EMBO
Molecular Medicine

Manuscript EMM-2011-00602

Probucol ameliorates renal and metabolic sequelae of primary CoQ deficiency in *Pdss2* mutant mice

Marni J. Falk, Erzsebet Polyak, Zhe Zhang, Min Peng, Rhonda King, Jonathan S. Maltzman, Ezinne Okwuego, Oksana Horyn, Eiko Nakamaru-Ogiso, Julian Ostrovsky, Letian X. Xie, Jia Yan Chen, Beth Marbois, Itzhak Nissim, Catherine F. Clarke and David L. Gasser

Corresponding author: Marni Falk, The Children's Hospital of Philadelphia and University of Pennsylvania

Review timeline:

Submission date:	19 January 2011
Editorial Decision:	07 March 2011
Revision received:	21 March 2011
Editorial Decision:	19 April 2011
Revision received:	27 April 2011
Accepted:	28 April 2011

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 07 March 2011

Thank you for the submission of your manuscript "Probucol ameliorates renal glomerular disease, tissue CoQ content, and PPAR-mediated metabolic sequelae of CoQ deficiency in Pdss2 mutant mice" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

In particular, reviewer #3 highlights that the lifespan of treated mutant mice should be assessed. In addition, reviewer #2 points out that additional insight into the mechanism behind the observed probucol effects would be required to strengthen the manuscript especially since some observations appear to be at odds with previous studies.

On a more editorial note, we agree with reviewer #3 that the manuscript in its present form might be overwhelming for the non-specialist reader. We would thus encourage you to edit the text and title to make the manuscript easier accessible to the non-expert.

Please note that EMBO Molecular Medicine requires submission of microarray data to the ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) or GEO (http://www.ncbi.nlm.nih.gov/geo/) databases, and provision of accession numbers within the manuscript. We would kindly ask you to include the accession numbers in your revised manuscript.

Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if

© European Molecular Biology Organization

you can convincingly address the issues that have been raised within the space and time constraints outlined below.

Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor.

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

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #1 - Comments on Novelty/Model system:

Overall the authors have performed clearly considered experiments in appropriate animal models. The use of Probucol is novel and the findings have potential significant impact for not only individuals with inherited mitochondrial disease, but for others with more common disorders that are associated with mitochondrial dysfunction.

Referee #1 – Other Remarks:

Falk and colleagues report the effects of treatment of a mitochondrial mouse model of kidney failure with Probucol. This paper is carefully considered and well written and the findings are important (and supported by copious data), especially given the lack of effective therapeutics for mitochondrial disorders. In particular, the upregulation of the PPAR pathway signaling could be particularly significant for mitochondrial patients; improved mitochondrial biogenesis has the potential to ameliorate patient symptoms. The favorable comparison of Probucol to CoQ10 is also interesting. Although CoQ10 supplementation is commonly employed as a treatment for mitochondrial disease, effects have been disheartening. Many questions remain, but perhaps the findings reported in this manuscript at least begin to shed some light on this issue.

My comments are relatively minor in nature:

1) Do you have hypotheses related to the differential response and characteristics between male and female mice?
2) You state "As subsequent evaluation of Mpv17-/- mice suggested their "kidney disease could no longer be documented" (Spinazzola et al, 2006), the B6.Pdss2kd/kd mouse may be the only available probucol treatable animal model of renal glomerular disease caused by a nuclear gene encoded mitochondrial defect." Is the renal disease in Mpv17-deficient animals different or are the authors suggesting that the kd/kd mice are unique because of isolated renal disease, instead of the primary hepatic disease present in the other model?
3) The Figure 1 legend states "Near normal glomerular histology is evident upon hematoxylin and eosin staining of renal sections from male mice shown at 100X magnification." Presumably this refers to the probucol treated kd/kd animals.
4) If CoQ biosynthetic pathway enzymes were not upregulated, why did CoQ levels increase?
5) "As probucol was previously reported to treat a mouse model of mitochondrial-based glomerular disease caused by mutations in Mpv17 (Binder et al, 1999) we added 1% probucol to standard mouse chow (TestDiet) fed to B6.Pdss2kd/kd missense mutant mice." A bit more background on Probucol would be helpful, especially given the association of the MPV17 mouse model with oxidant damage, which seems to be less of an issue with the kd/kd mouse. Why choose this agent - just because MPV17-deficient mice responded to Probucol therapy?

© European Molecular Biology Organization

Referee #2:

