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Controlled induction of human pancreatic progenitors produces functional beta-like cells in vitro.

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1st Editorial Decision 12 February 2015

Thank you very much for submitting your study on the in-vitro induction of presumptive functional beta-cells for consideration to The EMBO Journal editorial office.

Two expert scientists reviewed the study and their comments reveal certain general interest in the paper. Their recommendation indicates that despite some precedence from the very recent publications on this topic, the methodological advance, respective additional molecular insights coming fro your data justify further consideration of your study. However, it also becomes obvious that further functional corroboration and molecular characterizations are requested for this and before being able to fully commit to publication at The EMBO Journal.
In the interest of rather timely pursuit, I kindly ask you to consider your options/experimental capabilities since we might be able to alternatively offer more rapid consideration at one of our less-mechanistically demanding sister title (e.g. EMBOreports).

Conditioned on these, I am happy to formally invite revisions/relevant amendments to your paper before final assessment.

Therefore, please do get in touch to possibly discuss feasibility and anticipated timeline as to act efficiently on this paper (due to time constrains preferably via e-mail).

REFEREE REPORTS

Referee #1:

Russ et al provide a new protocol that makes a step forward towards generating functional, mature human beta cells by keeping developmental mechanisms in focus.
The majority protocols that aim for human ES cell differentiation result in polyhormonal cells lakcing key beta cell transcription factors and glucose responsiveness in vitro. Several months in vivo maturation of in vitro obtained pancreas progenitor cells does, however, result in glucose responsive beta-like cells. Maturation and function of in vitro obtained insulin-producing cells was focus of two similar differentiation protocols that allowed the generation of functional beta-like cells of which relatively few were multihormonal (Rezania et al, 2014 and Pagliuca et al, 2014).
Russ et al. hypothesized that the in vitro generation of polyhormonal cells in previous in vitro differentiation protocols resulted from a premature assignment to the endocrine fate and related this to the timing of the induction of Neurogenin 3 expression. They show that BMP inhibitors, part of most of the current hES-to-beta cell differentiation protocols, promote precocious induction of endocrine differentiation resulting in the formation of polyhormonal cells. Exclusion of BMP inhibitor from the culture medium allows for efficient generation of PDX1+/NKX6.1+ progenitors without precocious NGN3 activation.
Using this new protocol 23% human C-peptide+ cells that express critical markers for beta cell function and show the ultrastructure of human beta-like cells can be obtained within 3 weeks.
On a per cell basis, grafted hESC-derived beta-like cells secreted 1/3 of the insulin secreted from primary human beta cells.

Major concerns
- The recent success in beta cell differentiation protocols by Rezania et al (2014) and Pagliucia et al (2014) is attributed to a focus on late differentiation steps. Why Rezania et al obtained more beta-like and less polyhormonal cells following addition of Vitamin C from day 3 to 8 that resulted in reduced expression of NGN3 at the early stages of pancreas differentiation? The effects of Vitamin C and a possible relation with BMP signaling should be assessed since the present paper focuses on the subtle and time-dependent regulation of NGN3 gene expression as critical for efficient endocrine cell differentiation.
- Whereas generation of PDX1+ and PDX1+/NKX6.1+ progenitor cells is very efficient, endocrine differentiation remains restricted to a smaller population of cells. Is this due to inexact expression of NGN3 that apparently is more complex than just through lateral specification by Notch inhibition?
- The function of the hESC-derived beta-like cells should be tested in mice with diabetes or, at least, hyperglycemia.

Minor concerns
- In contrast to what is stated in the present manuscript, Rezania et al (2014) did affect the first steps of differentiation, namely by adding Vitamin C from day 3 to 8. The major (only?) novelty from the current manuscript compared to Rezania et al, is exclusion of BMP inhibition. Addition of Vitamin C may be easier than inhibition of BMP, it is not more efficient as it results in 3% polyhormonal cells versus 1% only in Rezania et al. This should be elaborated. Besides the study of the possible link between Vitamin C and BMP signaling (see first major question).

- The fold increase of secreted C-peptide by the hESC-derived beta-like cells is similar to that of human islets. How do the absolute amounts compare?
- Similarly, what is the amount of proinsulin per cell as compared to human beta cells?
- Why the amount of glucose in IPGTT is as high as 3g/kg BW?

