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JoVE

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Dear Dr. Wu,

Thanks for overseeing the review of our manuscript. We are pleased to see the positive evaluation by the reviewers and appreciate all the constructive Editorial and reviewer comments. As discussed in the following point-by-point response, we have addressed all of the comments in the revised manuscript. We feel that these changes have improved our work and hope that you and the reviewers will find the revised version suitable to publication in JoVE.

Best regards,

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EDITORIAL COMMENTS:

The manuscript has been modified and the updated manuscript, 59587_R0.docx, is attached and located in your Editorial Manager account. Please use the updated version to make your revisions.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

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Not applicable.

3. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Done.

4. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) in yellow that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Done.

5. Please use h, min, s for time units.

Done.

REVIEWER #1:

Manuscript Summary:
Well written clear presentation of a useful methodology. Last paragraph of Discussion (lines 417-431) is excellent.

Major Concerns:

The manuscript states that there can be significant inter-lobe differences in bile duct density within a liver, and the authors strongly emphasize that, therefore, there is a consequential need for using a method that evaluates across the entire liver. Whereas there is no doubt that a full-liver analysis is good, the authors do not establish in their presentation that this concern is, indeed, valid, nor that a real

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(significant) need for this exists. The data presented do not support this mandate. To support this concern, I would need to see a an analysis of inter-lobe bile duct density variance in a substantial set of livers. If the authors cannot clearly establish that this is a true statistically significant need, and that erroneous conclusions are likely to arise from more restricted (e.g., single-lobe) analyses, then the language needs to be adjusted. Otherwise, there is a risk that this publication will mandate unnecessary overkill, and future research submissions in the field reporting completely robust conclusions might be rejected for not following an unnecessarily exhaustive prescription.

I would like to re-emphasize that this is a very good submission, and regardless of the outcomes of a study on inter-lobe variance, should be published in JoVE. However, the language used needs to be supported by data presented. In the absence of demonstrating a significant need, the authors could simply re-word their presentation to indicate that the method presented is an improvement over more restricted analyses, rather than presenting it as a need.

We fully agree with the reviewer. In the revised manuscript, we have adjusted the language to address this point. We removed the "variability" argument from the Introduction. Also, in the Discussion, we now write "We use a microscopy slide covering all liver lobes for each animal and systematically quantify the bile duct to portal vein ratio across the whole slide. While this is not essential, it ensures that ample portal tracts are analyzed for each animal regardless of age and liver size (60-90 PVs per liver depending on the genotype and age). Covering all liver lobes for each animal can also decrease variability in measurements if a given mutations does not affect bile duct development uniformly across the liver."

Minor Concerns:
Does use of the phrase: "Last but not least" (line 53) serve a purpose?

Rephrased as "In addition".

REVIEWER #2:

Manuscript Summary:
The authors describe a method to quantify biliary structures on tissue sections. By counting the average number of bile ducts associated with the clear lumen around each portal vein, they distinguished between wild-type and Jag1+/- mice.

Major Concerns:
Because the mutant obviously has abnormalities of the biliary structure, it is quite easy to show the difference against the wild type by any quantitative experiments. Therefore, the reviewer could not judge whether this method is useful to estimate abnormalities of the biliary structure.

In our previous paper (Thakurdas et al, Hepatology, 2016), we have shown that this method not only shows the difference between BD numbers of wild-type and Jag1 heterozygous mutant, but can also detect a statistically significant but modest difference (20% to 30%) in BD numbers caused by several other genetic manipulations. Therefore, the presented method does not need a major defect in the number of BDs in order to detect a difference. In the revised version, we refer to the above-

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mentioned observations.

Minor Concerns:
The authors should show how many PV areas they need to count to determine the number of bile ducts for each animal.

We count all the PVs on a section from the whole liver. Depending on the liver size, this allows us to analyze between 60 and 90 portal veins per animal. This is added to the revised version.

Why the authors need to use anti-CK8 antibody in addition to anti-CK19 to recognize biliary structures? Which antibodies did the authors use?