This is manuscript which examined the effects of therapeutic interventions in a mouse model of mitochondrial based glomerular disease caused by mutations in Mpv17, which is a glomerulopathy variant, which results from CoQ deficiency within glomerular podocytes. Previous work showed that high dose CoQ10 supplementation mitigates renal disease in these mice through approximately 140 days of life, but does not normalize albuminuria to control levels, prevent nephritis nor restore CoQ9 or CoQ10 content in renal tissue. The authors in the present manuscript have examined the efficacy of treatment with the lipophilic drug probucol, in these mice. The authors have treated with probucol at 1% by weight in the diet. They monitored albuminuria as an indice of glomerulonephritis. They also determined the histological nephritis score immediately following sacrifice. They compared probucol supplementation from weaning or day 100 of life, high-dose CoQ10 supplementation from weaning or day 100 of life, or vitamin E from weaning. The dose of CoQ10 that was utilized was 1mg/per ml, which the authors estimate resulted in a final daily dosage of approximately 400 mg of CoQ10/per kg of body weight. Vitamin E was provided from weaning of standard chow at a dose of 300 IU per kg of chow. The CoQ10 preparation was LiQsorb from Tishcon Corporation. It is somewhat surprising that the CoQ10 was water-soluble but this must be a special preparation. The authors observed that the probucol therapy significantly prevented renal disease whether administered from the time of weaning through at least six months of age or for only approximately three weeks prior to sacrifice. The probucol treated missense mutant group showed significantly higher urine albumin than gender-matched healthy controls. Vitamin E showed a minor effect in the females as assessed utilizing the urine albumin, but no effect in the males. Long-term CoQ10 significantly prevented renal disease manifestations when provided from weaning through six months and significantly improved renal histology and function was seen when CoQ10 treatment was initiated at the time of birth rather then upon weaning. The probucol provided a more longlasting and efficacious prevention of renal disease than did supplemental CoQ10. The short-term treatment from day 100 to sacrifice resulted in significantly greater improvement of both genders with probucol and relative to CoQ10 only in albuminuria but with equivalent effects on the nephritis score. The male missense mutant mice tended to respond similarly or better to probucol therapy, whereas the females responded similarly or better to CoQ10 supplementation. The authors also looked at the blood cholesterol levels, which were significantly increased in the Pdss2 mutants. Probucol treatments significantly decreased plasma cholesterol and triglyceride levels, in addition to the manifestations of albuminuria and nephritis relative to untreated mutant littermates. The authors also utilized another control, which was Simvastatin which neither prevented renal disease nor lowered blood cholesterol in these mice when given from time of birth. Although probucol did normalize the elevated blood cholesterol, this effect was insufficient to prevent the severe renal disease in these animals. The authors examined hepatic phospholipid levels using NMR analysis and found that cardiolipin content was 80% decreased in the liver of conditional knockout mutant mice, and normalized with probucol treatment. CoQ10 treatment had no effect on cardioplipin content in the mutant mice but modestly increased phosphatidylethanolamine and phosphatidylcholine. The authors did not observe any evidence of increased reactive oxygen species due to primary CoQ10 deficiency in either the Pdss2 missense or liver conditional knockout mutants. The total lipid peroxidation appears to be unaltered. Aconitase activity was also examined and did not show any difference following longterm CoQ10 therapy but was increased 15% with probucol. CoQ10 treatment increased MNSOD enzyme activity in the liver and kidney mitochondria from the missense mutant mice but it was unchanged by treatment with probucol. In the liver conditional knockout mutants, blood glucose was significantly increased and this was decreased by feeding the mice with probucol. They also carried out genome-wide transcriptional profiling in the liver from the missense mutant mice on standard chow or supplemented longterm with either probucol or CoQ10. They utilized a number of different analyses to determine what pathways seem to be altered. There was an upregulation of oxidative phosphorylation, the tricarboxcylic acid cycle, and cell defense pathways in liver conditional Pdss2 knockout mice and similar effects occurred in both the missense and liver conditional knockout Pdss2 mice. The Pdss2 missense mutants uniquely upregulate lipid biosynthesis and downregulate fatty acid metabolism. Interestingly, probucol largely reversed the global pattern of metabolic pathway alterations. It reversed expression alterations within the PPAR signaling pathway, fatty acid metabolism and glycolysis in both mutant mice. Probucol did not have any effect on OXPHOS pathway upregulation. There also was no effect on antioxidant genes or hemoxygenase. This latter finding is at variance with a number of other reports in the literature, which were published by Dr. Stocker et al. They consistently show that hemoxygenase was upregulated by probucol. It is unclear why there is a discrepancy in the present

© European Molecular Biology Organization

report. In addition, Dr. Stocker et al., recently demonstrated that hemoxygenase induces a pattern of gene transcription with elevation of genes scavenging reactive oxygen species. It is, therefore, curious that this was not observed by the present authors. The authors should address this discrepancy. The authors did observe that branched chain amino acid metabolism was upregulated. Steroid biosynthesis and the complement and coagulation cascade were the most downregulated pathways. The authors found that PPARgamma and PPARalpha transcription factors were decreased in the missense mutants and this was normalized by probucol treatment but unaltered by CoQ supplementation. It would be nice if the authors had a good interpretation of all these expression data which would explain the efficacy of probucol. Does it act on specific promoters, etc. Does it stimulate PGC-1 or Nrf2/ARE? A number of compounds that activate PPAR are not known to induce CoQ synthesis. In the unsupplemented mice, CoQ was exclusively oxidized whereas it was to a large extent reduced following treatment of the liver with probucol supplementation. The authors found that probucol supplementation increased total CoQ content which correlated with a rescue of kidney disease, as evidenced by decreased urine albumin and nephritis scores. In addition, the authors found that total CoQ9 content in kidney homogenates was significantly increased by short term probucol supplementation, but there was no significant difference in the liver.

Overall, this is an interesting manuscript which describes some novel affects of treatment with probucol in transgenic mice which are either deficient in CoQ synthesis throughout the body leading to renal dysfunction or selectively in the liver. Probucol was shown to successfully treat the mice after onset of disease as evidenced by albuminuria. Furthermore, prophylactic treatment from birth to presymptomatic animals clearly increased the CoQ9 and CoQ10 levels in the liver and prevented nephritis. One issue which the authors have not addressed, is whether the deficient mice have a reduction in brain CoQ10 levels. It would be of considerable interest to determine whether this is the case and whether probucol administration has any effects on brain CoQ10 levels. This is particularly the case since many of the clinical manifestations of CoQ10 deficiency in patients are due to abnormalities in the brain such as cerebellar ataxia and seizures. The authors did not observe any improvement to mitochondrial respiratory capacity in the Pdss2 mutants having primary CoQ deficiency. The inability of the authors to find evidence of oxidative stress is rather surprising. It is also surprising that there was no effect of probucol on ROS scavenging enzymes, which was previously reported in yeast.
Although probucol has lipid lowering effects, its beneficial effects were not replicated by reductions utilizing simvastatin suggesting that these are unlikely to mediate beneficial effects. The high dose CoQ10 supplementation in the present manuscript was also unsuccessful, which is somewhat surprising. The authors found that there was a relative increase in expression of MNSOD but aconitase activity in 4HNE lipid peroxidation was unchanged following treatment with CoQ10 in the mutant mice, as compared to age-matched controls. Interestingly, probucol supplementation did reverse transcriptional alterations that affected a number of areas of intermediary metabolism. The authors believe that altered expression of key PPAR pathway transcriptional factors may play an important role, and that probucol enhances the PPAR signaling which then induces CoQ content.
Overall, this is an interesting manuscript which has a number of very intriguing observations, the most important of which is that probucol seems to exert neuroprotective effects in a setting of CoQ10 deficiency. This may be due to its ability to increase tissue CoQ10 content and it appears to be independent of its hypolipidemic affects. The finding that CoQ supplementation was not successful is surprising. In looking at the data, however, it is unclear whether the CoQ10 supplementation was sufficient to normalize to the CoQ10 levels in these mice and I did not see CoQ10 levels in the brain. A number of observations were made for which there is no good explanation. Amongst these is why treating symptomatic animals with probucol for only two weeks significantly increased CoQ9 levels in the mutant kidney at day 100 and longterm CoQ10 supplementation did not result in an increase of either quinone species. It would be useful for the authors to address this. The most important observation of this manuscript is that treatment with probucol may be useful in the treatment of some patients who have mitochondrial dysfunction, although the mechanism is not clear from the present paper.