Referee #2:

This manuscript by Hebrok and colleagues reports a novel and simplified strategy for large-scale production of functional human β cells from hESC in vitro. By using sequential modulation of various signaling pathways in a three-dimensional cell culture system, they generated glucose-responsive, monohormonal insulin-producing cells that showed key features of a bona fide β cell. Thanks to a systematic analysis of established differentiation conditions, they found that BMP inhibition at early stage of hESC differentiation leads to premature Ngn3 expression, which in turn

favors the generation of polyhormonal pancreatic endocrine cells in culture.
This is a nice manuscript, which highlights the importance of precisely recapitulating the temporal induction of pancreatic progenitor fate (pdx1/nkx6.1) in order to generate fully differentiated β cells in vitro.
The novel strategy presented here represents a further improvement of the recently published protocols for directed differentiation of hESCs into fully mature β-like cells (Rezania et al. 2014; Pagliuca et al. 2014). Moreover, it provides an ex vivo model highly amenable for studying human pancreatic development. The experiments are well performed and support the conclusions of the authors.

Specific comments:
- Global gene expression analysis of the hES cell-derived pancreatic progenitors and mature β cells is missing. This data would have offered valuable insights into human pancreatic differentiation. In addition, it would have made possible an exhaustive comparison among the β-like cells generated here, previously published ones (Pagliuca et al. 2014) and human islets.

- In the in vivo transplantation experiment, the hES-derived β-like cells engrafted under the kidney capsule were not subjected to FACS-purification. It is unclear the reason why the authors did not include this step of purification, which might have helped the analysis of the phenotype. Is a mixed cell population required for in vivo survival of the cells? This point should be clarified.

- Mice transplanted with hES-derived β-like cells seem to have fasting C-peptide levels higher than those transplanted with human islets (see Fig. 6A). How do the authors explain the difference? Is it due to defective insulin-secretory control in the hES-derived β-like cells or to the presence of not fully mature endocrine cells in the graft?
Also, the graft should be further characterized by IF staining, for instance for the presence of polyhormonal endocrine cells and other endocrine hormones (eg. glucagon and somatostatin).
Minor points: the number of transplanted animals should be included.

-Finally, to fully investigate the functional properties of the hES-derived β-like cells, transplantation experiments should also be performed in a mouse model of diabetes. This is indeed an important proof of principle in efforts to translate this approach into a clinical setting.

-The Figure Legends section is not included in the manuscript file.

1st Revision - authors' response 18 March 2015

Reviewer #1:

Russ et al provide a new protocol that makes a step forward towards generating functional, mature human beta cells by keeping developmental mechanisms in focus. The majority protocols that aim for human ES cell differentiation result in polyhormonal cells lacking key beta cell transcription factors and glucose responsiveness in vitro. Several months in vivo maturation of in vitro obtained pancreas progenitor cells does, however, result in glucose responsive beta-like cells. Maturation and function of in vitro obtained insulin-producing cells was focus of two similar differentiation protocols that allowed the generation of functional beta-like cells of which relatively few were multihormonal (Rezania et al, 2014 and Pagliuca et al, 2014). Russ et al. hypothesized that the in vitro generation of polyhormonal cells in previous in vitro differentiation protocols resulted from a premature assignment to the endocrine fate and related this to the timing of the induction of Neurogenin 3 expression. They show that BMP inhibitors, part of most of the current hES-tobeta cell differentiation protocols, promote precocious induction of endocrine differentiation resulting in the formation of polyhormonal cells. Exclusion of BMP inhibitor from the culture medium allows for efficient generation of PDX1+/NKX6.1+ progenitors without precocious NGN3 activation. Using this new protocol 23% human C-peptide+ cells that express critical markers for beta cell function and show the ultrastructure of human beta-like cells can be obtained within 3 weeks. On a per cell basis, grafted hESC-derived beta-like cells secreted 1/3 of the insulin secreted from primary human beta cells.
We would like to thank the Reviewer for highlighting that our novel hES differentiation protocol focuses on exact developmental steps and as such, represents a step forward in generating

functional, mature human beta cells.

