SUPPLEMENTARY INFORMATION

1. HPAP LABELLING INDEX AND QUANTIFICATION

With a view to determining the percentage of labelled beta-cells in the pancreas of double transgenic mice, we quantified the area positive for HPAP or insulin in multiple digitally recorded images of pancreatic sections using ImageJ software.

First, we needed to employ an appropriate protocol to take into account the complex and heterogeneous organization of islets within the pancreas.[1,2] Thus, specific regional adaptation of the rat pancreas has been shown during pregnancy, which include an increased replication rate in the head of the pancreas (region adjacent to the duodenum) compared to the tail (region of the pancreas adjacent to the spleen). Although, this might not necessarily reflect what occurs in mouse, we decided to roll up the excised pancreas in a flat spiral, and to section it in the same plane as the flat spiral, namely, along a z-axis (Figure S1). Thus, the different anatomical parts of the pancreas (tail, body, and head) will be on the same section and each section will represent different levels of the organ. Secondly, to avoid bias in sections chosen for analysis, we numbered our sections sequentially by ascribing the number one to the first microtome section, the number two to the second and so forth. In total from five to seven hundred sections per pancreas were cut but only six sections per level were collected. One level corresponds to ten abutting sections. As the thickness of the section was ten micrometers, one level represented a thickness of approximately one to two hundred micrometers, about the size range of a typical intermediate islet. [3] Given the great number of sections produced per pancreas, it was not feasible to process every single one of them. For this reason, five sections representing different regions or levels of the pancreas along a z-axis, and separated from each other by at least five hundred micrometers to minimize the sampling of the same islet twice, were chosen at random.

To recapitulate, first we embedded the harvested organ in a flat spiral. Then,

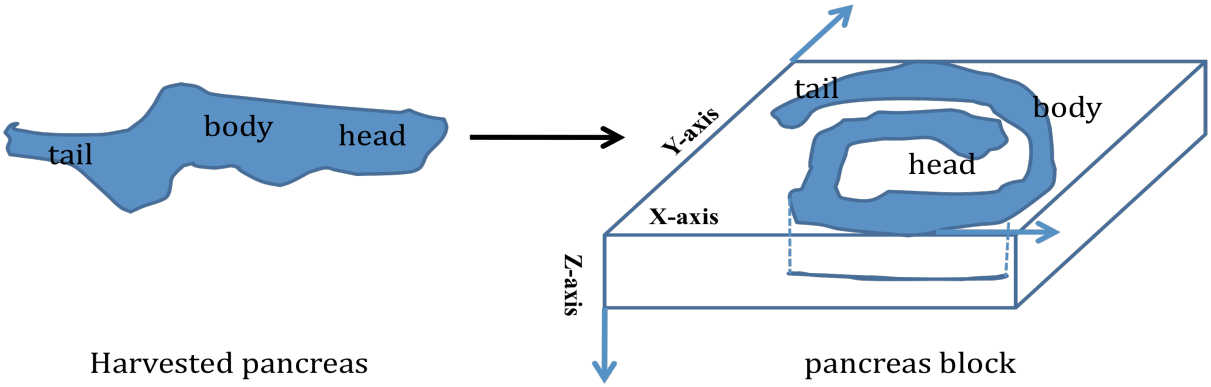


Figure S1. **Pancreas orientation before embedding and sectioning**. The pancreas is a heterogeneous organ which is divided in three regions, the head, body and tail. In order to extract information from this organ with little bias, the pancreata were rolled up in flat spiral before embedding and sectioning in the same plane as the flat spiral, i.e. along the z-axis.

it was sectioned along a z-axis and each section was numbered appropriately to ensure that in the selected analysed sections the same islets were not scored twice.

The second issue was to consider the HPAP and insulin area measurement procedure. In order to quantify the HPAP and insulin area in the section using ImageJ, we captured images of every immunostained pancreatic cross-sectional area of single β-cells, clusters of β-cells, and islets which were present in each section. Five sections per pancreas were entirely scanned using a confocal microscope (Leica, SP2). An average, two hundred and twenty one photos of islets or β-cells clusters per pancreas were captured, but four hundred and forty two images per pancreas or in the whole five sections were analysed, employing the measurement software, because the quantification of HPAP and insulin cross-sectionnal area had to be performed on separate images after immunostaining (i.e. red and green channels).

Overall, three thousand five hundred and thirty six images were processed for the whole experiment (4 paired animals; eight harvested pancreata in total) involving single pregnancy. In total two thousand one hundred and ninety eight images were analysed anonymously for the experiment (3 paired animals; 6 harvested pancreata in total) involving two pregnancies.

