rather accentuated.
Detailed suggestions:
1) First sentence: BMs are not fully “proteinaceous” but also contain important carbohydrate moieties, specifically heparan sulfates. These are very essential for the normal function of BMs, specifically the stabilisation of the connection between laminin- and collagen IV-networks (see also below).
2) Second paragraph, first sentence: LAMA5 is a gene, not a polypeptide, let alone a protein. Rephrasing may help here. The distinction between genes and proteins should also be made in several other instances throughout the manuscript.
3) The second sentence is not self-consistent and also needs rephrasing.
4) Second paragraph, line 7: Having performed aggregation studies at the level of individual proteins, Rupert Timpl and coworkers have arrived at the conclusion that nidogen constitutes the link between laminin- and collagen IV-networks (Fox et al. 1991). Although this notion has been generally accepted it has recently been proven as incorrect by studies at the level of supramolecular aggregates (Behrens et al. 2012). The role of an adapter between laminin- and collagen-networks has been assigned to aggregates of perlecan in the epidermal BM (other HS-proteoglycans may serve the same purpose in other BMs, see comment above). Here, the record should be set straight.
5) Results, first paragraph, line 5: ...vesiculourethral reflex... should read ...vesiculourethral reflux...?

Reviewer 2

Advance summary and potential significance to field

This paper provides insight into the significance of a human missense variant in the LAMA5 chain and its pathogenetic potential. The paper provides evidence to support that the variant interferes with LAMA polymerization. As such it expands our understanding of human disease pathogenesis.

Comments for the author

Summary:

This paper investigates the pathogenic effects of a laminin 5 (LAMA5) human missense variant on laminin polymerization and tissue formation. Previous work in genetic mouse models has demonstrated the critical roles that laminin plays in tissue and organ morphogenesis. In contrast, evidence conclusively demonstrating a relationship between human LAMA5 mutations and disease

has been sparse, in contrast to evidence related to other LAMA chains. Here, the authors report on a human homozygous missense mutation resulting in a predicted arginine to leucine residue change at position 291 in the amino terminal domain of LAMA5 next to the PLENGE sequence considered to be critical to LAMA polymer formation. The proband (the single patient affected patient reported) demonstrated multi-organ malformations and onset of renal failure. The authors used an in vitro LAMA polymerization assay to demonstrate that the missense mutation abrogates polymer formation. They next generated mice bearing the relevant human WT codon (different than mouse but encoding the same amino acid) as a control and a 2nd strain bearing the missense nucleotide variant. Of the seven mutant mice able to be identified, major abnormalities were identified across a variety of organs.
Perhaps in a manner not unexpected, mutant tissue phenotypes were often less severe than that found in mice with Lama5 deficiency. Malformations are demonstrated via several figures in a descriptive manner with more detailed analysis of lung and kidney. In kidney, focal glomerular abnormalities of endothelial vessels and glomerular basement membrane are described as well as postnatal hydronephrosis.

Comments:
At the level of developmental biology, this is a descriptive study in which the phenotypes associated with the human missense variant in LAMA5 are demonstrated. Other than the in vitro studies of LAMA polymerization there are no mechanistic studies. Thus, one is not able to conclude how the missense variant interferes with tissue morphogenesis beyond the inference that polymerization of laminin may be a problem. But even concerning the latter, it is not clear how this controls variable phenotype within and across diverse tissues.

The authors argue that vesicoureteral reflux results in medullary ablation in mutant mice. No evidence supporting this idea is provided. Indeed, other more tenable possibilities exist e.g. abnormal tissue morphogenesis other than the V-U junction but these are not at all investigated.

Reviewer 3

Advance summary and potential significance to field

The manuscript submitted by Lynelle Jones and colleagues focuses on the alpha 5 chain of laminin and proposes the identification of a new pathogenic variant in the LN domain. This variant is associated with a range of phenotypes including abnormalities in kidney and skeletal development. With functional studies of this variant the authors found defects in alpha 5 chain polymerization and using CRISPR-Cas9 firstly generated a humanized mouse and then a mouse with the human point mutation. The latter resulted in a range of phenotypes which were in common with the human clinical case presentation.

