TGF- β signalling prevents pancreatic beta cell death after proliferation

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Abstract

Objectives: Maintenance of functional beta cell mass is critical for prevention of diabetes. The transforming growth factor-beta (TGF- β) receptor signalling pathway plays an essential role in pancreatic development. However, its involvement in control of post-natal beta cell growth has only been recently reported. **Materials and methods:** Here, we studied the role of TGF- β receptor signalling in beta cell proliferation after 50% partial pancreatectomy (PPx), using beta cell-specific TGF- β receptor II (TBR2)-mutated mice.

Results: Consistent with previous reports, we found that inhibition of TGF- β receptor signalling in beta cells resulted in slightly higher beta cell mass 1 week after PPx, due to greater beta cell proliferation. However, beta cell mass in these beta cell-specific TBR2-mutated mice significantly decreased by 12 weeks after PPx, resulting from increase in beta cell apoptosis.

Conclusions: Our data thus suggest that TGF- β receptor signalling may be required for prevention of beta cell death after proliferation, and highlight this pathway as an essential regulator during post-natal beta cell homeostasis.

Introduction

Preservation and restoration of functional beta-cell mass are fundamental objectives in therapy for diabetes (1). Elucidation of molecular regulation of beta-cell mass in the adult pancreas may substantially boost diabetic research and treatment. During embryogenesis, beta-cell mass is

generated by both beta-cell proliferation and beta-cell neogenesis from pancreatic progenitor cells (2,3). However, post-natal beta-cell growth has been shown to be predominantly attributed to beta-cell replication (4–14).

The transforming growth factor-beta (TGF- β) receptor signalling pathway is essential for various biological processes (15–17). When a ligand binds to a type II TGF- β receptor (TBR2), it catalyses phosphorylation of a type I TGF- β receptor, which triggers phosphorylation of intracellular proteins, SMAD2 and SMAD3, to form heteromeric complexes with SMAD4. Activated SMAD complexes then translocate to the nucleus, where they regulate transcription of target genes (15–17). Previous studies have demonstrated that TGF- β receptor signalling plays a critical role in pancreatic organogenesis (18) and in pathogenesis of pancreatitis and pancreatic ductal adenocarcinoma (19,20). Moreover, although beta-cell mass and glucose metabolism seem not significantly affected by modification of TGF- β receptor signalling in the pancreas under physiological conditions (21,22), recent studies have suggested that TGF- β receptor signalling may play a role in conditions of stimulated post-natal beta-cell growth, for example, after partial pancreatectomy (PPx) (23,24), or after partial pancreatic duct ligation (24,25). Although these studies have extensively evaluated the role of TGF- β receptor signalling in post-natal beta-cell growth in the short term after stimulation (for example PPx), the role of TGF- β receptor signalling over the long term, in these models, has not been studied.

Here, we aimed to evaluate the role of TGF- β receptor signalling in beta cell proliferation and beta cell mass adaption, over the long term, after 50% PPx, using beta cell-specific TBR2-mutated mice. Consistent with previous results, we found that inhibition of TGF- β receptor signalling in beta cells slightly increased beta cell proliferation at early stages after PPx. However, beta cell mass in these beta cell-specific TBR2-mutated mice significantly decreased by 12 weeks after PPx, resulting from increase in beta cell apoptosis.

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Materials and methods

Mouse handling

All mouse experiments were approved by the Institutional Animal Care and Use Committee at the General Hospital of Ningxia Medical University (Animal Welfare Assurance). Mice were housed in a pathogen-free environment. All surgery was performed under ketamine/xylazine anaesthesia, according to the Principles of Laboratory Care, supervised by a qualified veterinarian. RIP-CreERT (4) and TGF- β receptor II (TBR2) *fx/fx* (26) mice were both purchased from Jackson, USA. RIP-CreERT mice were bred with TBR2*fx/fx* mice to generate RIP-CreERT/TBR2*fx/fx* animals. Cre-negative TBR2*fx/fx* littermates were used as controls. Tamoxifen solution (10 mg/ml) (Sigma, St. Louis, MO, USA) was dissolved in filter-sterilized corn oil. To induce depletion of TBR2 in beta cells, RIP-CreERT/TBR2*fx/fx* mice, 8 weeks of age, received intraperitoneal injections of 1 mg tamoxifen (100 μ l) or corn oil vehicle (control) once a day for 3 consecutive days (2 weeks before PPx). PPx was performed as previously described (27). 50% PPx was assured by ratio of weight of excised pancreas from PPx-mice to weight of complete pancreas of a littermate. For quantification of beta cell proliferation, 1 mg/ml BrdU (bromodeoxyuridine; Sigma, USA) was added into 1% sucrose drinking water and given to mice 24 h before sacrifice, as previously described (24,25,28,29). Ten mice were analysed in each experimental condition.

