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IGFBP1 Increases β-cell Regeneration by Promoting α- to β-cell Transdifferentiation

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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 25 October 2015

The comments of the three referees are shown below. As you will see, substantive points are raised by all the referees, which preclude publication of the work, at least at this time.

Referee 1 explicitly recommended against publication and classed the novelty and general interest of the findings as 'medium'. His/her concerns are wide ranging and some of the concerns question the strength of key data. In particular, point 2 regarding IGFBP1 overexpression. To address this point may require controls with lower expression levels. We understand that your data is complemented by loss of function data and would ask the other referees to comment on this issue.
Point 1 asks about liver - if data exists, that could indeed be interesting, but this is not a precondition for a revision in our view (see below).
The referee requests beta-cell ablation in older animals, which is a valid and addressable point.
A key point raised by this referee is the conundrum that in the absence of changes to alpha cell pool, it is hard to explain how transdifferentiation can yield more beta-cells. The referee recommend ablating alpha cells in these experiments. The referee is far from convinced that the alpha cells are the source for all beta cells in both fish and mouse and asks for insight into other sources of the beta cells. We understand this is not at all a trivial issue to address, but feel that the novelty of this manuscript does rest on the compelling description of the source of the insulin producing cells.
Finally, the referee requests addition of more definitive evidence for beta-cell identity of insulin producing cells

Referee 2 also recommends explicitly against publication, but this referee largely bases his/her view on a perceived lack on conceptual advance. In our view, the study is in principle sufficiently novel for publication, in the absence of the substantive points raised by the referees.
On a related not, we did note that the patient samples were based on your previous paper which already showed that low levels of IGFBP1 predicts typeII diabetes - i.e. the inverse of the present data.
Referee 2 takes issue with the mosaic data stating that it is unclear which cells produce the proteins under study. We would request that more compelling evidence would have to be presented in as far as it is experimentally possible.
We would also request a response to the question whether any beta cell associated transcript would be detected in the microarray screen.
Fig 2: more time points are requested for 2H.
Figure 3: the referee requests to increase the number of animals analyzed for more robust data.
Figure 4: consistent with referee 1, referee 2 concludes that there must be another source of beta-cells. It would be essential to clearly identify the origin of the beta cells as this is central to the conceptual advance of the work.

Referee 3 is more positively disposed and rates the quality and novelty of the work as 'high'.
However, like referee 1, the referee requests that the alpha cells are ablated. At the end of his/her report, the referee requests data on alpha cell regeneration.
Furthermore, the referee requires addition of data with regeneration after middle or long term diabetes to bolster the human relevance (see also end of the report).
Ref 3 requests additional proliferation markers, which is reasonable.
Importantly, the referee notes discrepancies to PMID 25141178 which would need to be discussed and accounted for. The referee suggests controls to bolster the zebrafish data.
The referee requires more compelling data on Pdx1 staining in figure 4A.
Ref 3 argues that the data do not support definitively the claim that inhibition of the Igf pathway stimulates beta cell regeneration in zebrafish, noting this contrasts previous mouse data.
A number of issues regarding the human data would have to be addressed: 'Discussion of the IGFBP1 levels and correlation with the risk of developing T2D should be elaborated further. The presented hypothesis of low IGFBP1, linking insulin deficiency and inhibition of IGF signaling does not hold up for individuals with IGT or recent/middle term onset T2D'.
Additional controls for 1D and 2D-F are requested.

We also noted at the editorial level that the IGFBP1 knockout mouse exists (a liver regeneration phenotype was reported, so this concept may apply in hepatocytes, as also pointed out by referees 1 - this may be another avenue to enhance the study). It is not clear to us if this mouse has diabetes, and if not, why this is not observed. We noted that the IGF1 ko mouse does have diabetes.

I am afraid that the referees request a large amount of new data. We feel obliged to pass on the majority of these requests as they concern the fundamental strength of the data. Also, we note that given the substantive literature on the topic (as pointed out by ref 2), we need to expect a highly developed dataset. For example, we noted previous data on transdifferentiation relevant to beta-cell expansion (Thorel, 2010, Ye, 2015).

Should you be able to address these criticisms in full, we would in principle be open to consider a revised manuscript. However, such a revision would have to satisfy substantively our present referee panel.
I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I do realize that addressing all the referees' criticisms outlined will require a lot of additional experimental effort and be technically challenging. I would therefore understand if you wish to publish the manuscript more rapidly elsewhere, in which case please let us know so we can withdraw it from our system.

If you decide to comprehensively revise the manuscript for the EMBO Journal, please include a detailed point-by-point response to the referees' comments. Please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.embo.org/embo-press

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication.
Again, I am very sorry for the excessive delays in this case and hope the report we ultimately obtained is seen as valuable feedback on your work.

REFEREE REPORTS

Referee #1:

In this manuscript, Lu et al. report a role of IGFBP1 in inducing beta-cell regeneration in zebrafish. The proposed mechanism would be a- to b-cell transdifferentiation.
While this referee recognises the work performed and the putative interest of the data, s/he cannot recommend this manuscript for publication. Indeed, this work is rushed and the conclusions are too hasty.

Major concerns :
- A very strong expression of IGFBP1 is reported in the liver : what is the effect of the overexpression of IGFBP1 in this organ? Could it play a role in beta-cell regeneration?
- Most of the analyses are done on larvae expressing 80 times more IGFBP1 than controls! Can one trust the data from such a strong over-expression system in which numerous physiological pathways must be very strongly altered?
- beta-cell ablation is performed during development, not in older animals : why? Would these early beta-cells be more plastic compared to older ones?
- most important concern : the authors propose alpha cell as a source for new beta cells : where do the alpha-cells come from? One can assume the conversion of endogenous alpha cells into beta-cells, but more alpha-cells would be required to replace lost beta-cells : where do the new alpha-cells come from? Why can't you detect increased proliferation using EDU ?
- You tried ablating delta-cells, why not try ablating alpha-cells to check whether those are replaced or not before to become beta-cells ?
- One would wish to see many more beta-cell markers or functional assays to ensure that regenerated insulin+ cells are beta-cells.
- Now, concerning the insulin/glucagon co-expression as a proof of transdifferentiation : this is no proof... Seeing twice more than very few cells is far from convincing! Lineage tracing also show twice more b-cells with a-cell origin : this is nothing! Where do the other cells come from then?
- Mouse work : a 3 fold increase in ins+/glu+ cells is noted : again, this is really not much to show a proof of transdifferentiation! going from 1-2 cells per islet to 3-6 cells (at best) per islet is no proof at all!
- The human data are of interest, but no proof of transdifferentiation, again.

Referee #2:

Embo Journal Lu et al.

Lu and colleagues employ a model of near-total induced ablation of beta-cells in the zebrafish to perform a screen for factors that might increase beta-cell regeneration. They perform a microarray expression analysis of control to beta-cell depleted islets. They then test 11 candidate genes by non-targeted (whole animal) but mosaic overexpression, and find that IGFBP1 has a positive effect on beta-cell number. Using lineage tracing studies, they conclude that this occurs via alpha to beta-cell transdifferentiation in zebrafish. They also treat mouse and human islets in vitro with IGFBP1 and find more bi-hormonal cells; in this case no lineage tracing was performed, so it is possible that beta-cells express glucagon after IGFBP1 treatment.

The novelty of the study is limited, there are multiple papers (more than 80!) on over-expression of IGFBP1 on islet cell behavior in the literature, often with divergent results. See for instance:

Diabetologia. 1996 Nov;39(11):1249-54.
Effects of insulin-like growth factors (IGF) on pancreatic islet function in IGF binding protein-1 transgenic mice.
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Specific comments:

Data presentation:
In several of the figures, the authors represent two state comparisons as bar graphs instead of scatterplots with means or medians. See discussion of this issue in: Weissberber, TL, PloS Biology, April 22, 2015. Likewise, non-parametric statistical tests should be used when comparing data sets with small n, as a normal distribution of the data cannot be assumed.