Overall this a very interesting and well-presented study. I predict it will have wide appeal to but of particular interest to developmental biologists, clinicians and protein biochemists.

Comments for the author

I have only minor comments and questions for clarification:

Comments and questions:
1. Were any other disease associated variants identified in the WGS that could be relevant for the phenotype (e.g. SRNS gene in the kidney, skeletal genes?).
2. It is interesting that the in vivo studies demonstrate incorporation of the laminin alpha 5 into basement membranes in the Lama5PM mice. Do the authors think that these are isolated trimers? If so- it is surprising that there is not more disruption of the basement membrane. For the discussion, it would be useful to add to details about the secretory pathway for laminin and the stage at which trimer is assembled. Can the authors speculate about the functions of laminin alpha 5 that are preserved with this variant?
3. Mouse studies- genetic background is indicated but not the sex of the mice in the methods section. This would be helpful to know. Was there a sex difference in the phenotype?

4. Figure 2 shows the assay for laminin polymerization and provides strong evidence for the role of the LN domain but these studies are with laminin 111. Can the authors indicate why this would also be true for the alpha5 chain?

First revision

Author response to reviewers' comments

Response to Reviewers Comments and Questions:
Reviewer 1
Although the artificial recombinant LN111-aggregating system is entirely unfunctional after introduction of the patient mutation, the deposition of the mutated laminin into BMs seems to occur in an appropriate way, both, in mice in situ as well as in cell cultures. This potentially confusing fact is not commented in the present form of the manuscript, but it should be, especially at the end of the first paragraph of the discussion (last two sentences) where the contradiction is rather accentuated.
We have not studied the deposition of the mutant laminin in cultured cells as suggested (we presume the Reviewer is referring to Figure 2B, which is LM111) but we agree that the deposition of the mutant laminin is one of the most interesting aspects of the work. We had refrained from speculating about these observations but, in retrospect, we agree with this Reviewer (and Reviewer 3) that greater time should be devoted to addressing this point.

Regarding the "normal" localisation of our mutant alpha 5 protein, we would note that polymerisation per se is not required for the function or BM localisation of many laminins. Indeed 8 out of the 16 laminin trimers are predicted to be unable to undergo this higher order organisation because one or more of their subunits lack the requisite LN domains. Nonetheless they play important developmental and homeostatic roles. We have no reason to suspect that the mutation we describe alters alpha/beta/gamma trimerisation and the finding that secretion of alpha 5 is unaffected by this change supports this view. Localisation of laminins to the BM/ECM and integration into the BM are not necessarily dependent on 100% perfect polymerisation, as binding to other BM proteins such as nidogen, agrin, perlecan and to cell surface receptors (integrins, dystroglycan, sulphated glycolipids) would link the imperfectly polymerised laminins to collagen IV and to cells. A well described example is the Lama-<dy2J> allele that causes muscular dystrophy due to a polymerization defect, but a less severe disease than observed in Lama2 null mutants. We have now included further discussion as suggested by the Reviewer.

This section now reads:
"Taken together with biochemical studies of the variant protein showing that its' capacity to polymerize is severely abrogated, this strongly supports the identification of a new syndromic condition specifically associated with a hypomorphic mutation in LAMA5. Notably though, the mutant LAMA5 protein is normally localised in BM's in multiple tissues. This finding suggests that trimerization and secretion of the protein is unaffected in our mouse model but also that incorporation of LAMA5 into the BM is not wholly dependent on its capacity to form higher order polymerised networks on its own. In this respect, the localisation of laminins and their stable integration into the BM is likely also mediated by interactions with a range of cellular and ECM components including cell surface receptors, sulphated glycolipids in the cell membrane and through interactions mediated by intermediary proteins like AGRIN and NIDOGEN. From our mouse studies it appears that BM integrity is only compromised in tissues which we propose are under greater mechanical stress as a consequence of rapid changes in tissue morphology (limb, lung) or acquisition of function (glomerulus)."

Detailed suggestions:

1) First sentence: BMs are not fully "proteinaceous" but also contain important carbohydrate moieties, specifically heparan sulfates. These are very essential for the normal function of BMs,

specifically the stabilisation of the connection between laminin- and collagen IV-networks (see also below).

