Rebuttal JoVE59096

We are thankful for the editorial and reviewer comments for improving our manuscript. We have modified the protocol as well as **Figure 1**, per the comments, and have a point-to-point response below.

Response to Editorial Comments:

We would like to thank the editor for her excellent comments which are addressed in a point-by-point response below.

1) ***"Protocol detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. mention surgery steps and tools) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol."*** - Steps 2.1, 2.1.1, 2.1.2, and 2.1.3 have been edited to include specific details on identifying and extracting the pancreas.
2) ***"Protocol detail: 2.1: unclear how the pancreas is dissected? Please add necessary steps to describe how sterility is maintained. For surgical steps please mention how and where incisions are made along with tools used. Mention any instructions to identify the pancreas and avoiding damage."*** - Steps for the dissection of the pancreas have been added (2.1, 2.1.1, 2.1.2, 2.1.3). For maintaining sterility, penicillin-streptomycin is included in the HBSS that the pancreas is put into immediately following dissection (noted in steps 1.6, 1.8.2, and 2.1.3). Step 2.1.3 also notes that while the dissection is done on a benchtop the remaining steps should be carried out with sterile technique under a laminar flow hood.
3) ***"Protocol Detail: 2.4: mention pipette tip size."*** - Use of a 5 mL pipet was added to step 2.4. We found additional steps that could be clarified by mentioning the pipet used and therefore also added this information to steps 2.6, 2.7, 2.8, and 2.9.
4) ***"Protocol Detail: 3.2, 3.3: mention the virus used (and cite a reference for its use)"*** - The specific adenovirus used was added to step 3.2 and the table of materials. Step 3.3. was moved to the discussion to compare and contrast adenoviral and lentiviral infection.
5) ***"Protocol Detail: 4: Since you do not show how to embed the cells in matrigel, please delete the references to Matrigel in 2.10.1, 3.2, 3.3. Alternatively, mention steps to***

perform embedding in Matrigel." - Step 4.3 was added to explain embedding in Matrigel (noted as basement membrane matrix in the manuscript).

6) ***"Protocol Detail: 4.2: Mention the stimulus required for ADM generation. Also mention dosage/concentration."*** - The stimulus used was 50 ng/ml TGF-α and this was added to steps 4.2.2 and 4.4, as well as the table of materials and Figure 2's legend.
7) ***"Results: Since you discuss both Matrigel and collagen embedding, I recommend adding steps mentioning Matrigel embedding in the protocol (currently only collagen embedding is described."*** - Steps detailing basement membrane matrix (Matrigel) embedding were added (4.3).
8) ***"Discussion: Please ensure that the discussion covers the limitations of the technique."*** - Limitations were added to the discussion, to include differences in collagen and basement membrane matrix in terms of experimental design. The discussion section 'Significance and Applications' was expanded to 'Significance, Applications, and Limitations'.
9) ***"Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Matrigel, parafilm, polybrene."*** - "Matrigel" was changed to "basement membrane matrix", "polybrene" was changed to "viral infection enhancer reagent", and "parafilm" was changed to "plastic paraffin film". "Ibidi" was also removed from the manuscript. We did not detect any other commercial language.
10) ***"Commercial Language: Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names."*** - Ctrl+F was used to find all instances of "Matrigel", "Polybrene", "Parafilm", and "Ibidi" to replace as noted in comment #9.
11) ***"Commercial Language: Please replace the product name "Matrigel" from Fig 1, 2 with a generic alternative (e.g. basement membrane matrix)"*** - "Matrigel" was replaced with "basement membrane matrix" both of the figures and figure legends.
12) ***"Table of Materials: Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials/software in separate columns in an xls/xlsx file. Please include items such as adenovirus and lentivirus (both viruses mention the catalog number),etc."*** -The

table of materials was revised to include all essential components with catalog numbers. The adenovirus was added, as was the kit for generating lentivirus.

13) ***"Table of Materials: Please remove the footnotes and use the "Comments" column instead."*** - The footnotes were removed and put into the "Comments" column.
14) ***"Table of Materials: Please list the materials in alphabetical order (Name column)."*** - The materials were revised to be in alphabetical order by name.
15) ***"Please define all abbreviations at first use."*** - The following abbreviations were identified and edited to include a definition at first use: TGF-α, MMP-7, RANTES, TNF-α, NF-κB, EGFR, and EGF.
16) ***"If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*** - All figures and tables are original.

Response to Reviewer #1 Comments:

We would like to thank the reviewer for her/his comments describing the manuscript as ***"timely"*** as well as ***"detailed and easy to follow"***. There were no additional comments to address.

Response to Reviewer #2 Comments:

We would like to thank the reviewer for her/his comments, noting that ***"The manuscript clearly describes the procedure and provides information needed to make necessary preparation."*** Below is a point-by-point response to the reviewer's comments.

1) ***"Please check the nomenclature for genes and proteins [human and mice],"*** - The nomenclature for genes and proteins was corrected in the manuscript, notably in the introduction, but also checked throughout the manuscript.