Figure 1.

The design of the microarray expression screen is puzzling. Essentially, the comparision performed should identify ANY beta-cell enriched transcript as differentiatl, as the beta-cell ablated islets have a much lower percentage of betqa-cells. The design of the mosaic overexpression screen is also suboptimal, as the authors have no control over which cell type makes the protein in question, and how much is being made.

Figure 2: panel H. The effects on glucose levels are marginal, only one time point shows any difference.

Figure 3: Regarding the effects of IGFBP1 on beta-cell replication, the authors conclude there is none (results section). However, the graph shown in panel H suggests a more than three-fold increase in beta-cell replication during development. Yes, the data are of low quality and the error bars are very large, but, that simply means the authors have to increase the number of animals analyzed.

Figure 4: The genetic lineage tracing does demonstrate that some Gcg expressing cells activate Pdx1. However, the number of cells does not match the increase in cell number shown in Figure 2. Thus, there must be another source of beta-cells.

Figure 6: The direction of effect assumed was not proven. It is just as likely that Beta-cells turned on the Gcg promoter. This needs to be acknowledged in the text and figure.

Referee #3:

Lu et al. provide interesting data on a possible role for IGFBP1 to increase beta cell regeneration upon injury. Using an elegant genetic screening technique in zebra fish they are able to narrow down

a list of upregulated genes upon beta cell ablation to 11 candidate genes which they subsequently overexpress in transgenic larvae. In this section it is indicated that mosaic-overexpression experiments give higher igfbp1a overexpression than stable transgenic lines igfbp1a, but this point is not sufficiently discussed.
The specificity and functional relevance of the igfbp1a effect on beta cell regeneration and an absence of delta cell regeneration are shown, but an additional testing of alpha cell regeneration upon ablation and igfbp1a overexpression would be of major interest.
All the regeneration experiments, however, are performed in very recent onset diabetes recovery of 3 days only. It would be a major upgrade for the article if regeneration after middle or long term diabetes were to be evaluated since this would make the process far more clinically relevant.
The effect of igfbp1a is subsequently shown to be independent of beta cell proliferation, however, as shown in PMID 24026213, nucleotide analogues may have caused an underestimation of beta cell proliferation as the re-division potential of beta cells is inhibited, ideally another proliferation marker should be includes (eg. pH3, Ki67).
The increase in beta cell regeneration is subsequently shown to depend on alpha to beta cell transdifferentiation. The authors should include in their discussion that these findings are in contrast to the age-dependent process seen in mouse in PMID 25141178, where before puberty no detectable α-cell conversion is seen upon ablation, and the juvenile adaptability is shown to depend on somatostatin-to-insulin delta-cell conversion. As in the current study zebra fish larvae are studied, one would have expected delta cell transdifferentiation and not alpha cell transdifferentiation. It is also intriguing that the absolute number of delta cells is higher than the number of beta cells upon ablation (Figure 2F), does this arise from a less efficient ablation or from a disturbed islet architecture. Here, also non ablated controls would be informative to correlate the non-ablated and the ablated number of beta cells to delta cells
In figure 4A the Pdx1 staining is of insufficient quality, cytoplasmic staining is seen in A', which is abnormal and rather surprisingly Pdx1+ insulin- cells are observed: this should be reexamined.
Next, the authors show that inhibition of the Igf pathway mimics the stimulatory effect of Igfbp1a on beta cell regeneration. However, using picropodophyllin the regeneration is less effective and one should be cautious to conclude from this experiment that inhibition of the Igf pathway stimulates beta cell regeneration in zebrafish. Especially since these findings contrast mouse studies (PMID 18663428) where IGF1 overexpression is shown to promote islet cell regeneration through increased beta cell proliferation (through p27, CDK-4 analysis).
As a very interesting proof-of-concept the authors continue to study the effect of IGFBP1 on human islets in vitro. The statement that the most reproducible effect on the increased prevalence of bihormonal cells is achieved with 1µg/ml should be illustrated by box-and-whisker plots rather than by a bar chart as this would allow interpretation of variability.
In the last experimental section the association of IGFBP1 levels and the risk for developing type 2 diabetes (T2D) are studied. Here the authors show an interesting correlation, however some important points of discussion need to be addressed in this section. As baseline IGFBP1 fasting levels were lower in the group that later developed T2D, a control for body mass index and insulinemia should be added. If BMI/insulinemia is significantly higher in this group, one could argue that the lower IGFBP1 levels are the direct result of the former and the correlation with T2D risk is indirect. The sex differences are remarkable and should be further discussed, especially since the absolute IGFBP1 levels are much higher in women, this could be related to estrogen levels and again a confounder for the observed effect on T2D risk.
In the discussion a study of Igf1 activation resulting in resistance to HFD induced and STZ induced diabetes is mentioned, however also the above mentioned study (PMID 18663428) should be discussed.
One important point of discussion is the presentation by the authors of T2D as a disease with insulin deficiency. The presented hypothesis of low IGFBP1, linking insulin deficiency and inhibition of IGF signaling may be relevant in a beta cell regeneration model of insulin deficiency, but its extrapolation to individuals with impaired glucose tolerance (IGT) or recent/middle term onset of type 2 diabetes - who are characterized by hyperinsulinemia (insulin deficiency is relative, not absolute) - doesn't hold up and should be corrected. In this light the above mentioned elaboration on regeneration experiments for middle or longstanding diabetes are obligatory.

Major points:
- Include a second proliferation marker to exclude an effect of igfbp1a on beta cell proliferation.
- Pdx1 staining as shown in Figure 4A' is of insufficient quality to make quantitative statements as shown in Figure 4C.

- Discussion of the IGFBP1 levels and correlation with the risk of developing T2D should be elaborated further.
- The presented hypothesis of low IGFBP1, linking insulin deficiency and inhibition of IGF signaling does not hold up for individuals with IGT or recent/middle term onset T2D

Minor points:
- Igfbp1a is claimed to robustly increase beta cell regeneration (fig1E) - controls should include non-ablated fish so as to give an indication of the degree of regeneration of the beta cell mass (% or absolute n of beta cells), this would likewise be informative for the delta cell ablation experiments (fig2 D-F).
- Effect igfbp1a overexpression on alpha cell regeneration would be interesting before making a statement of beta cell specificity of the regenerative processes
- Regeneration experiments for middle or longstanding diabetes would make the setup more clinically relevant.
- Discussion of contrast with findings in mouse in PMID 25141178

1st Revision - authors' response 15 February 2016

Referee #1:

In this manuscript, Lu et al. report a role of IGFBP1 in inducing beta-cell regeneration in zebrafish. The proposed mechanism would be a- to b-cell transdifferentiation.
While this referee recognises the work performed and the putative interest of the data, s/he cannot recommend this manuscript for publication. Indeed, this work is rushed and the conclusions are too hasty.

Major concerns :
- A very strong expression of IGFBP1 is reported in the liver : what is the effect of the overexpression of IGFBP1 in this organ? Could it play a role in beta-cell regeneration?

To the revised manuscript we have added experiments using a liver-specific promoter mediating 2.5 times the endogenous expression of *igfbp1a*, which (like widespread overexpression) also increased the regeneration of beta cells (Fig 1H). Thus, liver-specific overexpression of *igfbp1a* is sufficient to potentiate beta-cell regeneration.

- Most of the analyses are done on larvae expressing 80 times more IGFBP1 than controls! Can one trust the data from such a strong over-expression system in which numerous physiological pathways must be very strongly altered?

We have now performed many additional experiments using a stable transgenic line expressing 16 times the normal level of *igfbp1a*, see for example Fig 3A-L, Fig 4A-C and Fig 6A-E. Moreover, even in the previous version of the manuscript we showed that stable transgenic lines expressing 4-10 times the normal level of *igfbp1a* had an increase in beta-cell regeneration (Fig EV1C-F).

- beta-cell ablation is performed during development, not in older animals : why? Would these early beta-cells be more plastic compared to older ones?

