Response to Reviewer Comments

Title: Neo-islet formation in liver of diabetic mice by helper-dependent adenoviral vector mediated gene transfer

We thank the reviewers for their comments, critiques and suggestions. We have strived to answer all of these and incorporated them into the revised manuscript.

Reviewer 1:

Summary

This manuscript describes techniques used in studies published in 2009 (Dev Cell and Endocrinology), in which tail vein injection of helper-dependent adenovirus expressing neurogenin and betacellulin is reported to correct diabetes in mice, by neogenesis of new islet-like tissue in the liver. The present manuscript contains detailed procedural information for viral preparation, along with some additional information on how the mouse studies were performed. However, key information is also missing.

Major Concerns

1. The majority of the technique section is devoted to virus preparation, with relatively little emphasis on relevant mouse techniques. Certain key procedures, such as tail vein injection and saphenous vein blood collection, are not described at all. In current form, this manuscript would be better entitled "Production of helper-dependent adenovirus" than "Neo-islet formation…"

We have added the relevant mouse techniques and describe them in detail in the revised manuscript.

2. No mention is made of whether these viral vectors and cell lines are commercially available; if not available, the technique will not be of value to prospective readers/viewers

All the vectors and cell lines are available and can be requested from the NIH-funded Diabetes Research Center Viral Vector Core at Baylor College of Medicine. In addition, these are also commercially available and this information is now presented in the revised manuscript. We agree that this makes the manuscript more useful to the readers and thank the reviewer for pointing this out.

3. The manuscript lacks discussion of critical controls for this technique. For example, demonstration that the gene products delivered by adenovirus are actually overexpressed in the liver is not included in this manuscript, and seems also to be missing from both cited works. Since the virus is delivered systemically this seems important to show. Another critical element, verification that pancreatic insulin secretion remains low, was demonstrated in their cited works but is not mentioned in the present manuscript. Controls that are vital to the overall interpretation of experimental data should be included.

Confirming the expression of the vectors is an important preliminary step, as rightly pointed out by the reviewer. As previously shown in our prior study (Yechoor et. al. Dev Cell 2009, supplemental figures), expression of Ngn3, Btc, and in addition, Pdx-1 and Nkx6.1 immunostaining is now included in the Fig. 7 of the revised manuscript.

4. The Results and Discussion sections are underdeveloped; these should include a detailed description of controls and steps to verify the technique has been performed correctly.

As suggested by the Reviewer, this has now been expanded to include the controls needed and the methods to validate the technique.

Minor Concerns

1. Instructions for resuspension and storage of STZ are not include (this is a pH dependent drug that will lose activity if not handled correctly)

   We appreciate the reviewer pointing this out. We have described this important point in more detail in the revised manuscript.

2. Did the investigators really fast the mice every day?

   The mice were fasted weekly and this has now been clearly indicated in the revised manuscript.

3. The list of Specific Reagents and Equipment is lacking the majority of reagents and disposables necessary to perform these techniques. As a methods description work, these details are critically important.

   We apologize for this. We have now added all the reagents and equipment that are specific to this method in the table.

4. Typo in Keywords section ‘neuogenin’

   We thank the reviewer for bringing this to our attention. This has now been corrected.

5. Overuse of abbreviations makes this difficult to read: ‘Overlay 0.5ml CVL supplemented with HV at 200 vp/cell…’ None of these are traditional abbreviations known to non-virus users, and finding the translations proved slow.

   We have now limited the use of abbreviations. When non-standard abbreviations are used we provide the full name of the abbreviation with the first appearance in the manuscript. We have also indicated the common abbreviations used in the title page of the manuscript.

Reviewer 2:

Summary

Well-described summary of generation of helper-dependent adenoviral vectors expressing Neurogenin 3 and Betacellulin. These vectors direct insulin expression in mouse liver when injected intravenously, and reverse STZ-induced diabetes. This is a method that is innovative and addresses an important need in both beta-cell replacement and gene therapy. The description and rationale are clear.

Minor Concerns

1. Neurogenin is misspelled on p. 1, first para of the long abstract.

   We thank the reviewer for pointing this out and this has been corrected.

2. On page 7 (3.1.3), the combination group is described as 5x1011 vp Ngn3 + 1x1011 vp for the combination group" which appears to be a typo (should be "….+1x1011 vpBtc").

   We thank the reviewer for pointing this out and this has also been corrected.

3. It would be useful to describe/specify where the HV is on the CsCl gradients.

   We added in the statement "Some time, faint helper virus band can be seen below the vector band".

4. For steps 3.2.2 and 3.2.3 RNA and insulin extraction from the liver are described but no results are presented.

   The assessment of insulin and c-peptide content is described and now presented as part of Fig. 6.

5. If one transgene encodes a transcription factor that inhibits replication and packaging and the other a growth factor that enhances it, then one would expect the protocols should be somewhat different. This is mentioned in 2.4.12 but is this the only difference in the protocol?

   The protocols we follow are the same for all the viruses except for this step (2.4.12). As the reviewer astutely points out, we also believe the likely reason for the low Ngn3 virus yield is related to its growth and replication inhibition properties. We have tried different variations with this protocol, at multiple steps, to optimize the quantity of Ngn3 virus yield; however, this current protocol gives the best yield with the lowest helper contamination.

6. Although this is a methods paper, the findings would be strengthened further by 1) Co-staining for insulin and Ngn3 or Btc; 2) demonstrating presence of transcription factors or other identifiers of beta-cell lineage, such as pdx-1.

We have added the Ngn3 and Btc stain, though it has to be noted that the liver also expresses some Btc normally. We have also added the Pdx-1 and Nkx6.1 immunostains to demonstrate the periportal ‘neo-islets’ as being of the β-cell lineage.