Referee #3 - Comments on Novelty/Model system:

This extensive study suggests that probucol ameliorates the renal phenotype of mice with primary CoQ deficiency. The work is novel and technically solid. Evidence for some conclusions could be

© European Molecular Biology Organization

strengthened (see comments to authors). The findings have obvious therapeutic implications for patients with CoQ deficiencies. The manuscript presents a large amount of data, which may be overwhelming for non-specialists.

Referee #3 – Other Remarks:

Falk et al. have performed an extensive study of the effects of probucol on mice with coenzyme Q (CoQ) deficiency due to a defect of CoQ biosynthesis (Pdss2 mutation). Probucol is a lipophillic antioxidant and hyper-lipidemic diphenol linked by a disulfide bond. The CoQ-deficient mice develop nephropathy (glomerulopathy with proteinuria) that is fatal. Probucol treatment appears to ameliorate that nephropathy in the mice more effectively than CoQ supplementation. The mechanism of probucol therapy is unknown, but CoQ deficiency and altered transcriptional profiles (particularly PPAR pathway signaling) in liver of Pdss2 mutant mice are ameliorated by probucol. The work is novel and technically solid. Evidence for some conclusions could be strengthened (see below). The findings have obvious therapeutic implications for patients with CoQ deficiencies. The manuscript presents a large amount of data, which may be overwhelming for non-specialists.

1. The authors claim that CoQ deficiency in the Pdss2 mutant mice "both preventable and treatable with oral probucol therapy". To strengthen this claim, the authors should assess whether probucol extends the short lifespan of the mutant mice.
2. It would be useful to know the levels of probucol in tissues and plasma or serum of treated mice.
3. The authors note that antioxidant effects of probucol "appear to be related to its sulfur atoms more so than its phenol rings". If so, reduction of the disulfide bond will generate two separate phenol rings that may be utilized as substrates for CoQ biosynthesis (i.e. substrates for Coq2) leading to increased CoQ levels. Have the authors considered this possibility? If so, the resulting CoQ-like product would have a very different ring structure from normal CoQ.
4. Were wild-type mice treated with probucol and CoQ10? If so, what were the effects of these compounds on CoQ level and transcript levels?
5. Page 4: The sentence "As subsequent evaluation of Mpv17-/- mice suggested..." should be moved to the Discussion.
6. The authors note that 2 female mutant mice were outliers because of extremely high albuminuria. Were effects of CoQ and probucol therapies on female mutant mice significant after excluding the outliers?
7. The authors' claim that probucol's therapeutic effect is mediated by reversal of altered PPAR pathway signaling (e.g. reduced PPAR expression), transgenic overexpression of PPAR would be necessary (but beyond the scope of this manuscript). Thus, this claim seems rather speculative and should be described as a possible mechanism.

1st Revision - Authors' Response 21 March 2011

RESPONSE TO EDITORIAL and REVIEWER CRITIQUES

EDITORIAL CONCERNS:

1. Thank you for the submission of your manuscript "Probucol ameliorates renal glomerular disease, tissue CoQ content, and PPAR-mediated metabolic sequelae of CoQ deficiency in Pdss2 mutant mice" to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript. You will see that they find the topic of your manuscript potentially interesting. However, they also raise significant concerns on the study, which should be addressed in a major revision of the manuscript.

We are grateful for the very helpful critiques. We have now addressed all concerns raised by the Reviewers, as detailed below and highlighted in the revised manuscript.

2. In particular, reviewer #3 highlights that the lifespan of treated mutant mice should be assessed.

We have added additional data on probucol-treated missense animals beyond 200 days old (Table 1), as well as include data on probucol-treated liver conditional knockout mutants beyond 330 days old (Table 4). These data show that probucol-treated missense mutant animals have no renal disease or early demise relative through at least 200-215 days old (Fig 6 and Supp File 12), which is in stark

© European Molecular Biology Organization

contrast to untreated missense mutants that typically die well before this age of their nephritis. The results of this experiment are now included in Table 1, lines 13 and 14.

We agree that lifespan study extending possibly to 3 years of age would be interesting. However, we do not feel that analysis will alter the major and novel finding that is the main focus of this manuscript as related to the preventative and therapeutic efficacy of probucol in an otherwise lethal renal disease.

3. In addition, reviewer #2 points out that additional insight into the mechanism behind the observed probucol effects would be required to strengthen the manuscript especially since some observations appear to be at odds with previous studies.

We have now detailed a careful response to each point raised by Reviewer #2, which we believe clarifies any issues related to concerns of discrepant observations.

4. On a more editorial note, we agree with reviewer #3 that the manuscript in its present form might be overwhelming for the non-specialist reader. We would thus encourage you to edit the text and title to make the manuscript easier accessible to the non-expert.

We appreciate the suggestion and have now changed the Manuscript TITLE to emphasize the major finding of this work, "Probucol ameliorates renal and metabolic sequelae of primary CoQ deficiency in Pdss2 mutant mice". We have also now abbreviated the Results section by moving original Table IV to the Supplementary Files (now Supp File 2), and have renumbered the eleven subsequent Supplementary Files accordingly.

We acknowledge the "copious" mechanistic studies detailed in this work, as astutely described by Reviewer #1, but consider these multi-faceted investigations to be necessary to explore the means by which probucol causes such dramatic therapeutic benefits, which we have tried to present in an organized and concise fashion. Indeed, the very nature of this work speaks to the need to address areas of relevance to a broad audience.