The area evaluation was performed on pancreatic sections immunostained with anti-HPAP and anti-insulin antibodies (as described in Materials and Methods). Fluorescein and

Alexa fluo 633, which are fluorescent dies bound to secondary antibodies, were used to reveal the primary antibodies respectively. Using ImageJ, the HPAP and insulin area can be measured either automatically or manually after adjusting an image intensity threshold with a slider. In our case, the threshold was set manually for each picture, as it proved more accurate for distinguishing positive HPAP or insulin immunoreactivity from "background". After adjusting the threshold, the HPAP or insulin area was measured. The adjustment of the threshold was repeated three times on the same object, so that three counts per object were carried out and the three computed values were averaged. It is of interest to point out that ImageJ measures the area by counting the number of pixels constituting the object on the picture. Thus, the HPAP and insulin area determination represent the total number of pixels counted. Importantly, all such measurements were conducted blind. The images used to quantify the HPAP or insulin area were all anonymously coded. The experimenter had no knowledge of the condition being observed, i.e. whether the images analysed were from a pregnant or non-pregnant animal or from a given replicate. Using this tool (ImageJ), we were able to evaluate the HPAP and insulin area in each beta-cell cluster and islet, and to calculate the percentage of HPAP labelled beta-cells, the "HPAP labelling index" in the pancreas of each mouse. The HPAP labelling index was computed by forming the ratio of the total pixel cross-sectional area positive for HPAP over the total pixel cross-sectional area positive for insulin in the whole five sections of each animal. Alternatively, beta-cells positive for HPAP or insulin were counted manually in each picture using ImageJ. Each such measurement was repeated, to obtain three separate counts for each image.

The beta-cell mass was calculated by forming the ratio of the total number of pixels of insulin cross-sectional area to the total number of pixels of the pancreatic tissue cross-sectional area in the five sections in each pancreas, multiplying by the pancreas weight of the given mouse.[4]

Similarly, the proportion of beta-cell area positive for HPAP or the HPAP labelling index was calculated by forming the ratio of total number of pixels of cross-sectional area immunostained for HPAP to total number of pixels cross-sectional area immunostained for insulin, in all five sections in the given mouse from the x40 images.

Alternatively, the HPAP labelling index was also calculated by forming the ration of total number of beta-cells positive for HPAP over total number of beta-cells positive for insulin in all five sections in the given mouse from the x40 images.

The beta-cell area was calculated by forming the ratio of total number of pixels cross-sectional area positive for insulin over total number of beta-cell positive for insulin, in all five sections in the given mouse from the x40 images.