We perform experiments during larval development because it is a stage that allows for efficient and robust analysis. We now include new experiments in Fig 6 that show that overexpression of *igfbp1a* increases beta-cell regeneration in 1-month-old zebrafish as well (Fig 6A-E). It is hard to tell whether early alpha-cells are more or less plastic than older ones on the basis of these experiments, but we can conclude that there is enough plasticity for major effects on regeneration also at older stages.

- most important concern : the authors propose alpha cell as a source for new beta cells : where do the alpha-cells come from? One can assume the conversion of endogenous alpha cells into beta-cells, but more alpha-cells would be required to replace lost beta-cells : where do the new alpha-cells come from? Why can't you detect increased proliferation using EDU ?

We have now used EdU incorporation to determine the effect of *igfbp1a* on alpha-cell proliferation, and performed ductal-cell lineage tracing during beta-cell regeneration to search for newly formed *igfbp1a*-induced alpha-cells. We find that *igfbp1a* does not increase alpha-cell proliferation. In addition, lineage tracing using *Tg(Tp1:H2BmCherry)* showed that *igfbp1a* does not increase the number of alpha-cells deriving from the intrapancreatic duct. However, the extrapancreatic duct (which *Tg(Tp1:H2BmCherry)* does not label) has also been shown to contain pancreatic progenitors (Dong et al., 2007)—indeed, we do see a doubling of alpha-cells along the extrapancreatic duct by regular immunofluorescence, suggesting that the newly formed alpha cells may originate there. However, because there are currently no tools for genetic lineage tracing of the extrapancreatic duct (which would formally prove the origin of new alpha cells), we have refrained from speculating about the origin of new alpha-cells in this manuscript.

- You tried ablating delta-cells, why not try ablating alpha-cells to check whether those are replaced or not before to become beta-cells ?

We previously tried to make an alpha-cell ablation model by using the glucagon promoter to drive expression of nitroreductase. However, the glucagon promoter does not express a high enough level of nitroreductase in all alpha-cells to mediate efficient ablation. Moreover, the glucagon promoter is active in the brain, such that it can cause brain trauma and other adverse side effects. Therefore, we instead used a morpholino targeting the transcription factor *arx* to block the development of alpha-cells. Using this strategy we now show that the regenerative effect of *igfbp1a* is abolished in zebrafish larvae depleted of alpha-cells, indicating that alpha-cells are necessary for *igfbp1a* to potentiate beta-cell regeneration (Fig 3 J-L).

- One would wish to see many more beta-cell markers or functional assays to ensure that regenerated insulin+ cells are beta-cells.

We now show that the regenerated beta-cells in the islet express the functional beta-cell marker pcsk1 (the enzyme that processes proinsulin to its active form, and is therefore considered a requirement for a functional beta-cell), by generating a new transgenic line, *Tg(pcsk1:GFP)* (see new Fig 2H). There are currently no good antibodies against additional markers of functional beta-cells in zebrafish, so it is hard to examine many more functional markers. As for functional assays, we now show that not only genetic overexpression of *igfbp1a*, but also injection of recombinant igfbp1 protein lowers glucose levels in zebrafish larvae during beta-cell regeneration (new Fig 2J).

- Now, concerning the insulin/glucagon co-expression as a proof of transdifferentiation : this is no proof... Seeing twice more than very few cells is far from convincing! Lineage tracing also show twice more b-cells with a-cell origin : this is nothing! Where do the other cells come from then?

Co-expression of insulin and glucagon, as well as glucagon and pdx1, are often used as an indication of transdifferentiation. We agree with the reviewer that co-expression is no proof, only an indication of transdifferentiation, and we have now clarified this in the text. It is not possible to obtain lineage tracing evidence in human tissues and, despite this, reviewer 3 considers our co-expression findings a “very interesting proof-of-concept”.

As for the lineage-tracing results in zebrafish, one must take into account differences between species in regenerative capacity. We use the zebrafish model because of its prominent regenerative capacity – this capacity enables discovery of potent, endogenous regenerative signals (Fig 1A), but at the same time limits the fold increase in regeneration that can be induced by an added stimulus. In the beta-cell-ablation zebrafish model we use, the origin of around 80% of the beta-cells regenerated during early stages has been attributed to alpha-cells (Ye et al., 2015), through alpha- to beta-cell transdifferentiation. This means that a doubling of beta-cells with an alpha-cell origin results in a marked (~80%) increase in beta-cell regeneration. This situation differs from that in the mouse, which does not have as prominent a regenerative response and in which it is therefore possible to induce a much larger fold-increase in alpha- to beta-cell transdifferentiation – one that would nevertheless translate to a smaller absolute increase in beta-cell regeneration compared to that in zebrafish. How important alpha- to beta-cell transdifferentiation is in humans, and whether the situation is more similar to that in zebrafish or that in mice, remains to be shown.

Investigating other possible origins of beta cells, we have now examined the effects of *igfbp1a* overexpression on two other main cellular mechanisms of beta-cell regeneration: beta-cell neogenesis from ductal cells and beta-cell proliferation. We find that there is no increase in

neogenesis of beta-cells from ductal cells after *igfbp1a* overexpression (new data in Fig 3A-C). In addition, cumulative beta-cell proliferation is not significantly increased by *igfbp1a* overexpression (Fig 3D-F), though we do see an increase in a transgenic marker of beta-cell proliferation, *Tg(ins:Venus-zGeminin),* at the end of the regenerative period (6 dpf) (Fig 3G-I). However, even if there is a small increase in beta-cell proliferation at this late stage, the vast majority of beta-cell regeneration occurs through alpha- to beta-cell transdifferentiation at a time closer to beta-cell ablation (when there are few beta-cells present producing insulin, which can inhibit alpha- to beta-cell transdifferentiation at later stages, once a substantial number of beta-cells have been regenerated). Thus, alpha- to beta-cell transdifferentiation appears to drive the bulk of beta-cell regeneration, which is then complemented by beta-cell proliferation at a later stage.

- Mouse work : a 3 fold increase in ins+/glu+ cells is noted : again, this is really not much to show a proof of transdifferentiation! going from 1-2 cells per islet to 3-6 cells (at best) per islet is no proof at all!
- The human data are of interest, but no proof of transdifferentiation, again.

Please see answer above regarding co-expression and indication/proof of transdifferentiation. Additionally, we do not know how long the ins+glu+ co-expression lasts. If such co-expression is fleeting then we may be detecting merely a small proportion of an ongoing, alpha- to beta-cell transdifferentiation, meaning that a small number of bihormonal cells at any one time in fact reflects the generation of a substantial number of beta cells over a longer period.

Referee #2:

Embo Journal Lu et al.

Lu and colleagues employ a model of near-total induced ablation of beta-cells in the zebrafish to perform a screen for factors that might increase beta-cell regeneration. They perform a microarray expression analysis of control to beta-cell depleted islets. They then test 11 candidate genes by non-targeted (whole animal) but mosaic overexpression, and find that IGFBP1 has a positive effect on beta-cell number. Using lineage tracing studies, they conclude that this occurs via alpha to beta-cell transdifferentiation in zebrafish. They also treat mouse and human islets in vitro with IGFBP1 and find more bi-hormonal cells; in this case no lineage tracing was performed, so it is possible that beta-cells express glucagon after IGFBP1 treatment.

The novelty of the study is limited, there are multiple papers (more than 80!) on over-expression of IGFBP1 on islet cell behavior in the literature, often with divergent results. See for instance:

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Effects of insulin-like growth factors (IGF) on pancreatic islet function in IGF binding protein-1 transgenic mice.
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Indeed, there are many papers published about IGFs and IGFBP1 with regard to glucose regulation and islet-cell behavior, and we thank the reviewer for the additional references listed (we now

include two of them in the discussion). Nevertheless, neither IGFs nor IGFBP1 have previously been implicated in alpha- to beta-cell transdifferentiation. In fact, there are only around 10 papers published in the field of alpha- to beta-cell transdifferentiation as a whole.