We agree and had not meant to imply otherwise. We have altered the sentence to read “protein rich” and the revised Discussion now addresses the important contributions of the other BM components to development and protein localisation (see comments above).

2) Second paragraph, first sentence: LAMA5 is a gene, not a polypeptide, let alone a protein. Rephrasing may help here. The distinction between genes and proteins should also be made in several other instances throughout the manuscript.

We have used (or attempted to use), the HGNC guidelines for human nomenclature for laminin alpha 5 (LAMA5 (ital.) for the human gene, LAMA5 for the human protein) and the MGI guidelines for mouse (Lama5 (ital.) for the mouse gene, LAMA5 for the mouse protein). Based on these accepted guidelines LAMA5 is a protein. However, we apologise that in some cases these rules have not been uniformly applied and we have reviewed the manuscript and made appropriate corrections as needed.

3) The second sentence is not self-consistent and also needs rephrasing.

This sentence has been re-written.

4) Second paragraph, line 7: Having performed aggregation studies at the level of individual proteins, Rupert Timpl and coworkers have arrived at the conclusion that nidogen constitutes the link between laminin- and collagen IV-networks (Fox et al. 1991). Although this notion has been generally accepted it has recently been proven as incorrect by studies at the level of supramolecular aggregates (Behrens et al. 2012). The role of an adapter between laminin- and collagen-networks has been assigned to aggregates of perlecan in the epidermal BM (other HS-proteoglycans may serve the same purpose in other BMs, see comment above). Here, the record should be set straight.

We respectfully disagree with Behrens conclusions and the idea that this is “generally accepted”, which is subjective. The skin study to which the Reviewer refers proposed that PERLECAN spot welds collagen to LAMININ (based on the detection of paired components in extracts). However, the Perlecan knockout, published some years earlier, showed that most tissues and their BMs in development were normal (except for the absence of PERLECAN) and importantly that COLLAGEN-IV was present. As with the Nidogen knockouts, it could be argued that diffusion prevents collagen loss. However, that means that the Perlecan claim suffers from the same weakness as that of no Nidogen. Our cell culture studies that evaluated LAMININ/COLLAGEN-IV/NIDOGEN/PERLECAN assembly (McKee et al. 2007, 2009, 2017) reveal: (a) PERLECAN will not assemble in BMs unless both LAMININ and NIDOGEN are co-incubated (i.e. PERLECAN binds through NIDOGEN as previously reported) and (b) LAMININ does recruit COLLAGEN-IV so long as nidogen is present, and occurs similarly if PERLECAN is also present. Our feeling, from our work and that of others including knockout data, is that NIDOGEN is a key linker of LAMININ to COLLAGEN-IV; however, there are weaker interactions as well including a direct one between LAMININ and COLLAGEN (McKee et al. 2007).

All of this is somewhat academic as it isn’t critical to the work we present in this paper. However, we have changed this part of the paper to reference the Behrens work, acknowledge that this is an area of disagreement and reinforce the potentially important roles of interacting proteins in controlling the deposition of BM components such as LAMA5. This sentiment is then echoed in the new part of the Discussion, helpfully suggested by the Reviewer, which refers to the normal deposition of the LAMA5 mutant in the BM (see above).

This section now reads:
“The laminin complex is linked to (and integrated with) a second major BM protein network composed principally of COLLAGEN IV. Early investigations provided evidence that this is mediated through a shared association with NIDOGEN (Fox et al., 1991), however a more recent study in the skin suggests that PERLECAN may also arbitrate these linkages (Behrens et al., 2012). It seems

likely that such proteoglycan linking is a common feature of BMs generally and that these may be dependent on the tissue in question."

5) Results, first paragraph, line 5: ...vesiculourethral reflex... should read ...vesiculourethral reflux...?

Quite right, this should be vesicoureteral reflux; this has been corrected.