Major concerns

- The recent success in beta cell differentiation protocols by Rezania et al (2014) and Pagliucia et al (2014) is attributed to a focus on late differentiation steps. Why Rezania et al obtained more beta-like and less polyhormonal cells following addition of Vitamin C from day 3 to 8 that resulted in reduced expression of NGN3 at the early stages of pancreas differentiation? The effects of Vitamin C and a possible relation with BMP signaling should be assessed since the present paper focuses on the subtle and time-dependent regulation of NGN3 gene expression as critical for efficient endocrine cell differentiation.

We agree with the Reviewer that it is important to take advantage of our novel differentiation protocol to test the potential inhibiting effect of Vitamin C (Vit.C) on endocrine differentiation. To address this question, we compared the impact of removing BMP inhibitors to the effect of Vit.C addition on mRNA expression levels of the endocrine marker NGN3 and its downstream target NKX2.2. As shown in the revised Supplementary Figure 1, cells differentiated in the absence of BMP inhibitors at the pancreatic endoderm induction stage (R treatment) exhibit significantly lower levels of NGN3 mRNA when compared to RCN, thus confirming the protein data presented in the original manuscript. Importantly, addition of AA to R or RCN does not have a significant effect on either NGN3 or NKX2.2 transcript levels, indicating that the removal of BMP inhibitors is a more efficient way to prevent precocious endocrine induction in our system.

We added the following sentence on page 8: "qPCR analysis at day 8 of NEUROG3 and its downstream target NKX2.2 mRNA transcripts revealed significantly lower levels of these endocrine markers with R treatment when compared to the commonly employed RCN condition (Supp. Fig. 1D). Notably, addition of Vitamin C, recently shown to reduce endocrine differentiation of hESCs1, did not significantly lower NGN3 or NKX2.2 transcripts in our suspension culture system during RCN or R treatment (Supp. Fig. 1D)."

- Whereas generation of PDX1+ and PDX1+/NKX6.1+ progenitor cells is very efficient, endocrine differentiation remains restricted to a smaller population of cells. Is this due to inexact expression of NGN3 that apparently is more complex than just through lateral specification by Notch inhibition?

We agree with the Reviewer that one critical, yet poorly understood aspect of present human stem cell-based beta cell direct differentiation protocols is the induction of endocrine differentiation. It is noteworthy to point out that endocrine differentiation *in vivo* is also poorly understood and occurs in individual cells, rather than in a large cell population at a given time during pancreas development. However, it has been shown that reduction in Notch signaling results in enhanced endocrine differentiation *in vivo* and *in vitro2*. To assay if direct inhibition of Notch signaling has an additional effect on endocrine induction in our system, we tested the gamma secretase inhibitor XX (GSI-XX) employed in the two recent studies demonstrating the generation of functional beta-like cells[1,3] Treatment of cultures with compound GSI-XX at day 10 to 14 actually resulted in a marginal reduction in GFP+ cells at day 19 (0.94±0.16 fold) when compared to controls. Importantly, treatment with compound GSI-XX further increased cell death and impaired cluster structure in many spheres. Thus, other not yet defined factors need to be included to further promote endocrine differentiation and counteract the well-recognized toxicity of GSI-XX.

- The function of the hESC-derived beta-like cells should be tested in mice with diabetes or, at least, hyperglycemia.