5. Please note that EMBO Molecular Medicine requires submission of microarray data to the ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) or GEO (http://www.ncbi.nlm.nih.gov/geo/) databases, and provision of accession numbers within the manuscript. We would kindly ask you to include the accession numbers in your revised manuscript.

We have now completed submission of all microarray data in this study to GEO and included the accession number for the 20 Illumina WG6 v2.0 arrays performed on missense mutant mouse liver (#GSE27954) in the revised manuscript.

6. Given the balance of these evaluations, we feel that we can consider a revision of your manuscript if you can convincingly address the issues that have been raised within the space and time constraints outlined below. Revised manuscripts should be submitted within three months of a request for revision. They will otherwise be treated as new submissions, unless arranged differently with the editor. I look forward to seeing a revised form of your manuscript as soon as possible.

We are grateful for the opportunity to submit the attached Revision for consideration, and have tried to do so as expeditiously as possible. We believe we have satisfactorily addressed the very thoughtful and insightful critiques of the three expert Reviewers, as detailed below and have indicated all changes in TRACK CHANGES in the Revised Manuscript.

Referee #1 - Comments on Novelty/Model system:

Overall the authors have performed clearly considered experiments in appropriate animal models. The use of Probucol is novel and the findings have potential significant impact for not only individuals with inherited mitochondrial disease, but for others with more common disorders that are associated with mitochondrial dysfunction.

We thank the Reviewer for their recognition of the novel nature and broad significance of this work.

Referee #1 – Other Remarks:

© European Molecular Biology Organization

Falk and colleagues report the effects of treatment of a mitochondrial mouse model of kidney failure with Probucol. This paper is carefully considered and well written and the findings are important (and supported by copious data), especially given the lack of effective therapeutics for mitochondrial disorders. In particular, the upregulation of the PPAR pathway signaling could be particularly significant for mitochondrial patients; improved mitochondrial biogenesis has the potential to ameliorate patient symptoms. The favorable comparison of Probucol to CoQ10 is also interesting. Although CoQ10 supplementation is commonly employed as a treatment for mitochondrial disease, effects have been disheartening. Many questions remain, but perhaps the findings reported in this manuscript at least begin to shed some light on this issue.

We thank the Reviewer for their recognition of the relevance of this work.

My comments are relatively minor in nature:

1) Do you have hypotheses related to the differential response and characteristics between male and female mice?

The Reviewer highlights an intriguing question. Significant gender effects on urine albumin levels were apparent following nearly all treatment durations and regimens, which were typically greater effect in males treated with probucol and in females treated with CoQ10 (Tables 1 and 2). A gender effect was also evident when comparing CoQ_9 and CoQ_{10} liver levels, which were both approximately 50% greater in females but increased following long-term probucol treatment in both genders (Supp File 12). While a gender effect is evident, we are not certain of its physiologic basis. Possible contributing factors may of course be hormonal. Indeed, steroid biogenesis occurs within the mitochondria and steroid biogenesis was the most significantly downregulated pathway by probucol (Zhang Z et al, *Mol Gen Metab*, 2010). Interestingly, male rats have been found to be more responsive than female rats to peroxisome proliferators, and PPAR-alpha mRNA and protein levels were both higher in rat liver of males compared to females (Jalouli et al., *Endocrinology* 144, 101-109, 2003). Thus, it is tempting to speculate that the probucol-mediated restoration of PPARalpha expression in the *Pdss2* mutant mice might be responsible for its enhanced responsiveness in male mice as compared to females. However, further investigations will be needed to explore gender contributions to therapeutic effects.

2) You state "As subsequent evaluation of Mpv17-/- mice suggested their "kidney disease could no longer be documented" (Spinazzola et al, 2006), the B6.Pdss2kd/kd mouse may be the only available probucol treatable animal model of renal glomerular disease caused by a nuclear gene encoded mitochondrial defect." Is the renal disease in Mpv17-deficient animals different or are the authors suggesting that the kd/kd mice are unique because of isolated renal disease, instead of the primary hepatic disease present in the other model?

While this manuscript was under review, we became aware of a publication that we had previously overlooked (Viscomi et al., 2009). Additional insights into the $Mpv17^{-/-}$ defect were described in that paper, along with the updated information that these mice do indeed develop the kidney disease that was previously observed if the animals are maintained until 2 years of age. The reason for the discrepancy between the 1999 and 2009 papers was attributed to differences in the genetic background of the mice as the transgene was successively backcrossed. We have corrected our oversight, and have now expanded our Discussion of Mpv17 and how it relates to the probucol effect.

3) The Figure 1 legend states "Near normal glomerular histology is evident upon hematoxylin and eosin staining of renal sections from male mice shown at 100X magnification." Presumably this refers to the probucol treated kd/kd animals.

We thank the Reviewer for bringing our attention to this oversight and have now corrected the Figure 1 legend, as suggested.

4) If CoQ biosynthetic pathway enzymes were not upregulated, why did CoQ levels increase?

While we did not detect significant alterations in gene expression of the CoQ biosynthetic pathway or rate limiting pathway enzyme by transcriptional profiling, it is possible that probucol's effect is more directly related to post-translational modification or perhaps stabilization of CoQ species and/or biosynthetic enzymes. Future work is needed to fully explore these intriguing possibilities.

5) "As probucol was previously reported to treat a mouse model of mitochondrial-based glomerular disease caused by mutations in Mpv17 (Binder et al, 1999) we added 1% probucol to standard mouse chow (TestDiet) fed to B6.Pdss2kd/kd missense mutant mice." A bit more background on

© European Molecular Biology Organization

Probucol would be helpful, especially given the association of the MPV17 mouse model with oxidant damage, which seems to be less of an issue with the kd/kd mouse. Why choose this agent - just because MPV17-deficient mice responded to Probucol therapy?

Kindly see response to #2, above. Although there have long been many unanswered questions related to probucol effects, we have systematically addressed in the Discussion each of the possible mechanisms that have previously been raised in the literature, including an extensive discussion on its antioxidant properties. We have now further updated and expanded our discussion on why probucol is interesting as related to the Mpv17-deficient mice.