Reviewer 2

Comments:
At the level of developmental biology, this is a descriptive study in which the phenotypes associated with the human missense variant in LAMA5 are demonstrated. Other than the in vitro studies of LAMA polymerization, there are no mechanistic studies. Thus, one is not able to conclude how the missense variant interferes with tissue morphogenesis beyond the inference that polymerization of laminin may be a problem. But even concerning the latter, it is not clear how this controls variable phenotype within and across diverse tissues.

While we agree that aspects of the study are descriptive, we respectfully disagree that this somehow renders the work less important. This is the first convincing description of a disease-causing variant in LAMA5 and it provides significant functional insights into the role of the LN domain of the protein in regulating tissue differentiation and development. With regards to mechanism, the studies modelling the change in LM111 demonstrate convincingly that the variant in question affects laminin polymerisation. Taken together with the observations in mouse models showing that specific developmental functions of LAMA5 are affected (or not affected) by this mutation, we provide very clear evidence for the mechanism by which the LAMA5 LN domain mediates embryonic development and BM function.

The authors argue that vesicoureteral reflux results in medullary ablation in mutant mice. No evidence supporting this idea is provided. Indeed, other more tenable possibilities exist e.g. abnormal tissue morphogenesis other than the V-U junction but these are not at all investigated.

Our argument that the mutation causes VUR in mice is based on the observation that the patient was assessed clinically and was found to have this defect. Given the otherwise extensive phenotypic overlap between the mouse model and the patient this does not seem to us to be an unreasonable conclusion to draw. For this reason, we also fail to see how other possibilities are "more tenable", but accept that there remains a chance that the same phenotype in humans and mice may arise through completely different mechanisms. To better detail the reasoning behind drawing this conclusion the text in the revised manuscript has been modified to read:

"Given the extensive clinical correlates with our mouse model this suggests that the development of hydronephrotic phenotypes in LN mutant mice does not arise because of blockage of the urinary tract. However, we cannot definitively exclude the possibility that the same overall phenotype (hydronephrosis) arises through completely different mechanisms.

Reviewer 3

Comments and questions:

1. Were any other disease associated variants identified in the WGS that could be relevant for the phenotype (e.g. SRNS gene in the kidney, skeletal genes?).

The simple answer to this question is no - our analysis did not identify any other Likely Pathogenic or Pathogenic variants in disease associated genes. However, as with any consanguineous pedigree there are a few homozygous missense gene variants present in the patient. From a functional perspective (i.e. residue conservation) the most compelling of these are in TSC22D1 (a regulator of bone marrow homeostasis) and TLE1 (a regulator of cerebellar development and biomarker for synovial carcinoma). From what is known of their biology, neither are compelling drivers of the patient phenotypes, which in any event are largely modelled by mutation of LAMA5. The possible exception is a potential link between the emergent patient post-transplant lymphoproliferative

disorder and altered TSC22D1 function, but without extensive modelling this seemed to us too tenuous an association to make or mention.

2. It is interesting that the in vivo studies demonstrate incorporation of the laminin alpha 5 into basement membranes in the Lama5PM mice. Do the authors think that these are isolated trimers? If so- it is surprising that there is not more disruption of the basement membrane. For the discussion, it would be useful to add to details about the secretory pathway for laminin and the stage at which trimer is assembled. Can the authors speculate about the functions of laminin alpha 5 that are preserved with this variant?

This is a point also raised by Reviewer 1 and the subject of expanded consideration in the revised Discussion. Laminin trimers are assembled intracellularly and for the most part the proteins are secreted as trimers. The normal deposition of the protein into the BM indicates that trimerization is unlikely to be perturbed by the mutation. While it is formally possible that it can polymerise to some small extent, our assays using the LM-111 system suggest that this would be very limited. However, that is not to say that mutant LAMA5 molecules are not stabilised by interactions with other components of the BM niche. The most obvious are those with cell surface receptors and the conserved and unperturbed LG domains of the protein, but could also include cell membrane sulphated glycolipids, dystroglycans and bridge proteins (like Agrin) which mediate interactions between cells and BM components. As detailed in the revised Discussion, we propose that some normal cellular interactions of LAMA5, such as those mediated by LG domains, remain unperturbed by the mutation. However, in tissues undergoing extensive remodelling or subject to stress (lung pleura, GBM, limbs) structural weakness in the BM is apparent and derived from an absence of polymers. These possibilities are now addressed through modification of the 1st paragraph of the Discussion.