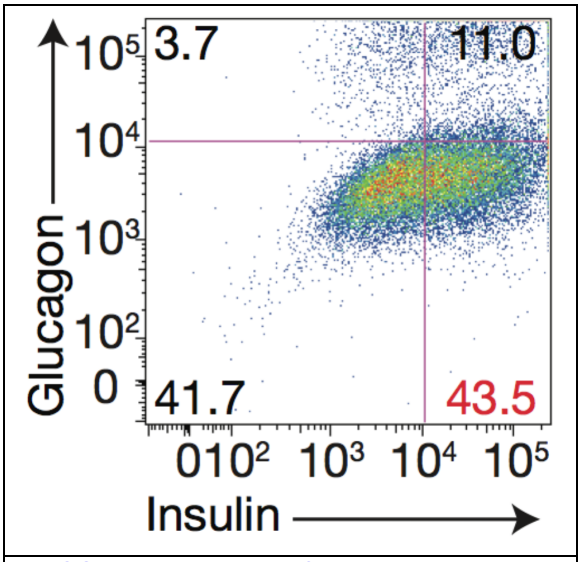
To address this important point raised by both Reviewers, we performed additional experiments where day 19 cells from two independent experiments were transplanted into streptozotocin (STZ) treated animals. Importantly, these new data (now included in Figure 6D) show that mice who received beta-like cell grafts exhibit significantly reduced blood glucose (BG) levels at all time points analyzed when compared to control animals. While blood glucose levels are significantly reduced in graft-bearing mice, they exhibit hyperglycemic BG values at the end of the experiment. This is likely due to the limited number of clusters containing beta-like cells that can be transplanted under the kidney capsule. Indeed, according to a previous study, 4,000 human islets are required to establish euglycemia in mice rendered diabetic, while 1,500 human islets fail to lower BG levels 7 days post transplantation4. Since we transplanted ~1.15x106 beta-like cells, a number higher than the ~0.75x106 beta cells present in 1,500 human islets but significantly lower than the ~2.0x106 beta cells present in 4.000 human islets, we expected a reduction in BG levels in mice bearing transplants but not necessarily a full rescue of diabetes.

We describe these new results on page 14 of the manuscript as follows: "To further investigate the

functional properties of hES derived beta-like cells *in vivo*, we transplanted clusters under the kidney capsule of mice rendered diabetic through treatment with the beta cell toxin streptozotocin. Mice that received grafts exhibit significantly reduced blood glucose (BG) levels at all time points analyzed when compared to control animals (Fig. 6D). While BG levels were significantly reduced in graft-bearing mice, they continued to exhibit hyperglycemic BG values over time. This is likely due to the limited number of beta-like cells that can be transplanted under the kidney capsule in one mouse. It has previously been shown that 4,000 human islets are required to establish long-term euglycemia in diabetic mice. Transplantation of a smaller number of human islets (1,500 islets) reduce blood glucose levels only for 7 days post transplantation, after which hyperglycemia returned 4. Our surgical procedure permits the transplantation of ~1.15x106 beta-like cells, substantially less than the ~2.0x106 beta cells present in the 4,000 human islets previously found to be required for the long-term reversal of diabetes. Hence the observed reduction in BG levels, but lack of complete diabetes reversal in mice bearing hES-derived transplants is not unexpected given this technical constraint."
Taken together our new *in vivo* data further emphasizes the functional properties of beta-like cells generated by our novel approach and highlights their potentialtherapeutic value.

Minor concerns
- In contrast to what is stated in the present manuscript, Rezania et al (2014) did affect the first steps of differentiation, namely by adding Vitamin C from day 3 to 8. The major (only?) novelty from the current manuscript compared to Rezania et al, is exclusion of BMP inhibition.

Addition of Vitamin C may be easier than inhibition of BMP, it is not more efficient as it results in 3% polyhormonal cells versus 1% only in Rezania et al. This should be elaborated. Besides the study of the possible link between Vitamin C and BMP signaling (see first major question).



FACS plot duplicated from Rezania et al. 2014 for expression of insulin and glucagon in S7 cells showing 11% polyhormonal cells.

We respectfully disagree with the Reviewer on the first comment. Indeed, our simplified protocol results in ~3% polyhormonal cells while Rezania et al. report the generation of 11% polyhormonal cells, not only 1% (Please see FACS plot duplicated from Figure 5C of Rezania et al. 2014). In addition, we now show new data demonstrating that exclusion of BMP inhibition lead to a greater reduction in NGN3 mRNA levels when compared to RCN and RCN + Vit.C. Thus, we conclude that our exact differentiation protocol represents an important improvement over existing strategies and is of great interest to the research community.

- The fold increase of secreted C-peptide by the hESC-derived beta-like cells is similar to that of human islets. How do the absolute amounts compare?
- Similarly, what is the amount of proinsulin per cell as compared to human beta cells?
We agree with the Reviewer that the evaluation of absolute amounts of insulin, C-peptide and proinsulin in beta-like cells is important. We present new data in figure 5 quantifying insulin, human c-peptide, and proinsulin levels in beta-like cells as ~2.5 ug, ~0.32ug, and ~310ng per ug DNA, respectively. These values are comparable to ~2.8ug, ~0.55ug, and ~150ng per ug DNA for human islets recently published by the Kieffer group 1. These data demonstrate that beta-like cells generated with our novel protocol efficiently process proinsulin and contain insulin and C-peptide levels comparable to human beta cells.