Referee #2:

This is a manuscript which examined the effects of therapeutic interventions in a mouse model of mitochondrial based glomerular disease caused by mutations in Mpv17, which is a glomerulopathy variant, which results from CoQ deficiency within glomerular podocytes. Previous work showed that high dose CoQ10 supplementation mitigates renal disease in these mice through approximately 140 days of life, but does not normalize albuminuria to control levels, prevent nephritis nor restore CoQ9 or CoQ10 content in renal tissue. The authors in the present manuscript have examined the efficacy of treatment with the lipophilic drug probucol, in these mice. The authors have treated with probucol at 1% by weight in the diet. They monitored albuminuria as an indice of glomerulonephritis. They also determined the histological nephritis score immediately following sacrifice. They compared probucol supplementation from weaning or day 100 of life, high-dose CoQ10 supplementation from weaning or day 100 of life, or vitamin E from weaning. The dose of CoQ10 that was utilized was 1mg/per ml, which the authors estimate resulted in a final daily dosage of approximately 400 mg of CoQ10/per kg of body weight. Vitamin E was provided from weaning of standard chow at a dose of 300 IU per kg of chow. The CoQ10 preparation was LiQsorb from Tishcon Corporation. It is somewhat surprising that the CoQ10 was water-soluble but this must be a special preparation.

We appreciate the Reviewers' careful description of the general study scope and methods, but respectfully point out that the animals' disease results from a mutation in the CoQ biosynthetic pathway gene, *Pdss2*. The preparation of CoQ10 used is a special-order preparation that we chose based on its solubility and its currently being the standard of care preparation in human mitochondrial disease therapy.

The authors observed that the probucol therapy significantly prevented renal disease whether administered from the time of weaning through at least six months of age or for only approximately three weeks prior to sacrifice. The probucol treated missense mutant group showed significantly higher urine albumin than gender-matched healthy controls. Vitamin E showed a minor effect in the females as assessed utilizing the urine albumin, but no effect in the males. Long-term CoQ10 significantly prevented renal disease manifestations when provided from weaning through six months and significantly improved renal histology and function was seen when CoQ10 treatment was initiated at the time of birth rather then upon weaning. The probucol provided a more long-lasting and efficacious prevention of renal disease than did supplemental CoQ10. The short-term treatment from day 100 to sacrifice resulted in significantly greater improvement of both genders with probucol and relative to CoQ10 only in albuminuria but with equivalent effects on the nephritis score. The male missense mutant mice tended to respond similarly or better to probucol therapy, whereas the females responded similarly or better to CoQ10 supplementation. The authors also looked at the blood cholesterol levels, which were significantly increased in the Pdss2 mutants. Probucol treatments significantly decreased plasma cholesterol and triglyceride levels, in addition to the manifestations of albuminuria and nephritis relative to untreated mutant littermates. The authors also utilized another control, which was Simvastatin which neither prevented renal disease nor lowered blood cholesterol in these mice when given from time of birth. Although probucol did normalize the elevated blood cholesterol, this effect was insufficient to prevent the severe renal disease in these animals. The authors examined hepatic phospholipid levels using NMR analysis and found that cardiolipin content was 80% decreased in the liver of conditional knockout mutant mice, and normalized with probucol treatment. CoQ10 treatment had no effect on cardioplipin content in the mutant mice but modestly increased phosphatidylethanolamine and phosphatidylcholine. The authors did not observe any evidence of increased reactive oxygen species due to primary CoQ10 deficiency in either the Pdss2 missense or liver conditional knockout

© European Molecular Biology Organization

mutants. The total lipid peroxidation appears to be unaltered. Aconitase activity was also examined and did not show any difference following longterm CoQ10 therapy but was increased 15% with probucol. CoQ10 treatment increased MNSOD enzyme activity in the liver and kidney mitochondria from the missense mutant mice but it was unchanged by treatment with probucol. In the liver conditional knockout mutants, blood glucose was significantly increased and this was decreased by feeding the mice with probucol.

We sincerely appreciate the Reviewers' careful description of the multi-faceted study findings.

1. They also carried out genome-wide transcriptional profiling in the liver from the missense mutant mice on standard chow or supplemented long-term with either probucol or CoQ10. They utilized a number of different analyses to determine what pathways seem to be altered. There was an upregulation of oxidative phosphorylation, the tricarboxcylic acid cycle, and cell defense pathways in liver conditional Pdss2 knockout mice and similar effects occurred in both the missense and liver conditional knockout Pdss2 mice. The Pdss2 missense mutants uniquely upregulated lipid biosynthesis and downregulated fatty acid metabolism. Interestingly, probucol largely reversed the global pattern of metabolic pathway alterations. It reversed expression alterations within the PPAR signaling pathway, fatty acid metabolism and glycolysis in both mutant mice. Probucol did not have any effect on OXPHOS pathway upregulation. There also was no effect on antioxidant genes or hemoxygenase. This latter finding is at variance with a number of other reports in the literature, which were published by Dr. Stocker et al. They consistently show that hemoxygenase was upregulated by probucol. It is unclear why there is a discrepancy in the present report. In addition, Dr. Stocker et al., recently demonstrated that hemoxygenase induces a pattern of gene transcription with elevation of genes scavenging reactive oxygen species. It is, therefore, curious that this was not observed by the present authors. The authors should address this discrepancy.

As the Reviewer clearly points out, we fully expected heme oxygenase and ROS scavenging genes to have altered expression following probucol therapy, as we had originally cited from Dr. Roland Stocker's work (Stocker R, *Curr Opin Lipidol*, 2009). We have previously discussed this result directly with Dr. Stocker, and agree that is anomalous. However, this apparent discrepancy may relate to our investigation of this drug in the *Pdss2* mutant, whereas prior analyses of probucol effect have been in other models and wild-type mice. All that we believe is reasonable to conclude from this analysis is that altered expression levels of heme oxygenase and ROS scavenging enzymes are not the primary means by which probucol efficacy in Pdss2 mutant mice can be explained.