Specific comments:

Data presentation:
In several of the figures, the authors represent two state comparisons as bar graphs instead of scatterplots with means or medians. See discussion of this issue in: Weissberber, TL, PloS Biology, April 22, 2015. Likewise, non-parametric statistical tests should be used when comparing data sets with small n, as a normal distribution of the data cannot be assumed.

We thank the reviewer for the comments on both points. We believe that scatterplots are indeed good to use when there are reasons for visualizing the individual values, e.g. when values cluster into two groups or the sample size is very small. For instance, we do use scatterplots when we have a very small sample size (see Fig EV1), in accordance with the instructions for *The EMBO Journal* ("the actual individual data from each experiment should be plotted if $n<5$"). As for the rest of the data, we have larger groups and do not see a skewed distribution, and think that scatterplots would only divert attention from the message of the result. However, we can convert all graphs to scatterplots if required. As for the statistical analyses, we have reanalyzed our data using non-parametric statistical tests for experiments with small n, defined as $n<10$.

Figure 1.

The design of the microarray expression screen is puzzling. Essentially, the comparision performed should identify ANY beta-cell enriched transcript as differentiatl, as the beta-cell ablated islets have a much lower percentage of betqa-cells. The design of the mosaic overexpression screen is also suboptimal, as the authors have no control over which cell type makes the protein in question, and how much is being made.

Yes, beta-cell transcripts are downregulated as the beta-cells are ablated. That is one of the reasons why we focus solely on upregulated transcripts (the other reason is that we hypothesize that a regenerative response in islet cells other than beta-cells could upregulate a secreted factor that induces beta-cell regeneration).

Regarding the level and source of *igfbp1a* overexpression: We have now performed many experiments using a stable transgenic line overexpressing 16 times the endogenous level of *igfbp1a*, i.e. with control over the level of overexpression. Moreover, we have since cloned *igfbp1a* downstream of a liver-specific promoter, and when we use this construct we know that the exogenous *igfbp1a* is coming from the liver (Fig 1H). The data show that liver-specific overexpression of *igfbp1a* is sufficient to promote beta-cell regeneration.

Figure 2: panel H. The effects on glucose levels are marginal, only one time point shows any difference.

The effects on glucose are perhaps not as large as one would expect from the increase in regeneration. However, this might be due to the prominent regenerative capacity of zebrafish, resulting in a quite rapid recovery of the glucose level even in the control larvae, such that it is hard to obtain larger effects of accelerated regeneration. Nevertheless, we have now performed another experiment in which we monitor the glucose level during beta-cell regeneration, and show that not only genetic overexpression of *igfbp1a* but also injection of recombinant igfbp1 protein lowers glucose levels (Fig 2J).

Figure 3: Regarding the effects of IGFBP1 on beta-cell replication, the authors conclude there is none (results section). However, the graph shown in panel H suggests a more than three-fold increase in beta-cell replication during development. Yes, the data are of low quality and the error bars are very large, but, that simply means the authors have to increase the number of animals analyzed.

The whole of Fig 3 has now been replaced with new data generated with a stable transgenic line overexpressing 16 times the endogenous level of *igfbp1a*. This figure now contains data addressing

all three main regenerative mechanisms, i.e. beta-cell proliferation, beta-cell neogenesis, and alpha- to beta-cell transdifferentiation. We find that while overexpression of *igfbp1a* does not lead to a cumulative increase in beta-cell proliferation during the regenerative period (as assessed by EdU incorporation; Fig 3D-F), it does stimulate beta-cell proliferation towards the end of the regenerative period (at 6 dpf; as assessed by a transgenic marker of beta-cell proliferation, *Tg(ins:Venus-zGeminin)*; Fig 3G-I). Moreover, the additional experiments show that overexpression of *igfbp1a* does not significantly affect beta-cell neogenesis from ductal progenitors (Fig 3A-C), and that the net increase in beta-cell regeneration is mainly due to alpha- to beta-cell transdifferentiation (Fig 3J-L).

Figure 4: The genetic lineage tracing does demonstrate that some Gcg expressing cells activate Pdx1. However, the number of cells does not match the increase in cell number shown in Figure 2. Thus, there must be another source of beta-cells.

It is true that the numbers are not the same, but that is because co-expression of glucagon and pdx1 is transient. Once an alpha-cell has transdifferentiated to a beta-cell, glucagon expression is lost. Regarding the genetic lineage-tracing data, the established efficiency of the approach is such that only ~20% of alpha-cells are labeled (Ye et al., 2015), meaning that the numbers in Fig. 4 should be multiplied by 5 if they are to be compared to those in Fig. 2. This has now been clarified in the text.

Figure 6: The direction of effect assumed was not proven. It is just as likely that Beta-cells turned on the Gcg promoter. This needs to be acknowledged in the text and figure.

We now include the following text to clarify that co-expression does not reveal in which transdifferentiation direction a bihormonal cell is heading: “The presence of such bihormonal cells indicates that transdifferentiation is taking place, though it cannot distinguish between α- to β-cell transdifferentiation and the converse, β- to α-cell transdifferentiation.” (in the main text), and “Co-expression of insulin and glucagon is indicative of transdifferentiation, which could reflect either α cells transdifferentiating to β cells or vice versa.” (in the figure legend).

Referee #3:

Lu et al. provide interesting data on a possible role for IGFBP1 to increase beta cell regeneration upon injury. Using an elegant genetic screening technique in zebra fish they are able to narrow down a list of upregulated genes upon beta cell ablation to 11 candidate genes which they subsequently overexpress in transgenic larvae. In this section it is indicated that mosaic-overexpression experiments give higher igfbp1a overexpression than stable transgenic lines igfbp1a, but this point is not sufficiently discussed.

We have now elaborated on this point in the text, as well as performed many experiments using a stable transgenic line overexpressing 16 times the normal level of *igfbp1a* (rather than 80 times). There seems to be a selection against high levels of overexpression in stable lines, perhaps because they do not grow and breed as well as wild-type zebrafish. Alternatively, it may be that overexpression is higher in mosaic overexpression because it involves several genetic integrations, whereas overexpression in the stable lines involves only one genetic integration. Nevertheless, we show that three different stable transgenic lines expressing 4-16 times the normal level of *igfbp1a* also had an increase in beta-cell regeneration (Fig 3; Fig EV1C-F).

The specificity and functional relevance of the igfbp1a effect on beta cell regeneration and an absence of delta cell regeneration are shown, but an additional testing of alpha cell regeneration upon ablation and igfbp1a overexpression would be of major interest.

Please see our response below, where these points are reiterated.

All the regeneration experiments, however, are performed in very recent onset diabetes recovery of 3 days only. It would be a major upgrade for the article if regeneration after middle or long term diabetes were to be evaluated since this would make the process far more clinically relevant.

Please see our response below, where these points are reiterated.

The effect of igfbp1a is subsequently shown to be independent of beta cell proliferation, however, as shown in PMID 24026213, nucleotide analogues may have caused an underestimation of beta cell proliferation as the re-division potential of beta cells is inhibited, ideally another proliferation marker should be includes (eg. pH3, Ki67).

Please see our response below, where these points are reiterated.

The increase in beta cell regeneration is subsequently shown to depend on alpha to beta cell transdifferentiation. The authors should include in their discussion that these findings are in contrast to the age-dependent process seen in mouse in PMID 25141178, where before puberty no detectable α-cell conversion is seen upon ablation, and the juvenile adaptability is shown to depend on somatostatin-to-insulin delta-cell conversion. As in the current study zebra fish larvae are studied, one would have expected delta cell transdifferentiation and not alpha cell transdifferentiation. It is also intriguing that the absolute number of delta cells is higher than the number of beta cells upon ablation (Figure 2F), does this arise from a less efficient ablation or from a disturbed islet architecture. Here, also non ablated controls would be informative to correlate the non-ablated and the ablated number of beta cells to delta cells

We have now added a non-ablated control to Fig 2F. It is hard to know whether there is more regeneration or less efficient ablation of the delta cells since we lack genetic tools for marking the delta cells prior to ablation, e.g. as we did for beta cells by using *ins:Kaede* (Fig 2A-C). See discussion below regarding previous findings on delta-cell transdifferentiation in prepubescent mice (PMID 25141178).