We thank the reviewer for raising this point. We should have clarified the reason for this choice in the manuscript. CK19 marks mature biliary cells. In contrast, CK8 marks both mature and immature cholangiocytes. We use a 50:50 mixture of anti-CK18 and anti-CK19 antibodies for two reasons. First, even in wild-type animals, there are a number of PVs (especially in the peripheral areas of the liver) that do not have a large enough portal vein to be identified by immunofluorescent or immunohistochemical staining. In mutants affecting BD development, this can be even a bigger issue. If we only use CK19 staining, it will be difficult to differentiate those PVs from central veins, and this will distort the BD to PV ratio. To confidently distinguish PVs from central veins, a second antibody needs to be used together with CK19. We have chosen CK8, because it enhances the overall staining of BDs (please see the second reason) and because it will also provide an assessment of the degree of ductular reaction. An alternative strategy would be to co-stain the liver sections with CK19 and glutamine synthetase (GS), which exclusively marks the hepatocytes surrounding the central veins (Kuo et al, Molecular and Cellular Biology, 1988). Second, in our hands, individual CK19 or CK8 staining of the biliary cells generates a relatively weak signal and is associated with background staining. Mixing the two antibodies results in a consistently strong signal in biliary cells and therefore facilitates the quantification. In the revised Discussion, we have added a new paragraph to address this issue.

REVIEWER #3:

Manuscript Summary:
This is a manuscript describing a method for determining bile duct density in the mouse liver. The experimental protocol was simple and clear. The methods described in this paper can quantify the density of bile duct formation. However, several points in this manuscript should be changed.

Major Concerns:

#1. As described by the authors in Page 8, Line 378-380, there are differences in the sizes of portal vein between the hilum and peripheral portion of liver. Authors described that "every portal vessel across the liver is imaged": however, one portal vein is cut in cross-sectional, and another is cut in longitudinal axis. To improve reproducibility of this method, authors should define the inclusion and/or exclusion criteria of portal vein diameter to count the number of bile ducts.

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The reviewer makes an important point. We occasionally see portal vessels that are cut longitudinally. In those cases, there is usually a neighboring bile duct that is also cut longitudinally and appears like a long open tube. To ensure reproducibility, we count these long tubes as a single bile duct. We have described this in the second paragraph of Discussion in the revised manuscript.

#2. Page 8, Line 383-386. Authors described that "this can sometimes result in one structure being called a bile duct by an investigator but not by another colleague, … we recommended that all samples related to a specific project be analyzed by the same person". It seems to be scientifically strange.
To improve reproducibility of this method, another colleague should assess the result of bile duct density using same specimens, but not by a single researcher. Authors should change the description about this point.

Thanks for raising this point. What we wrote was meant to address an important issue about consistency of measurements across genotypes in a given project. However, having read the reviewer's comment, we realized that the original sentence did not take into account another equally important issue, namely reproducibility. We fully agree that having more than one investigator perform the counts is important. In fact, we have done this in our lab. When multiple individuals in our lab assessed bile duct counts of the same samples, we observed a variation of 5-10% which is consistent across all genotypes for each person. In other words, some investigators counted 5-10% fewer bile ducts for all genotypes compared to other investigators. Therefore, in the first submission we recommended that one person perform all of the quantifications for a project. In the revised manuscript, we have rephrased to suggest that at least two individuals perform all the counting of all samples to ensure both consistency and reproducibility.

Minor Concerns:
#3. Page 9, Line 413. "it in sensitive" is a spelling error.

Spelling error is corrected.

REVIEWER #4:

The authors describe a method to calculate the average number of bile ducts associated with each portal vein in sections covering all lobes of a mouse liver. In this method, the bile ducts are identified via light microscopy of cholangiocytes stained with antibodies to two different cytokeratins. These bile ducts are then counted and divided by the total number of portal veins present in the liver section, providing a quantitative relative assessment of bile duct to portal vein ratio across the liver. This method was able to clearly distinguish between liver sections of wild type mice and a mouse model of Alagille syndrome. This study makes a valuable contribution to the methodology available to study bile duct differentiation and formation in mouse models.

Specific comments:

1. The manuscript does not state clearly whether EVERY section needs to be stained and counted. They clearly make the point that the observed phenotype can vary across different liver lobes, but is it really necessary to stain every section? How many sections would that amount to in a typical situation (e.g., an

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8 week old adult liver, or a postnatal day 6 liver). Having done my own sectioning, I know very well that you sometimes lose some. How would this be accounted for? I feel that some additional details along these lines are essential for the manuscript.

We apologize for not clarifying this issue. Not every section needs to be stained and counted. We continue the sectioning until all lobes are present on a given slide. We then stain that single slide and quantify all PVs and bile ducts on it. In other words, for each animal we use one slide covering all lobes for quantification. We have updated the phrasing to make this clear.

2. The abbreviation wsCK is used multiple times in the manuscript prior to it being defined at the bottom of page 6 (first paragraph of the Representative Results section).

We have defined wsCK in the Introduction upon first use.

3. Line 371 (suggested change in capital letters): Thus, to ANALYZE bile duct development

Changed analysis to analyze as suggested.

4. Line 413: previously reported that it IS sensitive.

Corrected.

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