2. RESULTS. SUPPLEMENTARY FIGURES

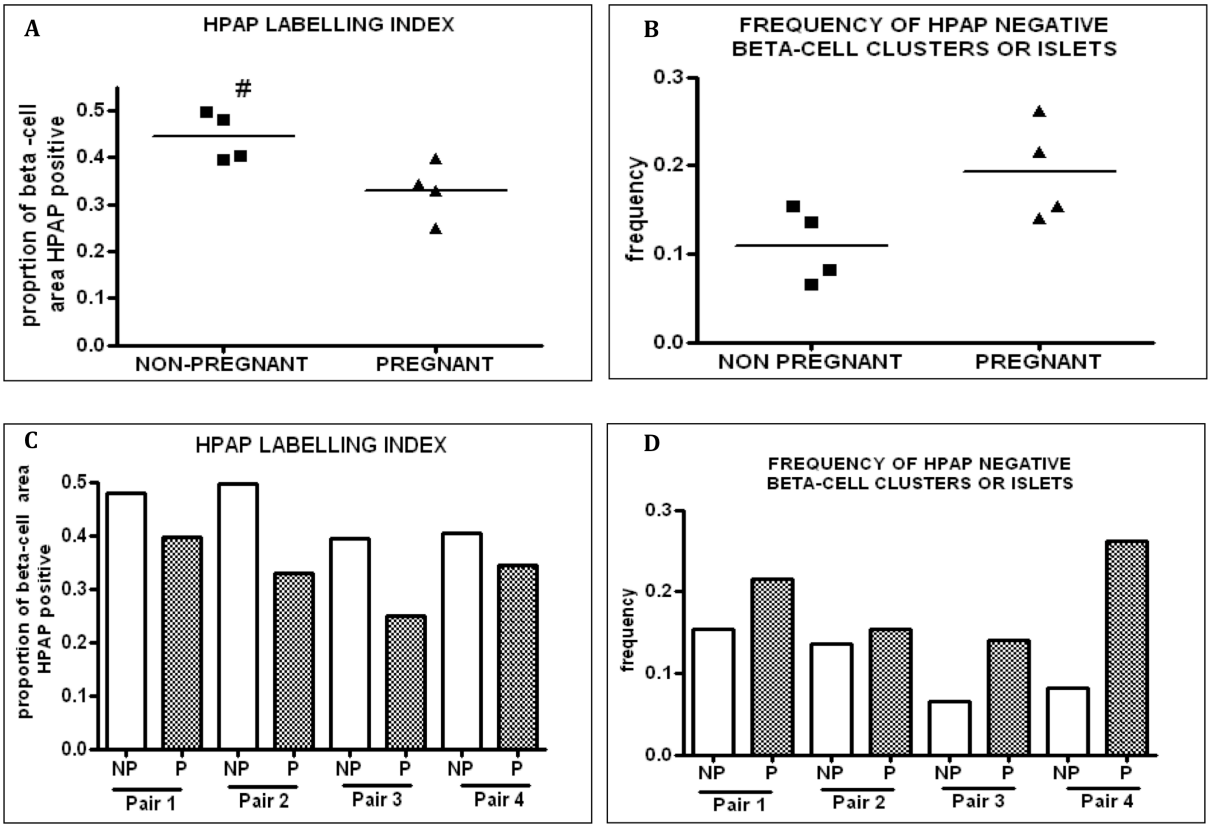


Figure S2 **Lineage tracing in single pregnancy**. (A), The HPAP labelling index in non-pregnant control and pregnant female animal pancreata was calculated by forming the ratio of total number of pixels of cross-sectional area immunostained for HPAP to total number of pixels cross-sectional area immunostained for insulin, using Image J. The mean labelling index in the pancreata of non-pregnant animals is higher (0.44±0.05) than in the pregnant mice (0.33±0.06). (B) The proportion of islet sections completely negative for HPAP is calculated by forming the ratio of the HPAP-negative islet cross-sectional area to the total islet cross-sectional area in the whole of 5 sections for each pancreas. The mean proportion of islet sections HPAP negative in the pregnant group is (0.19± 0.06) is higher than in their age-matched, non-pregnant control counterparts (0.11±0.04) as seen in the graph. (C) and (D) represent the HPAP labelling index and the frequency of HPAP negative β-cell cluster or islets in pregnant and non-pregnant animals respectively in each pair. # designates a P-value <0.05 by the paired two-tailed student t-test. Non-pregnant (NP), pregnant (P).