In figure 4A the Pdx1 staining is of insufficient quality, cytoplasmic staining is seen in A', which is abnormal and rather surprisingly Pdx1+ insulin- cells are observed: this should be reexamined.

Please see our response below, where these points are reiterated.

Next, the authors show that inhibition of the Igf pathway mimics the stimulatory effect of Igfbp1a on beta cell regeneration. However, using picropodophyllin the regeneration is less effective and one should be cautious to conclude from this experiment that inhibition of the Igf pathway stimulates beta cell regeneration in zebrafish. Especially since these findings contrast mouse studies (PMID 18663428) where IGF1 overexpression is shown to promote islet cell regeneration through increased beta cell proliferation (through p27, CDK-4 analysis).

Please see our response below, where the point regarding mouse findings (PMID 18663428) is reiterated. In addition, we have now downplayed our conclusions regarding the mechanism of Igfbp1-mediated inhibition of IGFs, to allow for the possibility that additional mechanisms are at play.

As a very interesting proof-of-concept the authors continue to study the effect of IGFBP1 on human islets in vitro. The statement that the most reproducible effect on the increased prevalence of bihormonal cells is achieved with 1µg/ml should be illustrated by box-and-whisker plots rather than by a bar chart as this would allow interpretation of variability.

This part of the main text has been rephrased and no longer contains such a statement.

In the last experimental section the association of IGFBP1 levels and the risk for developing type 2 diabetes (T2D) are studied. Here the authors show an interesting correlation, however some important points of discussion need to be addressed in this section. As baseline IGFBP1 fasting levels were lower in the group that later developed T2D, a control for body mass index and insulinemia should be added. If BMI/insulinemia is significantly higher in this group, one could argue that the lower IGFBP1 levels are the direct result of the former and the correlation with T2D risk is indirect.

We have now added a figure (Fig EV4) showing that both BMI and fasting insulin values inversely correlate with IGFBP1 (all values were recorded at baseline when all subjects had a normal oral glucose tolerance test). However, this inverse correlation exists in both those who later developed

T2D and those who remained healthy. Although, we agree with the reviewer that BMI/insulinemia may nevertheless be a confounding factor, we cannot adjust the analysis for these factors because they correlate with insulin sensitivity, which in turn affects how much insulin signaling the α cell can sense (and thus also alpha- to beta-cell transdifferentiation, according to our model).

The sex differences are remarkable and should be further discussed, especially since the absolute IGFBP1 levels are much higher in women, this could be related to estrogen levels and again a confounder for the observed effect on T2D risk.

We now include in the manuscript a discussion of the sex differences in IGFBP1 levels. Estrogen levels are of course much lower in men than in premenopausal women (they are comparable to those in postmenopausal women), and it is therefore possible that these sex-dependent differences in estrogen levels explain why IGFBP1 levels are higher in women. However, the evidence for a relationship between estrogen and IGFBP1 levels is conflicting: Whereas oral administration of estrogen to postmenopausal women increases their IGFBP1 levels (Heald et al., 2005; Isotton et al., 2012), circulating estrogen levels in premenopausal women have been shown to correlate *inversely* with IGFBP1 levels (Unden et al., 2005). Regardless of a possible influence of estrogen on IGFBP1 levels, we do not consider it a confounder for this study because the association between high IGFBP1 levels and reduced risk for developing T2D is seen in both men and women.

In the discussion a study of Igf1 activation resulting in resistance to HFD induced and STZ induced diabetes is mentioned, however also the above mentioned study (PMID 18663428) should be discussed.

We have now included the abovementioned study in the discussion, writing that "IGFs robustly promote proliferation (and thus regeneration) of rodent β cells".

One important point of discussion is the presentation by the authors of T2D as a disease with insulin deficiency. The presented hypothesis of low IGFBP1, linking insulin deficiency and inhibition of IGF signaling may be relevant in a beta cell regeneration model of insulin deficiency, but its extrapolation to individuals with impaired glucose tolerance (IGT) or recent/middle term onset of type 2 diabetes - who are characterized by hyperinsulinemia (insulin deficiency is relative, not absolute) - doesn't hold up and should be corrected. In this light the above mentioned elaboration on regeneration experiments for middle or longstanding diabetes are obligatory.

Please see our response below, where these points are reiterated.

Major points:
- Include a second proliferation marker to exclude an effect of igfbp1a on beta cell proliferation.

We have now redone all the experiments previously shown in Fig 3. We now show data from experiments in which we assessed beta-cell proliferation with two different approaches: EdU incorporation and a new transgenic marker of beta-cell proliferation, *Tg(ins:Venus-zGeminin)*. Although we do find that *igfbp1a* overexpression significantly increases beta-cell proliferation (as assessed by *Tg(ins:Venus-zGeminin)*) at 6 dpf (Fig 3G-I), it fails to significantly increase the cumulative amount of beta-cell proliferation (as assessed by EdU incorporation) during the 4-6 dpf duration of regeneration (Fig 3E-F). Therefore, it is likely that proliferation accounts for only a small proportion of *igfbp1a*'s regenerative effect. Moreover, we also examined the number of phospho-histone H3 expressing beta-cells at 6 dpf and did not detect an increase after overexpression of *igfbp1a*. We have not included this result in the manuscript because the low baseline level of phospho-histone H3-positive beta-cells was incompatible with appropriate statistical analysis.

- Pdx1 staining as shown in Figure 4A' is of insufficient quality to make quantitative statements as shown in Figure 4C.

Thank you for pointing this out – we have redone the experiment and now provide better-quality images.

- Discussion of the IGFBP1 levels and correlation with the risk of developing T2D should be elaborated further.

We have now elaborated on the association between IGFBP1 levels and the risk of developing T2D in several places in the manuscript, with regards to both the relevance of α- to β-cell transdifferentiation and possible confounding effects. For example,
- in the end of the results section:
"Because increasing BMI and insulin levels are associated with lower insulin sensitivity, and α cells become insulin resistant during the development of T2D (Lee et al., 2014), we propose a model in which the α cells sense neither insulin nor IGFs when IGFBP1 levels are high, thereby enabling the α cells to transdifferentiate to β-cells (Fig 7G)."

- in the discussion:
"An alternative explanation could be that the association between IGFBP1 and T2D is due to differences in *IGFBP1* expression as a result of altered insulin response/sensitivity or BMI (Brismar et al., 1991; Kotronen et al., 2008). Indeed we found that both fasting insulin levels and BMI correlate inversely with IGFBP1 levels. However, because these factors are indicators of insulin sensitivity (and we favor a model in which α- to β-cell transdifferentiation occurs when the α cells are insulin resistant) the relevance of these correlations are hard to interpret."

- The presented hypothesis of low IGFBP1, linking insulin deficiency and inhibition of IGF signaling does not hold up for individuals with IGT or recent/middle term onset T2D

(We presume the reviewer means "high IGFBP1" and not "low IGFBP1".)

We now describe in greater detail how insulin resistance in prediabetes can fit into a model wherein both insulin and IGF signaling are blunted, allowing transdifferentiation of α- to β-cells to occur, i.e. because the α-cells themselves also are insulin resistant.

"Despite individuals with impaired glucose tolerance or prediabetes having hyperinsulinemia, recent findings indicate that development of T2D is associated with the appearance of insulin-resistant α cells (Lee et al., 2014). Thus, we propose that in individuals who have high levels of IGFBP1, a combined lack of insulin and IGF signaling in α cells in the prediabetes state allows transdifferentiation of α to β cells and thereby counteracts the development of T2D."

Minor points:
- Igfbp1a is claimed to robustly increase beta cell regeneration (fig1E) - controls should include non-ablated fish so as to give an indication of the degree of regeneration of the beta cell mass (% or absolute n of beta cells), this would likewise be informative for the delta cell ablation experiments (fig2 D-F).