2. The authors did observe that branched chain amino acid metabolism was upregulated. Steroid biosynthesis and the complement and coagulation cascade were the most downregulated pathways. The authors found that PPARgamma and PPARalpha transcription factors were decreased in the missense mutants and this was normalized by probucol treatment but unaltered by CoQ supplementation. It would be nice if the authors had a good interpretation of all these expression data which would explain the efficacy of probucol. Does it act on specific promoters, etc. Does it stimulate PGC-1alpha; or Nrf2/ARE? A number of compounds that activate PPAR are not known to induce CoQ synthesis.

We agree with the Reviewer that it is important to investigate the central regulators for the observed pathway alterations. Indeed, we have begun several studies based on these data to investigate the transcriptional activators of the observed pathway changes. Our preliminary studies suggest that expression of multiple transcription factor target pathways are significantly altered in Pdss2 mutant liver. Interestingly, GSEA and rVista analyses of transcription factor pathways in the same expression data sets as reported in this manuscript suggest missense mutant mice significantly upregulate 20 and downregulate 7 transcription factor target pathways, several of which have normalized expression following either probucol or CoQ10 therapy. However, the validation, biologic role, and relevance to mitochondrial biology of many of these suggested transcription factor pathway alterations are not yet known. Therefore, we feel inclusion of a section exploring these data in this manuscript would not be appropriate at this time. Rather, premature conjecture on such data would likely make it significantly more overwhelming to non-specialists and detract from the manuscript's main findings.

3. In the unsupplemented mice, CoQ was exclusively oxidized whereas it was to a large extent reduced following treatment of the liver with probucol supplementation. The authors found that probucol supplementation increased total CoQ content which correlated with a rescue of kidney disease, as evidenced by decreased urine albumin and nephritis scores. In addition, the authors found that total CoQ9 content in kidney homogenates was significantly increased by short term

© European Molecular Biology Organization

probucol supplementation, but there was no significant difference in the liver. Overall, this is an interesting manuscript which describes some novel affects of treatment with probucol in transgenic mice which are either deficient in CoQ synthesis throughout the body leading to renal dysfunction or selectively in the liver. Probucol was shown to successfully treat the mice after onset of disease as evidenced by albuminuria. Furthermore, prophylactic treatment from birth to presymptomatic animals clearly increased the CoQ9 and CoQ10 levels in the liver and prevented nephritis.

We thank the Reviewer for concisely summarizing some of the most important findings of this work.

4. One issue which the authors have not addressed, is whether the deficient mice have a reduction in brain CoQ10 levels. It would be of considerable interest to determine whether this is the case and whether probucol administration has any effects on brain CoQ10 levels. This is particularly the case since many of the clinical manifestations of CoQ10 deficiency in patients are due to abnormalities in the brain such as cerebellar ataxia and seizures.

We commend the reviewer for this insightful comment. We have indeed tested these mice for neuromuscular coordination, and have observed significant deficiencies in all of the behavioral tests that we used. Reviewer #3 correctly observed that the present manuscript in its present form "may be overwhelming for non-specialists". Thus, we thought it best to describe the neuromuscular findings in a separate manuscript, which is currently in preparation but has not yet been submitted.

5. The authors did not observe any improvement to mitochondrial respiratory capacity in the Pdss2 mutants having primary CoQ deficiency. The inability of the authors to find evidence of oxidative stress is rather surprising. It is also surprising that there was no effect of probucol on ROS scavenging enzymes, which was previously reported in yeast. Although probucol has lipid lowering effects, its beneficial effects were not replicated by reductions utilizing simvastatin suggesting that these are unlikely to mediate beneficial effects. The authors found that there was a relative increase in expression of MNSOD but aconitase activity and 4HNE lipid peroxidation were unchanged following treatment with CoQ10 in the mutant mice, as compared to age-matched controls.

We had fully shared the Reviewers' expectation that mitochondrial oxidative stress would be increased in the Pdss2 mutants. However, careful analysis of multi-dimensional parameters, as the Reviewer concisely summarized, suggest that mitochondrial oxidative stress is not a gross component in all tissues of these mutant mice. We also analyzed all microarray data for expression alterations among major antioxidant pathways, including glutathione and thioredoxin. No significant expression alterations were seen either in individual genes or at the level of concordant pathways in either missense or alb/cre knockout mutants at baseline or following probucol therapy. These data are included as Supplemental File 6. Although global expression level alterations were not identified in these other antioxidant pathways, it is well-known that gene expression is not necessarily indicative of antioxidant response at the cellular level. The possibility remains that intracellular oxidative stress is increased in renal glomerular podocytes from Pdss2 missense mutant mouse, but was not directly testable with available methodologies.

Similarly, prior reports of probucol acting as an anti-oxidant agent raised the consideration that an antioxidant effect is a potential mechanism by which probucol prevents and mitigates renal disease in *Pdss2* mutant mice. However, we now appreciate that an antioxidant effect is not the primary mechanism of probucol action in these animals, since it does not appear to increase oxidant defenses or lipid peroxidation damage despite modestly increasing oxidant stress. Rather, our new observation that probucol significantly increases renal CoQ_9 levels (the primary CoQ isoform in mice) represents a more likely means by which probucol prevents and treats renal disease than does its purported function as an antioxidant agent. Similarly, while CoQ_{10} treatment clearly induces mitochondrial oxidant defense (MnSOD enzyme activity and expression), it alters neither oxidant damage (lipid peroxidation) nor oxidant stress (aconitase activity). These considerations are included in the Discussion.

The high dose CoQ10 supplementation in the present manuscript was also unsuccessful, which is somewhat surprising.

As we had previously reported (Saiki et al, AJP Renal, 2008), high dose CoQ10 supplementation does ameliorate disease, as was consistent with the data reported here in a different cohort of Pdss2 mutant animals. What we have further reported here, however, is that (1) clinical (renal) disease efficacy is higher with probucol than seen with CoQ10 therapy and (2) CoQ10 therapy does not replete endogenous CoQ10 content. In addition, we report novel data that chronic high-dose CoQ_{10}

© European Molecular Biology Organization

supplementation of *Pdss2* mutant mice in equivalent doses to that provided to many human patients does indeed induce antioxidant defenses, as assessed by both activity and relative expression assays of the prime mitochondrial antioxidant defense enzyme, MnSOD (Fig 2). We think these additional data provide novel insight of importance to understanding future therapeutic approaches in mitochondrial disease, which is clearly needed given the clinical observation astutely pointed out by Reviewer #1 that "although CoQ10 supplementation is commonly employed as a treatment for mitochondrial disease, effects have been disheartening".