- Why the amount of glucose in IPGTT is as high as 3g/kg BW?
The amount of glucose for injections was chosen to elicit a strong insulin secretion response in both experimental groups (mice transplanted with human islets or hES derived beta-like cells).

Reviewer #2:

This manuscript by Hebrok and colleagues reports a novel and simplified strategy for large-scale production of functional human β cells from hESC in vitro. By using sequential modulation of various signaling pathways in a threedimensional cell culture system, they generated glucose-responsive, monohormonal insulin-producing cells that showed key features of a bona fide β cell. Thanks to a systematic analysis of established differentiation conditions, they found that BMP inhibition at early stage of hESC differentiation leads to premature Ngn3 expression, which in turn favors the generation of polyhormonal pancreatic endocrine cells in culture. This is a nice manuscript, which highlights the importance of precisely recapitulating the temporal induction of pancreatic progenitor fate (pdx1/nkx6.1) in order to generate fully differentiated β cells in vitro. The novel strategy presented here represents a further improvement of the recently published protocols for directed differentiation of hESCs into fully mature β-like cells (Rezania et al. 2014; Pagliuca et al. 2014). Moreover, it provides an ex vivo model highly amenable for studying human pancreatic development. The experiments are well performed and support the conclusions of the authors.
We thank the Reviewer for the supportive comments acknowledging the novelty and quality of our work, which highlights the importance of precisely recapitulating the temporal induction of pancreatic progenitor fate (pdx1/nkx6.1) in order to generate fully differentiated β cells *in vitro*.

Specific comments:
- Global gene expression analysis of the hES cell-derived pancreatic progenitors and mature β cells is missing. This data would have offered valuable insights into human pancreatic differentiation. In addition, it would have made possible an exhaustive comparison among the β-like cells generated here, previously published ones (Pagliuca et al. 2014) and human islets.
We agree with the Reviewer that global gene expression analysis of our beta-like cells would be desirable. However, while we can sort beta-like cells by GFP expression, such populations still contain low levels of immature, polyhormonal cells also expressing GFP, which renders data interpretation and comparison to published data challenging. To circumvent this issue, we are actively collaborating with other investigators to develop an approach employing surface marker antibodies to purify distinct hormone expressing cell populations. Once this method is established, we plan to perform a global analysis of gene expression and epigenetic marks in sorted populations. However we do believe that such detailed study is outside the scope of this manuscript.

- In the in vivo transplantation experiment, the hES-derived β-like cells engrafted under the kidney capsule were not subjected to FACS-purification. It is unclear the reason why the authors did not include this step of purification, which might have helped the analysis of the phenotype. Is a mixed cell population required for in vivo survival of the cells? This point should be clarified.
We thank the Reviewer for raising this important point. We initially did perform FACS purification of GFP+ cells to explore the attractive possibility of transplanting only purified beta-like cells. However, we found that a large proportion of total cells die during sample preparation (38±6%, judged by DAPI staining, n=3, independent experiments). Furthermore, of all GFP+ sorted cells, 25±26%, undergo cell death within 30min while still in the collection tube during sorting (based on DAPI staining, n=3, independent experiments). Due to these technical limitations, we decided to use heterogeneous cell clusters to help protect beta-like cells from stress during the elaborate transplantation procedure. To specifically examine the properties of *in vitro* generated beta-like cells and to exclude the contribution of progenitor cells that differentiate into functional betalike cells only after several weeks post transplantation, we have confined our *in vivo* analysis to the first weeks following transplantation.