2) ***"Point 1.6 - please provide the concentration of penstrep,"*** - The amount of penicillin-streptomycin was added to step 1.6. This is using the penstrep noted in the table of materials which is a 100X solution, therefore it is used at 1X in step 1.6.
3) ***"Points 2.2 and 2.3 - in both cases HBSS with penstrep is used, please add penstrep to these sections,"*** - Penicillin-streptomycin was added to steps 2.2. and 2.3.
4) ***"Point 3.1 lists as an alternative use of low-attachment plates. They are different types of low attachment plates it would be great if Authors could provide in the material section information regarding manufacturer and catalog number."*** - While it is possible to use low attachment plates, using the lid of 35 mm plates works very well. We have removed wording mentioning low-attachment plates.
5) ***"Point 4.2, Authors suggested to add stimulus or inhibitor to the collagen-cell suspension. I was wondering if they can add an option of adding stimulus or inhibitor to the media as these can be change every three days, and maybe add short statement why adding directly to the cell mixture is more effective than to the media."*** - We add the stimulus or inhibitor directly to the cell suspension as well as in the media, with replacement of the media on the day after embedment and then every other day. We have updated the manuscript to make these details more clear (see step 4.4).
6) ***"Point 5.6. Here, the Authors provide information regarding last step of preparation of cells for analysis with snap-freezing the cells for storage or immediate use. It would be great if Authors could add some comments/suggestions how to prepare cells for immunofluorescence stain or FACS sorting."*** - Details on preparation for immunofluorescence were added to the discussion in the second paragraph of the 'Significance, Applications, and Limitations' section.
7) ***"Modifications section: I think I would prefer that this section is shown in the main protocol point by point as alternative for embedment in collagen. It will be easier to follow and understand differences between these two ways of embedding the cells."*** - We are thankful for this suggestion and in agreeance have included embedment in Matrigel within the protocol point by point and changed the modifications to address lentiviral infection.
8) ***"Figure 1 - The numbers of steps in this figure should match the numbering of the sections in the main text e.g. Step 6 on Figure 1 does not refer to any of the steps provided in the main text."*** – This is an excellent point. Figure 1 has been updated to match the numbering of the sections within the protocol.

Response to Reviewer #3 Comments:

We would like to thank this reviewer for her/his excellent comments and referring to our manuscript as ***"highly detailed"*** and ***"an important manuscript as slight differences in the isolation and culturing procedure have significant consequences"***. Below is a point-by-point response to the reviewer's comments.

1) ***"The source of collagenase greatly effects the time and extent of acinar cell isolation. Even from the same company, collagenase digestion can vary significantly in time. This should be included. Also, what is the expected yield in term of acinar number?"*** - Step 2.5.1 was modified to direct the reader to the 'critical steps and troubleshooting' section of the discussion, where there are tips on looking for adequate digestion and a warning to not over-digest the tissue. Additional comments were put into the 'critical steps and troubleshooting' section to highlight that different digestion times may be required. We also included an average pancreas weight with which we typically use this protocol and noted an increase in digestion may be required for larger pancreata. As the yield from this protocol is acinar clusters, we do not take a cell count, but rather adjust our digestion time based on the size of the pancreas, of which the weight is a good indicator.
2) ***"Assessment of pH is also important and should be monitored throughout the stages of digestion and isolation."*** - In our protocol we do not monitor pH throughout digestion and isolation, yet we achieve consistently reliable results. The pH of the solutions used in digestion and isolation, including the collagenase diluted in HBSS, ranges from 7.0-7.4. As assessing pH is not part of our protocol, we did not alter the manuscript to include this. Evidence of our reliable results lies in previous publications in which we employed this technique, such as:

   Liou, G. Y. *et al.* Protein kinase D1 drives pancreatic acinar cell reprogramming and progression to intraepithelial neoplasia. *Nat Commun.* 6:6200, (2015).

   Liou, G. Y. *et al.* Mutant KRas-Induced Mitochondrial Oxidative Stress in Acinar Cells Upregulates EGFR Signaling to Drive Formation of Pancreatic Precancerous Lesions. *Cell Rep.* 14(10):2325, (2016).
3) ***"The use of lentivirus to infect acinar cells is much less common in the literature and, from my interactions with several laboratories, much more involved than adenoviral infection. A nice addition to this proposal would be to talk of the differences between the different infection types and provide a very detailed methodology section on***

lentiviral infection." - We moved the lentiviral infection section of the protocol to the 'modifications' section of the discussion and have provided additional details on the differences between infection types. Within the modifications we describe our method for lentiviral infection of primary cells, which is not significantly more involved than adenoviral infection.

4) ***"Purity of the culture is often an issue and it would be good to know that amount of duct cells vs. acinar cells vs. islets that are obtained in this approach. It would also be of significant value to speak to the isolation of acini from KRAS expressing tissue, especially tissue already containing significant ADM. Do these structures come out in this protocol as well?"*** - In regards to the amount of duct vs acinar vs islet cells obtained in this approach, the protocol is specific to acinar isolation. Similar protocols for the isolation section of our protocol note that the filtration step through the 105 µm filter separates the acinar cells from the ducts and islets[1,2].