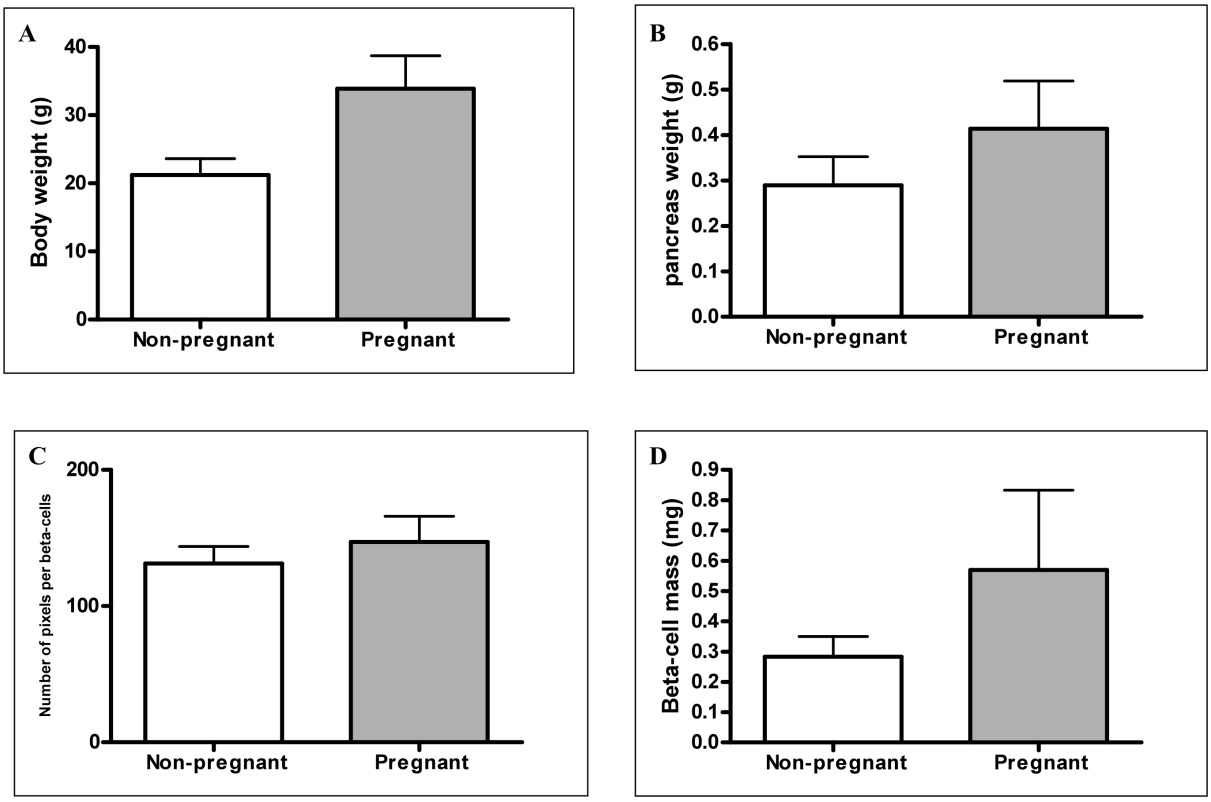
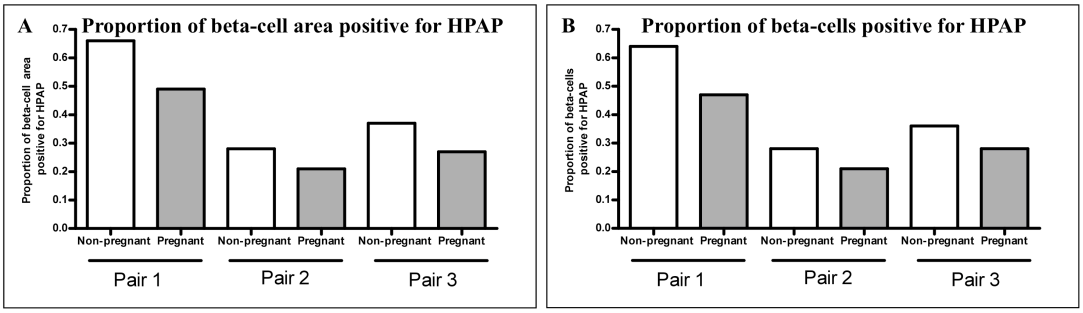


Figure S3 **Responses to a second pregnancy**. During a second pregnancy, the mean body weight increased by 60% (A), the mean pancreas weight by 53% (B), and the β-cell area increased about 12% (C) as compared with their age-matched non-pregnant control counterparts. Mean β-cell mass in pregnant animals doubled (D).



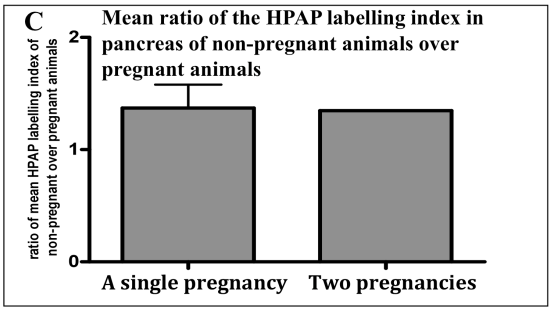


Figure S4 **Lineage tracing in pregnancy**. The HPAP labelling indices in pancreata of non-pregnant and age-matched pregnant animals during a second pregnancy, were calculated using data generated from either an area measurement (A) in pixel number using ImageJ, or manual cell counting (B). The HPAP labelling index values calculated from data produced by area measurement or generated by manual cell counting, for the same animal, are numerically similar. (C) The mean ratio of the HPAP labelling index in pancreata of non-pregnant controls over pregnant animals during one (1.37± 0.209) and two (1.35±004) rounds of pregnancy (for the latter case the area-based measurement was used).

SUPPLEMENTARY REFERENCES

1. Alanentalo T, Asayesh A, Morrison H, Loren CE, Holmberg D, Sharpe J, Ahlgren U. Tomographic molecular imaging and 3D quantification within adult mouse organs. Nat Methods 2007; 4:31-33.
2. Avril I, Blondeau B, Duchene B, Czernichow P, Breant B: Decreased beta-cell proliferation impairs the adaptation to pregnancy in rats malnourished during perinatal life. J Endocrinol 2002; 174:215-223.
3. Jo J, Choi MY, Koh DS. Size distribution of mouse Langerhans islets. Biophys J 2007; 93:2655-2666.
4. Huang C, Snider F, Cross JC. Prolactin receptor is required for normal glucose homeostasis and modulation of beta-cell mass during pregnancy. Endocrinology 2009; 150:1618-1626.