We now include an additional graph comparing the beta-cell regenerative effect in ablated *vs* non-ablated zebrafish (Fig 1I), quantified by confocal microscopy (because with the wide-field microscope used for screening we cannot count individual cells in an intact islet). Moreover, with the screening microscope we see only strongly expressing beta-cells, but with the confocal we see all beta-cells (including recent, weakly insulin-expressing, beta-cells); that is why the numbers differ between the graphs). Data from non-ablated controls are now also shown for the delta-cell ablation experiment Fig 2F).

- Effect igfbp1a overexpression on alpha cell regeneration would be interesting before making a statement of beta cell specificity of the regenerative processes

This question is related to a question by reviewer #1, so we reiterate our response:
We previously tried to make an alpha-cell ablation model by using the glucagon promoter to drive expression of nitroreductase. However, the glucagon promoter does not express a high enough level of nitroreductase in all alpha-cells to mediate efficient ablation. Moreover, the glucagon promoter is active in the brain, such that it can cause brain trauma and other adverse side effects. Therefore, we instead used a morpholino targeting the transcription factor *arx* to block the development of alpha-cells. Using this strategy we now show that the regenerative effect of *igfbp1a* is abolished in alpha-

cell depleted zebrafish larvae, indicating that alpha-cells are necessary for *igfbp1a* to potentiate beta-cell regeneration.
Moreover, we are not claiming that Igfbp1 potentiates solely, and specifically, beta-cell regeneration, because it has previously been shown to stimulate regeneration of hepatocytes in the mouse (Leu et al., 2003). In the discussion we write: "This indicates that Igfbp1 may be a regenerative signal with importance for both hepatocytes and β cells. However, we did not see an effect of Igfbp1 on δ-cell regeneration, suggesting that Igfbp1 can promote regeneration of some, but not all, cell types."

- Regeneration experiments for middle or longstanding diabetes would make the setup more clinically relevant.

One would need to use a transgenic mouse overexpressing *Igfbp1* to carry out these types of studies, since there is no good middle-longstanding-diabetes model in zebrafish. We believe this would be a very interesting avenue for future studies by researchers who have such transgenic mice. Likewise, it would be interesting to examine whether *Igfbp1*-deficient mice are more prone to develop diabetes after various challenges.
Nevertheless, using zebrafish we have now examined beta-cell regeneration in 1-month-old zebrafish overexpressing *igfbp1a*. As in the 6-day old larvae, 1-month-old zebrafish also have an accelerated regeneration of beta-cells (Fig 6).

- Discussion of contrast with findings in mouse in PMID 25141178

In the reference listed by the reviewer, delta-cells are shown to convert to beta-cells in young mice, whereas alpha-cells are the main cell-type converting to beta-cells in adult mice (as in our study in zebrafish). In zebrafish, Ye et al., 2015, have previously shown that there is no delta- to beta-cell conversion at early stages, using the same model of beta-cell ablation as we do. Thus, there seems to be a difference between zebrafish and mice in this regard; however, it is not know how this relates to humans. We now discuss these differences at the end of the manuscript.

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2nd Editorial Decision 25 March 2016

Thank you for submitting your revised manuscript for further consideration by the EMBO Journal. It has now been seen by the original three referees whose comments are enclosed.
As you will see, referees #1 and #3 express continued interest in your manuscript, while referee #2 suggests that the conclusion are not definitive and the novelty remains low (as also stated in the first report of this referee).
In our view, eventual publication remains an attractive prospect for the EMBO Journal, but this would necessitate further revision to address referee #3's constructive comments.
In particular, Ref #3 recommends:
1) a better control for the delta cell ablation experiment with cells expressing IR and/or IGF1R.
2) In fig 2, the provided glucose levels should be put into the context of normal free glucose levels in zebrafish and the interpretation adapted as suggested by ref #3.
3) The beta cell proliferation experiments need to be more definitive (ref #3)
4) Show total pancreas size and beta cell mass (ug) or volume (ul) measurements to support conclusions made in the revised manuscript. Include scale bars. (ref #3)
5) Higher dose for the induction of bihormonal cells (0.5ug/ml)
6) Adapt model and tone down conclusions ('indicate that IGFBP1 has a direct effect on the islet, rather than an indirect effect through modulation of IGF or insulin signaling in other organs' and medical application as suggested by ref #3 (i.e. 'Igfbp1 is correlated with diabetes risk reduction').

I would like to invite you to submit a revised version of the manuscript, addressing the comments of referee #3.

REFEREE REPORTS

Referee #1:

The authors have considerably improved the manuscript by adding numerous data following the three referees' remarks.
The authors clearly state the technical limitations they face to address the referees' comments when necessary.
The conclusions have also been reviewed and are less affirmative about the trans-differentiation effects of Igfbp1a in mice and human.
The analysis on the correlation between the protein levels and T2DM are interesting and the additional points developed concerning this part of the study increase the quality of the discussion.

Referee #2:

Unfortunately, the revised version of the article does not convince me of the conclusions of the authors. Overall, the findings made, i.e. that there is some plasticity among endocrine cell types in the pancreas are not novel, and are not at the level of impact typically seen at EMBO Journal

Referee #3:

The authors did significant efforts to improve the manuscript by additional experiments and analyses, however some questions remain:
The rebuttal letter states "the glucagon promoter is active in the brain, such that it can cause brain trauma and other adverse side effects", the same is of course is true for the insulin promotor.
Concerning the delta cell ablation experiment: delta cells do not have an insulin receptor or IGF1R. If one wants to determine specificity for beta cells within the endocrine pancreas a better control would be a cell type more resembling the beta cell with IR and/or IGF1R expression (to exclude indirect effects of perturbing insulin-IGF1-signaling) such as alpha cells. As the authors state in their rebuttal the glucagon promotor is indeed weak so the introductory and conclusion sentence of this paragraph should be adjusted.
Concerning the euglycemia restoring experiments (Fig2): to determine functional beta cell