6. Interestingly, probucol supplementation did reverse transcriptional alterations that affected a number of areas of intermediary metabolism. The authors believe that altered expression of key PPAR pathway transcriptional factors may play an important role, and that probucol enhances the PPAR signaling which then induces CoQ content.

We agree with the Reviewer that reversal of transcriptional alterations across many intermediary metabolic domains is an interesting finding from this work.

7. Overall, this is an interesting manuscript which has a number of very intriguing observations, the most important of which is that probucol seems to exert neuroprotective effects in a setting of CoQ10 deficiency. This may be due to its ability to increase tissue CoQ10 content and it appears to be independent of its hypolipidemic affects. The finding that CoQ supplementation was not successful is surprising. In looking at the data, however, it is unclear whether the CoQ10 supplementation was sufficient to normalize to the CoQ10 levels in these mice and I did not see CoQ10 levels in the brain.

We respectfully must point out that this manuscript details only the renal-protective effects of probucol in primary CoQ deficient Pdss2 mutant mice. Neurologic parameters at either the behavioral or metabolite level were not studied in this work but are under investigation for description in a separate manuscript focusing solely on an extensive series of experiments to define the neurologic manifestations of Pdss2 missense and cell-specific conditional knockout mice.

8. A number of observations were made for which there is no good explanation. Amongst these is why treating symptomatic animals with probucol for only two weeks significantly increased CoQ9 levels in the mutant kidney at day 100 and long-term CoQ10 supplementation did not result in an increase of either quinone species. It would be useful for the authors to address this.

One consideration to explain why CoQ10 supplementation did not increase either quinone species might be the tissue bioavailability of the preparation used. Although we used the commercial LiQsorb version of CoQ10 (ubiquinone) preparation that is commonly used to treat human mitochondrial disease patients, it is well-known that essentially all CoQ10 preparations that are currently available are inherently suboptimal. Indeed, there are clinical trials underway for "more bioavailable" preparations, such as trials of EPI-743 (Edison pharmaceuticals), MitoQ (Dr. Michael Murphy), and possibly, Idebenone; however, none of these are both proven to be safe and efficacious in humans and currently commercially available. Thus, even though some renal-protective benefit was seen with the CoQ10 supplementation (both in this work and previously by our group as published by Saiki et al, 2010), its incomplete efficacy may relate in part to these issues of tissue bioavailability. Our data can be interpreted as confirmation that long-term, high-dose ubuiquinone does not increase tissue quinone levels. These points are included in the Discussion in the section on 'effects of chronic CoQ10 supplementation in primary CoQ deficiency'.

9. The most important observation of this manuscript is that treatment with probucol may be useful in the treatment of some patients who have mitochondrial dysfunction, although the mechanism is not clear from the present paper.

We concur with the Reviewer that probucol does hold promise as a possible therapeutic agent to be rigorously studied in human mitochondrial disease. Data presented in this work would suggest its mechanism relates to increasing endogenous, reduced CoQ isoforms as well as transcriptional pathways (such as PPAR) that appear to mediate downstream sequelae of primary CoQ deficiency.

Referee #3 - Comments on Novelty/Model system:

This extensive study suggests that probucol ameliorates the renal phenotype of mice with primary CoQ deficiency. The work is novel and technically solid. Evidence for some conclusions could be

© European Molecular Biology Organization

strengthened (see comments to authors). The findings have obvious therapeutic implications for patients with CoQ deficiencies. The manuscript presents a large amount of data, which may be overwhelming for non-specialists.

Referee #3:

Falk et al. have performed an extensive study of the effects of probucol on mice with coenzyme Q (CoQ) deficiency due to a defect of CoQ biosynthesis (Pdss2 mutation). Probucol is a lipophillic antioxidant and hyper-lipidemic diphenol linked by a disulfide bond. The CoQ-deficient mice develop nephropathy (glomerulopathy with proteinuria) that is fatal. Probucol treatment appears to ameliorate that nephropathy in the mice more effectively than CoQ supplementation. The mechanism of probucol therapy is unknown, but CoQ deficiency and altered transcriptional profiles (particularly PPAR pathway signaling) in liver of Pdss2 mutant mice are ameliorated by probucol. The work is novel and technically solid. Evidence for some conclusions could be strengthened (see below). The findings have obvious therapeutic implications for patients with CoQ deficiencies. The manuscript presents a large amount of data, which may be overwhelming for non-specialists.

We thank the Reviewer for recognizing the value, novelty, and technical nature of the extensive studies performed to dissect the therapeutic mechanism of probucol in Pdss2 mice. We agree with the Reviewer that the manuscript presents a large amount of data as we believe is necessary to address the range of mechanisms by which dramatic therapeutic benefit of probucol might reasonably be expected to occur. However, we have ardently tried to present these data in as clear a fashion as possible, with only the most directly relevant findings present in the main text and all other analyses located in the Supplemental Files. We have now simplified the Title, as detailed in our response to Editorial Critique #4 (above), to draw attention to the main content of this manuscript. In addition, we have now moved Table IV that reports all metabolite data from the main manuscript to revised Supplementary File 2.

1. The authors claim that CoQ deficiency in the Pdss2 mutant mice "both preventable and treatable with oral probucol therapy". To strengthen this claim, the authors should assess whether probucol extends the short lifespan of the mutant mice.

As detailed in our response to Editorial Comment #2, above, we have added additional data on probucol-treated missense animals beyond 200 days old (Table 1), as well as include data on probucol-treated liver conditional knockout mutants beyond 330 days old (Supp File 2). These data show that probucol-treated missense mutant animals have no renal disease or early demise relative through at least 200-215 days old (Fig 6 and Supp File 12), which is in stark contrast to untreated missense mutants that typically die well before this age of their nephritis. We now include these new results in Table 1, lines 13 and 14.