- Mice transplanted with hES-derived β-like cells seem to have fasting C-peptide levels higher than those transplanted with human islets (see Fig. 6A). How do the authors explain the difference? Is it due to defective insulin-secretory control in the hES-derived β-like cells or to the presence of not fully mature endocrine cells in the graft? Also, the graft should be further characterized by IF staining, for instance for the presence of polyhormonal endocrine cells and other endocrine

hormones (eg. glucagon and somatostatin).
We agree with the Reviewer that fasting C-peptide levels in sera from mice bearing hES-derived beta-like grafts are slightly higher when compared to sera from mice transplanted with human islet. However, this difference is not statistically significant. As suggested by the Reviewer, one likely confounding factor is the presence of poly-homormonal cells lacking regulated insulin secretion and continuously secreting the hormone within grafts. This possible explanation is supported by new data generated following the Reviewer's suggestion to further characterize the grafts. Indeed, while immunofluorescence staining for glucagon, somatostatin and human C-peptide revealed that the majority of C-peptide positive cells in grafts are mono-hormonal, it also confirmed that a few poly-hormonal cells are present. These new data are now included in the revised Figure 6C. We also included a representative H&E staining in the new Figure 6B to visualize the structure and morphology of the grafted tissue.

Minor points: the number of transplanted animals should be included.
We apologize for this oversight. The number of transplanted mice has been included in the revised manuscript.

-Finally, to fully investigate the functional properties of the hES-derived β-like cells, transplantation experiments should also be performed in a mouse model of diabetes. This is indeed an important proof of principle in efforts to translate this approach into a clinical setting.
To address this important point raised by both Reviewers, we performed additional experiments where day 19 cells from two independent experiments were transplanted into streptozotocin (STZ) treated animals. Importantly, these new data (now included in Figure 6D) show that mice who received beta-like cell grafts exhibit significantly reduced blood glucose (BG) levels at all time points analyzed when compared to control animals. While blood glucose levels are significantly reduced in graft-bearing mice, they exhibit hyperglycemic BG values at the end of the experiment. This is likely due to the limited number of clusters containing beta-like cells that can be transplanted under the kidney capsule.
Indeed, according to a previous study, 4,000 human islets are required to establish euglycemia in mice rendered diabetic, while 1,500 human islets fail to lower BG levels 7 days post transplantation[4]. Since we transplanted ~1.15x106 beta-like cells, a number higher than the ~0.75x106 beta cells present in 1,500 human islets but significantly lower than the ~2.0x106 beta cells present in 4.000 human islets, we expected a reduction in BG levels in mice bearing transplants but not necessarily a full rescue of diabetes. We describe these new results on page 14 of the manuscript as follows: "To further investigate the functional properties of hES derived beta-like cells *in vivo*, we transplanted clusters under the kidney capsule of mice rendered diabetic through treatment with the beta cell toxin streptozotocin. Mice that received grafts exhibit significantly reduced blood glucose (BG) levels at all time points analyzed when compared to control animals (Fig. 6D). While BG levels were significantly reduced in graft-bearing mice, they continued to exhibit hyperglycemic BG values over time. This is likely due to the limited number of beta-like cells that can be transplanted under the kidney capsule in one mouse. It has previously been shown that 4,000 human islets are required to establish long-term euglycemia in diabetic mice. Transplantation of a smaller number of human islets (1,500 islets) reduce blood glucose levels only for 7 days post transplantation, after which hyperglycemia returned 4. Our surgical procedure permits the transplantation of ~1.15x106 beta-like cells, substantially less than the ~2.0x106 beta cells present in the 4,000 human islets previously found to be required for the long-term reversal of diabetes. Hence the observed reduction in BG levels, but lack of complete diabetes reversal in mice bearing hES-derived transplants is not unexpected given this technical constraint." Taken together our new *in vivo* data further emphasizes the functional properties of beta-like cells generated by our novel approach and highlights their potential therapeutic value.

-The Figure Legends section is not included in the manuscript file.
We apologize for this error. The figure legend is now included in the manuscript file.

1. Rezania, A. *et al.* Reversal of diabetes with insulin-producing cells derived in vitro from human pluripotent stem cells. *Nat. Biotechnol.* (2014). doi:10.1038/nbt.3033
2. Shih, H. P. *et al.* A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. *Development* **139,** 2488–2499 (2012).
3. Pagliuca, F. W. *et al.* Generation of Functional Human Pancreatic b Cells In Vitro. *Cell* **159,** 428–439 (2014).

4. Fiaschi-Taesch, N. M. *et al.* Induction of Human -Cell Proliferation and Engraftment Using a Single G1/S Regulatory Molecule, cdk6. *Diabetes* **59,** 1926–1936 (2010).