regeneration, glucose levels should return closer to normal homeostatic levels, i.e. not per se lower levels compared to control fish, the provided glucose levels should be put into the context of normal free glucose levels in zebrafish; when beta cells function properly they sense glucose levels and will only adjust insulin secretion when needed => if lower compared to control would be below homeostatic (=euglycemic) levels, this also is 'dysfunction' of the beta cell. Additionally, the effects of Igfbp1 on glucose levels should not be considered proof for beta cell regeneration as Igfbp1 increases insulin sensitivity and will in via this route decrease glucose levels (PMC3314358). Lastly, the additional experiments with injections of recombinant (fish?) Igfbp1 and its effect on free glucose levels do not make the point of beta cell regeneration more convincing.
The beta cell proliferation experiments: the current text seems to oversee that proliferation is going from 1 cell to 2 cells, not per se cell cycle activation as assessed by any single marker. If one marker shows no significant increase while another marker does, further validation is needed as via beta cell volume. I do agree with the authors that based on the currently provided data one should not attribute a major role to beta cell proliferation in the model.
Concerning the determination of insulin positive area and other morphometric analyses included in the revised manuscript: the material and methods section should be updated to include details on these measurements.
Further concerning these measurements: "we found that Igfbp1a overexpression did not change the number of counts (objects with coherent insulin-positive pixels), but it did significantly increase the average size of each count". What do the authors mean by objects with coherent insulin positive pixels? Are these INS+ cells? If so the conclusion should be that there is beta cell hypertrophy but no beta cell hyperplasia. Furthermore it is unclear whether the total pancreas size is comparable in igbp1a overexpressing and control fish, which is of course important as in the manuscript the authors make statements on increments in beta cell mass but no true beta cell mass (ug) or volume (ul) measurements are shown, only the percentage of INS+ area over total pancreas area. Related to this, all figures in the manuscript would benefit from including a scale bar.
Concerning the dose dependency of the induction of bihormonal (GLU+/INS+ cells) in mouse islets: the authors should include an additional dose of 0.5ug/ml to prove their point of dose dependency of the effect, since control and 0.3ug/ml do not differ significantly, the current results rather fit a threshold model. Why does the highest concentration of rIgfbp1a 25ug/ml lead to a reduction in this percentage of bihormonal cells?
- Concerning the proposed theoretical model: not per se sensing is affected in alpha cells, when IGFBP1 is high more of IGF will be bound, and the free fraction will be lower; better in my view to propose a model in which higher IGFBP1 lead to reduced IGF1 signaling to alpha cells and hereby enabling of the transdifferentiation
- In the discussion the author state "In this study we show that Igfbp1 promotes β-cell regeneration and may reduce the risk of developing diabetes". The current study is not designed or appropriate to address the latter statement on the prevention of diabetes. Alternatively, it can be stated that Igfbp1 is correlated with diabetes risk reduction.
- "Importantly, these in vitro effects also indicate that IGFBP1 has a direct effect on the islet, rather than an indirect effect through modulation of IGF or insulin signaling in other organs". This statement does no right to the remaining uncertainty of the mechanism. An indirect effect on the islet through modulation of IGF or insulin signaling within the islet is also possible, and to formally prove a direct effect additional experiments perturbing intra-islet IGF or insulin signaling should be done. In fact, the authors show that by inhibition of IGF signaling the regenerative effect is mimicked which would fit the above indirect, intra-islet effect hypothesis at least as well as the direct effect hypothesis.
- "Our results indicate that Igfbp1 plays a previously unappreciated role in expanding the β-cell mass, a role that may be important in protecting against the development of T2D and in regenerating the β-cell mass in T1D-and one that could perhaps be exploited therapeutically." This conclusion is not supported by the data provided, the authors should include beta cell volume/mass to prove this point and not relative % of INS+/total pancreas or size of INS+ counts.
- "In sum, we show that IGFBP1 is a secreted factor that can expand the β-cell mass by potentiating α- to β-cell transdifferentiation. Our findings also suggest that IGFBP1 may in this way protect against the development of diabetes and thus has potential as a treatment for diabetes." As stated above, this is an overinterpretation of the provided data.

2nd Revision - authors' response 30 May 2016

Referee #3:

The authors did significant efforts to improve the manuscript by additional experiments and analyses, however some questions remain:
The rebuttal letter states "the glucagon promoter is active in the brain, such that it can cause brain trauma and other adverse side effects", the same is of course is true for the insulin promotor.

In zebrafish there are two genes encoding insulin, *insa* and *insb*. The short, 1-kb promoter of *insa* that we use for marking beta cells is expressed only in beta cells (and not in the brain or anywhere else in the zebrafish). By contrast, *insb* is expressed in the brain.

Concerning the delta cell ablation experiment: delta cells do not have an insulin receptor or IGF1R. If one wants to determine specificity for beta cells within the endocrine pancreas a better control would be a cell type more resembling the beta cell with IR and/or IGF1R expression (to exclude indirect effects of perturbing insulin-IGF1-signaling) such as alpha cells.

According to unpublished single-cell RNA-seq data from human islet cells (generated by another group at the Karolinska Institutet), delta cells do express mRNA for both the insulin and the IGF receptor at similar levels to those in alpha cells (see attached figure for reviewers/editors). Unfortunately, owing to the previously mentioned reasons, we have not yet managed to make an ablation model for alpha cells, although we agree with the reviewer that such a model would be of interest.

As the authors state in their rebuttal the glucagon promotor is indeed weak so the introductory and conclusion sentence of this paragraph should be adjusted.

We now write that "*we lineage traced a proportion of the α cells during β-cell regeneration*", as well as limit our conclusions to "*the lineage-traced α cells*" specifically, and not α cells in general.

Concerning the euglycemia restoring experiments (Fig2): to determine functional beta cell regeneration, glucose levels should return closer to normal homeostatic levels, i.e. not per se lower levels compared to control fish, the provided glucose levels should be put into the context of normal free glucose levels in zebrafish; when beta cells function properly they sense glucose levels and will only adjust insulin secretion when needed => if lower compared to control would be below homeostatic (=euglycemic) levels, this also is 'dysfunction' of the beta cell.

We have now added baseline reference levels of free glucose to the graphs, showing that normal levels of free glucose are restored after 2-3 days of regeneration in zebrafish overexpressing igfbp1a or injected with mouse Igfbp1 protein.

Additionally, the effects of Igfbp1 on glucose levels should not be considered proof for beta cell regeneration as Igfbp1 increases insulin sensitivity and will in via this route decrease glucose levels (PMC3314358).

We have now downplayed the conclusion of this experiment, by including a phrase in the discussion about the possible effect on insulin sensitivity, as well as referencing the mentioned paper.

Lastly, the additional experiments with injections of recombinant (fish?) Igfbp1 and its effect on free glucose levels do not make the point of beta cell regeneration more convincing.

We agree to some extent but have kept the experiment in the figure as it confirms the effects of genetic overexpression of *igfbp1a*. Also, this experiment is more relevant now that we added the basal level of free-glucose (see answer to question above; as the free-glucose level is in fact restored to normal levels after injection of recombinant Igfbp1). It is stated in the methods and figure legend that it is recombinant mouse Igfbp1 protein that is injected.

The beta cell proliferation experiments: the current text seems to oversee that proliferation is going from 1 cell to 2 cells, not per se cell cycle activation as assessed by any single marker. If one marker

shows no significant increase while another marker does, further validation is needed as via beta cell volume. I do agree with the authors that based on the currently provided data one should not attribute a major role to beta cell proliferation in the model.

We now clarify in the text that cells can activate the cell cycle without completing it and actually dividing. To independently assess Igfbp1's effect on beta-cell proliferation, we have now also tested its effect on isolated mouse islets. We find that Igfbp1 does not increase mouse beta-cell proliferation, as assessed with two independent markers of proliferation: Ki67 and EdU (see new Fig. 7D).

Concerning the determination of insulin positive area and other morphometric analyses included in the revised manuscript: the material and methods section should be updated to include details on these measurements. Further concerning these measurements: "we found that Igfbp1a overexpression did not change the number of counts (objects with coherent insulin-positive pixels), but it did significantly increase the average size of each count". What do the authors mean by objects with coherent insulin positive pixels? Are these INS+ cells? If so the conclusion should be that there is beta cell hypertrophy but no beta cell hyperplasia. Furthermore it is unclear whether the total pancreas size is comparable in igbp1a overexpressing and control fish, which is of course important as in the manuscript the authors make statements on increments in beta cell mass but no true beta cell mass (ug) or volume (ul) measurements are shown, only the percentage of INS+ area over total pancreas area. Related to this, all figures in the manuscript would benefit from including a scale bar.

We have now updated the methods section, adding a description of the methods used to determine insulin-positive areas and other morphometric analyses:
"We exposed one-month-old Tg(ins:H2B-GFP);Tg(ins:Flag-NTR) zebrafish, with or without Tg(bactin:igfbp1a), to 1 mM of MTZ and 0.1% DMSO for 24 hours to ablate their β cells. We sacrificed the zebrafish 4 days after the MTZ treatment, and then analyzed their entire pancreas by confocal microscopy with a 10X objective. With this setup and resolution, a single β cell was typically visualized as 30 adjacent insulin-positive pixels. Insulin-positive pixels were quantified with ImageJ software, according to the user guide for statistical measurement of image data. We converted the stack of images from a single pancreas into a flattened projection, in which we then measured the total insulin-positive area, the number of units comprising adjacent insulin-positive pixels, and the average size of the units (which ranged from single β cells and β-cell clusters)."

To be more accurate in our descriptions, we have changed the term "*counts*" to "*units*". Also, we now clarify, in the main text, the figure text and methods, what we measure: "units comprising adjacent insulin-positive pixels (ranging from single β cells to β-cell clusters)".