We agree that lifespan study extending possibly to 3 years of age would be interesting. However, we do not feel that analysis will alter the major and novel finding that is the main focus of this manuscript as related to substantial preventative and therapeutic efficacy of probucol in an otherwise lethal renal disease for which no other therapy is known.

2. It would be useful to know the levels of probucol in tissues and plasma or serum of treated mice.

The estimated dose of probucol was 95 mg/kg/day, as detailed in the Methods section. However, probucol levels were not measured in the treated animals. There is no standard, easily performed assay to quantitate probucol levels, as we learned through detailed discussions with international probucol experts (George Rothblat, PhD and Roland Stocker, PhD). Nor is there a meaningful target blood or tissue "concentration" that we were aiming to reach. The goal of this manuscript was not to detail the pharmacokinetics of Probucol administration but rather its phenotypic effects. Indeed, consistent demonstration of ameliorated renal disease offers strong indication of phenotypic effect regardless of tissue levels achieved.

3. The authors note that antioxidant effects of probucol "appear to be related to its sulfur atoms more so than its phenol rings". If so, reduction of the disulfide bond will generate two separate phenol rings that may be utilized as substrates for CoQ biosynthesis (i.e. substrates for Coq2)

© European Molecular Biology Organization

leading to increased CoQ levels. Have the authors considered this possibility? If so, the resulting CoQ-like product would have a very different ring structure from normal CoQ.

This is an intriguing suggestion. However, it is important to note that the sulfur atoms in probucol are not in fact in disulfide linkage. Therefore, the mechanism that must be invoked to produce independent aromatic rings is not a straightforward reduction of a disulfide, and would require oxidation of the quaternary carbon that links the two sulfur atoms. Thus, while the idea is very novel, it seems too speculative to include in the discussion.

4. Were wild-type mice treated with probucol and CoQ10? If so, what were the effects of these compounds on CoQ level and transcript levels?

We have performed extensive analyses of CoQ levels in liver and kidney in B6 wild-type control mice (Saiki et al, AJP Renal, 2008). However, we have not analyzed CoQ levels nor transcript levels in these control mice on any supplements such as CoQ10 or probucol. All analyses following such treatments have been performed in Pdss2 mutant mice.

5. Page 4: The sentence "As subsequent evaluation of Mpv17-/- mice suggested..." should be moved to the Discussion.

We thank the Reviewer for bringing our attention to this point, and have now amended this sentence as detailed in our response to Reviewer #1, Critique #2.

6. The authors note that 2 female mutant mice were outliers because of extremely high albuminuria. Were effects of CoQ and probucol therapies on female mutant mice significant after excluding the outliers?

It is important to note that urine albumin is paradoxically very low in end-stage renal disease, which can account for outliers having low urine albumin despite high nephritis score. Thus, it may well be that the 2 female missense mutant 'outliers' were caught late in their disease just before glomerular degeneration. Nonetheless, we have now included in the Table 1 legend summarized urine albumin levels and nephritis score for the untreated female missense mutant mice. Effects of CoQ10 and probucol therapy did remain significant after excluding these two outliers.

7. The authors' claim that probucol's therapeutic effect is mediated by reversal of altered PPAR pathway signaling (e.g. reduced PPAR; expression), transgenic overexpression of PPAR; would be necessary (but beyond the scope of this manuscript). Thus, this claim seems rather speculative and should be described as a possible mechanism.

We fully agree with the Reviewer that PPAR overexpression would be a necessary future study, and have clarified the manuscript to emphasize this is one possible mechanism for the observed effects.

In summary, we thank the Editor and Reviewers for their careful reading of our manuscript. In responding to the suggestions and corrections raised we believe our manuscript is significantly improved. We are grateful for your consideration of this revised version for publication in *EMBO Molecular Medicine*.

2nd Editorial Decision 19 April 2011

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:

Reviewer #3 points out that he/she still has concerns about the length of the manuscript and the accessibility to a more general readership. We would thus encourage you to move some text (for example describing supporting material) to the Supplementary Information section.

Please also provide an index on the first page of the Supplementary Information.

Please amend the format of 'The Paper Explained' according to journal style. I attached a published

© European Molecular Biology Organization

EMBO Molecular Medicine paper for your information.

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

Yours sincerely,

Editor
EMBO Molecular Medicine

REFEREE REPORTS:

Referee #2:

I reviewed the manuscript and I think the changes the authors made are fine.

Referee #3 - Comments on Novelty/Model System:

I remain concerned that this very long, highly detailed, and data rich manuscript may be overwhelming for non-specialist. Nevertheless, the results appear reliable and are worthy of publication. I suggest moving Figure 3 to supplemental data.

Referee #3 - Other Remarks:

This is an extensive study of probucol treatment in Pdss2 mutant mice. The data indicate that probucol exerts beneficial effects and alters specific pathways in the mutant animals. Previous criticisms of reviewers have been addressed. This long and highly detailed paper may be overwhelming for non-specialists. The authors should consider moving figure 3 to the supplemental data.

2nd Revision - Authors' Response 27 April 2011

We are thrilled to have our work published in EMBO Molecular Medicine and thank you for your efforts regarding this manuscript.

We have worked to address the outstanding requests, particularly to streamline the manuscript results section. In particular, we have now combined 2 prior results section related to plasma lipid levels and liver phospholipids into a single paragraph, removing all details about the liver phospholipids to the Supplemental File 2 description. In addition, we have removed details related to gender effect of probucol supplementation on tissue CoQ levels to the Supplemental File 12 description. We have assured that all other discussion of supplemental files within the Results section is limited to a maximum of 1-2 sentences for each to introduce the relevance of its content. In addition, we have added an INDEX for the supplemental files, which we agree greatly facilitates the utility of this file. We have also amended the Paper Explained section per your guidelines, which we have attached here to determine if this is now acceptable.

If possible, we would like to request leaving Figure 3 in the main text. It's explanation within the Results text is very concise, yet the data visually conveyed in the figure we believe to be very helpful to elucidate the range and degree of metabolic alterations in the mutant mice, as well as their dramatic response to therapy. These findings are central to the paper.

Please let us know if these changes are acceptable, and we will gladly upload them to the online system.

Many thanks for your consideration,

© European Molecular Biology Organization