   [1] Gout, J. *et al.* Isolation and culture of mouse primary pancreatic acinar cells. *J Vis Exp.* 10.3791/50514 (78), (2013).

   [2] Williams, J. A. Isolation of rodent pancreatic acinar cells and acini by collagenase digestion. *Pancreapedia: Exocrine Pancreas Knowledge Base.* 10.3998/panc.2010.18, (2010).

   The modifications section was altered to include a discussion of isolation of acinar cells from *Kras*^{G12D} expressing mice. As noted in the revised section, we advise isolating cells from an LSL-*Kras*^{G12D} mouse followed by infection with adeno-cre to induce expression of KRas^{G12D}. This helps with reproducibility between experiments as there can be wide variation between fibrosis and lesions between transgenic mice. Of note: Our protocol is optimized for isolation of acinar cell to investigate the ADM process. The isolation of ADM and PanIN cells from *Kras*^{G12D}-expressing mice for organoid culture requires altered protocols.

5) ***"The centrifugation of the cells described during isolation seems fairly harsh. In our hands we use centrifugation at 100-300g, which yields a softer pellet with more viable cells that are easier to filter."*** - A note was added in the 'troubleshooting' section of the discussion such that if there is an issue with viability, the centrifugation in steps 2.4, 2.6, and 2.7 can be brought down to 300 x g. As we have not had issues using the centrifugation noted in the main protocol, we have not altered this detail there.

6) ***"Also, the figure provided showing ADM infected with GFP does not show GFP in 5 day old cultures. Why is this? The expression of GFP should be ubiquitous."*** –During

prolonged cell culture, we sometimes see a loss in fluorescence due to the stability of the protein and the potential for the signal to be diluted out upon proliferation.

7) ***"The definition of trans-differentiation should be provided as this is used in several different fashions. The original idea of trans-differentiation is direct conversion of one cell type to another without an intermediate phenotype. In the case of ADM, acinar cells revert to a progenitor like state, expressing progenitor cell markers such as SOX9 and PDX1, then to duct cells. This is called trans-differentiation by many laboratories but by the strict definition, is actually not. The authors should be clear about this. In particular the idea of "trans-differentiation" back to acinar cells is often referred to as re-differentiation."*** - "Transdifferentiation" was changed throughout the manuscript to "ADM" or "acinar cell differentiation to ductal cells".

8) ***"Line 65 - provide the full name for RANTES."*** - We modified the introduction to include the full name of RANTES and defined other abbreviations in that same section.

9) ***"Line 77 and 78 - "One caveat of using primary acinar cells is that they are more difficult to transfect than typical cancer cell lines." I am not aware of transfection working at all in acinar cells so this statement should be stronger."*** – We agree that the statement should be stronger and as such, have changed it to "One caveat of using primary acinar cells is that they cannot be transfected as typical cancer cell lines can."

10) ***"The first two points of the methodology (Lines 89-93) seem overly detailed. Maybe this is JoVE's expectation, but likely indicating the size of the mesh to be required would be sufficient."*** - These instructions are important for maintaining sterility. Folding the mesh allows the user to avoid handling the point at which cells are put through the mesh.

11) ***"Space between numbers and oC should be removed."*** - Per the JoVE instructions, there should be a space between numbers and °C.

12) ***"Not sure why the acinar cell isolation portion of the manuscript was highlighted. Is this typical?"*** - Instructions for submission to JoVE included highlighting material that would go in the video.

13) "***Line 148 - the actual amount, type and source of collagenase should be included here as this is a critical component of the protocol."*** - The concentration of the collagenase is given in step 1.7, which is when the collagenase is prepared. Step 2.5 references the use of the collagenase diluted in step 1.7 and details of the collagenase (type and source) are provided in the table of materials.

14) ***"Line 179-180 - some idea of the acinar cell number should be provided for infection as opposed to two infections from one pancreas. This will vary dependent on the age,***

size and genetic background of the animal." - The addition of step 3.1.2 addresses this. While we did not provide the cell number to use, we provided a guideline for the volume/plate size. Using animals outside of the provided age range or of a different genetic background may require a larger volume, as noted in the revised manuscript.

15) ***"Line 214 - regarding spatulas, it should be clear that one spatula is used per culture plate/well."*** - Step 5.1 has been modified to include altering spatula number according to the number of conditions.

16) ***"The isolation procedure described at the end is fairly extensive and long and will greatly affect gene expression. Some suggestions should be given regarding downstream analysis to mitigate these effects."*** - This limitation was added to the discussion and we suggested confirming results via immunofluorescence, as the fixation time is considerably less than that of harvesting for qPCR or Western blotting.

17) ***"As written, the protocol appears to suggest that pharmacological agents cannot be added after embedding. This should be clearer."*** - This is an important point. Please see our response to point #5 from reviewer #2. We have clarified the manuscript to note that stimuli or inhibitors are added both directly to the cell suspension as well as after embedding, in the media (step 4.4).