3. Mouse studies- genetic background is indicated but not the sex of the mice in the methods section. This would be helpful to know. Was there a sex difference in the phenotype?

We apologise for not including this information and thank the Reviewer for this observation. All the experiments were undertaken using embryos/mice of both sexes. To the extent we have been able to ascertain, there is no sexual dimorphism evident in their phenotypes. This was true with respect to male versus female syndactyly (which was fully penetrant), penetrance of lung fusion ($p= 0.062$, chi sq. test), Mendelian ratios at E18.5 ($p=0.138$, chi sq. test) or weights at E18.5 ($p=0.395$, Welch t-test). This data has now been included in the revised results section.

4. Figure 2 shows the assay for laminin polymerization and provides strong evidence for the role of the LN domain but these studies are with laminin 111. Can the authors indicate why this would also be true for the alpha5 chain?

There are several reasons why we believe what holds for polymerization of LM111 will be true for LAMA5 containing trimers. Firstly, there is very high homology between the subunit LN sequences and in the polymerization face where the mutation we have identified sits. Moreover, the residue is adjacent to the conserved PLENGE domain thought necessary for LN domain associations. Secondly, equivalent changes in different subunits have been found to have conserved effects for other mutations we have studied. For example, we have recently shown that beta1 S68R and the corresponding beta2 S83R are both defective for polymerization (Funk S.D., Bayer RH, McKee KK, Okada K, Nishume H, Yurchenco PD and Miner JH (2020), “A deletion in the N-terminal polymerizing domain of laminin β2 is a new mouse model of chronic nephrotic syndrome”, Kidney International, In press). Thirdly, we have used this approach to evaluate alpha 2 and beta 2 mutations that cause other human diseases (see McKee et al., Matrix Biol. 2018). In both cases we have observed strong correlations.

Second decision letter

MS ID#: DEVELOP/2020/189183

MS TITLE: A mutation affecting laminin alpha 5 polymerisation gives rise to a syndromic developmental disorder.

AUTHORS: Lynelle Jones, Rachel Lam, Karen McKee, Maya Aleksandrova, John Dowling, Stephen Alexander, Amali Mallawaarachchi, Denny Cottle, Kieran M Short, Lynn Pais, Jeff Miner, Andrew Mallett, Cas Simons, Hugh McCarthy, Peter Yurchenco, and Ian Smyth

I have assessed your response to the reviewers' comments and am satisfied that you have adequately addressed all the comments raised.

There is however one additional issue that I would like you to address that concerns homozygous variants, other than the reported *LAMA5* is the only gene involved in generating the patient's clinical features. Given this, it would be important to list the other homozygous variants identified in the patient as they are unlikely to be causing clinical features. This information might be useful if these variants are identified in other studies. Also, this would provide methodological and technical information concerning the whole genome data and its analysis, and data transparency more generally. I suggest that you provide information concerning the patient's homozygous variants as a supplementary table that at least includes the gene symbol, RefSeq identification, the variant and its minor allele frequency.

Provided you are able to fully address the above issue, we are positive about publication of your paper (we accept over 95% of revision submissions) and therefore hope you won't mind any extra work involved in reformatting your manuscript at this point.

Second revision

Author response to reviewers' comments

The requested addition of other homozygous variants in our patient is now incorporated as a Supplementary Table as requested. Reference to this list is now made in the results section describing the identification of the LAMA5 mutation

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Third decision letter

MS ID#: DEVELOP/2020/189183

MS TITLE: A mutation affecting laminin alpha 5 polymerisation gives rise to a syndromic developmental disorder.

AUTHORS: Lynelle Jones, Rachel Lam, Karen McKee, Maya Aleksandrova, John Dowling, Stephen Alexander, Amali Mallawaarachchi, Denny Cottle, Kieran M Short, Lynn Pais, Jeff Miner, Andrew Mallett, Cas Simons, Hugh McCarthy, Peter Yurchenco, and Ian Smyth
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.