As mentioned in the manuscript, the *igfbp1a*-overexpressing transgenics do not grow as well as their controls (meaning that the *igfbp1a*-overexpressing fish are smaller, as are their pancreata), perhaps because the targets of Igfbp1a are growth factors. Therefore, we calculated the beta-cell area as a function of the total pancreas area. In the updated version of Figure 6, you can now find three additional measurements: body length/fish, ins+ area/fish, and ins+ area/body length. As such, we show the analysis of β-cell regeneration in both absolute and relative terms, and conclude that: "*igfbp1a can potentiate β-cell regeneration also in 1-month-old zebrafish, at least when related to body length or whole-pancreas size*". And we now include scale bars in the accompanying images.

Concerning the dose dependency of the induction of bihormonal (GLU+/INS+ cells) in mouse islets: the authors should include an additional dose of 0.5ug/ml to prove their point of dose dependency of the effect, since control and 0.3ug/ml do not differ significantly, the current results rather fit a threshold model. Why does the highest concentration of rIgfbp1a 25ug/ml lead to a reduction in this percentage of bihormonal cells?

We did not intend to place such emphasis on a dose dependency, rather we tested several concentrations on mouse islets in order to identify one concentration that would be likely to work on human islets. This is because human islets are hard to come by, requiring us to limit the number of experimental setups used to study them. Given the goal of these experiments, we have now taken out the claim of dose dependency, rather than redoing the whole experiment. It is hard to know why the higher dose, 25ug/mL, has a slightly lower effect than the 1 or 10 ug/mL doses (though it is

common that treatments start to have a lesser effect once they reach a certain concentration); perhaps it is due to toxicity.

- Concerning the proposed theoretical model: not per se sensing is affected in alpha cells, when IGFBP1 is high more of IGF will be bound, and the free fraction will be lower; better in my view to propose a model in which higher IGFBP1 lead to reduced IGF1 signaling to alpha cells and hereby enabling of the transdifferentiation

Thank you for pointing this out. We agree and have adjusted the text to clarify this point.

- In the discussion the author state "In this study we show that Igfbp1 promotes β-cell regeneration and may reduce the risk of developing diabetes". The current study is not designed or appropriate to address the latter statement on the prevention of diabetes. Alternatively, it can be stated that Igfbp1 is correlated with diabetes risk reduction.

We have now changed the statement from "*may reduce the risk of developing diabetes*" to "*that high levels of IGFBP1 are associated with a reduction in the risk of developing diabetes*".

- "Importantly, these in vitro effects also indicate that IGFBP1 has a direct effect on the islet, rather than an indirect effect through modulation of IGF or insulin signaling in other organs". This statement does no right to the remaining uncertainty of the mechanism. An indirect effect on the islet through modulation of IGF or insulin signaling within the islet is also possible, and to formally prove a direct effect additional experiments perturbing intra-islet IGF or insulin signaling should be done. In fact, the authors show that by inhibition of IGF signaling the regenerative effect is mimicked which would fit the above indirect, intra-islet effect hypothesis at least as well as the direct effect hypothesis.

You make a very good point, thank you. We have now deleted the sentence about direct effects of IGFBP1.

- "Our results indicate that Igfbp1 plays a previously unappreciated role in expanding the β-cell mass, a role that may be important in protecting against the development of T2D and in regenerating the β-cell mass in T1D-and one that could perhaps be exploited therapeutically." This conclusion is not supported by the data provided, the authors should include beta cell volume/mass to prove this point and not relative % of INS+/total pancreas or size of INS+ counts.
- "In sum, we show that IGFBP1 is a secreted factor that can expand the β-cell mass by potentiating α- to β-cell transdifferentiation. Our findings also suggest that IGFBP1 may in this way protect against the development of diabetes and thus has potential as a treatment for diabetes." As stated above, this is an overinterpretation of the provided data.

We have now rephrased these sentences to downplay the potential medical application of our findings, as well as replaced "*β-cell mass*" with "*β-cell number*", which is what we analyze in many different experiments throughout the manuscript.

Additional Correspondence 14 June 2016

One key issue I would like to ask about upfront is the alpha cell control (ref 3, point 2): it is fine that this experiment was not included, but this implies that we need to have confidence the delta cells do indeed express IR and IGFR. Can the attached figure be included in the paper as evidence? This may require an authorship change.
This would be the most compelling way to resolve this issue, which seems fairly central to the paper.

I am raising this to make the paper as compelling as possible. The concerns the referee raised will also occur to other expert readers.

The other issue are all fine as addressed.

Also please take a look at fig 7 in particular. Some of the background areas appear sursprisingly void of signal. I understand this can easily be a threshold effect, but I'd suggest to check to ensure that the background was not 'blacked out'

Additional Correspondence 15 June 2016

Regarding the images, the lack of signal in the background areas is due to two factors: a threshold effect and the absence of cells in the black areas. We did increase the contrast of most images in order to more clearly visualize the overlap of the stainings of interest. We have not used anything like the "brush tool" in Photoshop to selectively modify the background or any other part of the image (the textbox is however black, but I guess you were not referring to that). Does this answer your question?

As for the delta-cell regeneration experiment, we are not aware of any findings suggesting that delta cells do not express insulin or IGF receptors. In fact, in the Human Protein Atlas one can find that the insulin and IGF1 receptor is expressed in all tissues

http://www.proteinatlas.org/ENSG00000171105-INSR/tissue

http://www.proteinatlas.org/ENSG00000140443-IGF1R/tissue

including the islets/pancreas

http://www.proteinatlas.org/ENSG00000171105-INSR/tissue/pancreas

http://www.proteinatlas.org/ENSG00000140443-IGF1R/tissue/pancreas

In any case, in our experiments we ablate the delta cells, so presumably it would be more important to know whether the cells giving rise to new delta cells express insulin or IGF receptors, rather than the delta cells themselves. (We have not, however, studied the cellular origin of delta-cell regeneration.) Moreover, we do not consider the delta-cell regeneration experiment to be central to the manuscript because we are not claiming that Igfbp1's effect on regeneration is limited to beta cells, as clarified in the discussion section of the manuscript:

"Igfbp1-deficient mice have no phenotype during development or adult homeostasis, but after hepatectomy they cannot regenerate their livers to the same extent as their wild-type littermates (Leu et al., 2003). This indicates that Igfbp1 may be a regenerative signal with importance for both hepatocytes and β cells. However, we did not see an effect of Igfbp1 on δ-cell regeneration, suggesting that Igfbp1 can promote regeneration of some, but not all, cell types."

Additional Correspondence 21 June 2016

It is fine to display the figures with the current contrast settings and I am not going to belabour this point beyond suggesting that in order to avoid any concerns being raised by (over)eager image integrity scouts, it might be a good idea to also display minimally processed images as 'SourceData' alongside the figure.
Source Data is something we in fact encourage as a matter of routine these days for all figures, as this really adds value to the dataset presented.
Please let us know if you are inclined to add any source data, including for figure 7.
I follow the arguments made for widespread expression of insulin and IGFR and that the key cells of interest are actually the regenerating cells, which have not been studied in detail in this body of work.
I do find the single cell analysis data rather useful and striking. I want to be reasonable about this issue: I would be pleased to add this data to the paper as it adds value to the work. If you are not planning to publish this data as part of another study, this ought to be a good opportunity in fact to share it.
Alternatively, one could reduce the scope of the dataset readily to much fewer cell types.

Accepted 27 June 2016

I am very pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal pending some minor additional information as discussed in our previous exchange:

1) please add source data minimally for the specific panels we discussed in fig 7 and any other key figure panels as you see fit. The idea is to enrichen your paper with data others can interpret and work with.
2) Please specifically discuss and cite the Cell Metabolism paper that you mentioned in your last response.
3) please add scale bars to all microscopy images

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Olov Andersson
EMBOJ
Manuscript Number: EMBOJ-2015-92903

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, page 31.
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Page 27 and 28.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse, page 24 and 27.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Zebrafish, page 24.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We comply.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Page 28.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Page 28.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	We have not used that section.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.