Islet isolation

Mouse pancreas was first perfused with 0.125 mg/ml LiberaseTL (Roche, Nutley, NJ, USA) from the biliary-pancreatic bile duct, then incubated in 0.125 mg/ml LiberaseTL in a 37 °C shaker at 200 times per minute for 45 min. Histopaque1077 and Histopaque1119 (Sigma, USA) were mixed, ratio 9:33 (vol:vol), to generate Histopaque1110. Centrifugation in Histopaque1100 at 500 *g* for 15 min with no brake (islets in the solution fraction), followed by hand-picking, was then performed to isolate islets.

Genomic DNA isolation and PCR

Genomic DNA was extracted from isolated islets using DNeasy kit (Qiagen, Hilden, Germany) and quantified with Nanodrop1000 (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Conventional polymerase chain reaction (PCR) was performed with TBR2 primer pairs (sense: 5'-GAAGGAAAAGAAAAGG G-3', antisense: 5'-CCAGCACTCGGTCAAAG-3', with a

product length of 1195 bp), or with GAPDH primer pairs (sense: 5'-AACTTTGGCATTGTGGAAGG-3', antisense: 5'-ACACATTGGGGGTAGGAACA-3', with a product length of 1288 bp). All targets were amplified for 30 cycles.

Protein isolation and western blotting

Protein was extracted from purified islets using RIPA buffer (Sigma) for western blotting. Supernatants were collected after centrifugation at 12000 $\times g$ at 4 °C for 20 min. Protein concentration was determined using BCA protein assay, and whole lysates were mixed with 4 $\times$ SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT and 0.2% bromophenol blue), 1:3. Samples were heated to 100 °C for 5 min and were separated on SDS-polyacrylamide gels. Separated proteins were then transferred to PVDF membranes and membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated secondary antibody, autoradiographs were prepared using an enhanced chemiluminescence system to visualize protein antigens. Signals were recorded using X-ray film. Primary antibodies for western blotting were anti-TBR2 and anti-GAPDH (all purchased from Cell Signaling, San Jose, CA, USA). Secondary antibody was HRP-conjugated anti-rabbit (Jackson Labs, Bar Harbor, ME, USA). Images shown in the respective figure were representative of three repeats. Densitometry of western blots was quantified using NIH ImageJ software (Bethesda, MA, USA).

Immunohistochemistry

Mouse pancreata were dissected out and fixed in 4% paraformaldehyde for 10 h, then cryo-protected in 25% sucrose overnight. After embedding and mounting, samples were sectioned at 6 μ M. Primary antibodies used for immunohistochemistry were guinea pig polyclonal anti-insulin and rat polyclonal anti-BrdU (Abcam, Cambridge, MA, USA). For BrdU staining, antigen retrieval was performed by incubation of sections in 1 mol/l HCl at room temperature for 45 min. Secondary antibodies were Cy3- and Cy2-conjugated antibodies for corresponding species (Jackson ImmunoResearch Labs, West Grove, PA, USA). TUNEL staining was performed using In Situ Cell Death Detection Kit. DAPI (4',6-diamidino-2-phenylindole) was used to stain nuclei.

Quantification of beta cell proliferation, beta cell apoptosis and beta cell mass

Insulin staining was used to identify beta cells. Quantification of BrdU⁺ beta cells or TUNEL⁺ beta cells was

based on five sections 120 μ m apart from each other. More than 1000 beta cells were counted for each animal. Ten mice were used for each experimental condition. Quantification of beta cell mass was performed as previously described (7). Briefly, pancreata were weighed then fixed in 4% paraformaldehyde for 10 h, then cyro-protected in 25% sucrose overnight, so as to allow longitudinal sections from tail to head of the pancreas, to be obtained. Sections at 120 μ m intervals from whole pancreata were immunostained for insulin and analysed using Image J software. Relative cross-sectional areas of beta cells were determined by quantification of cross-sectional area occupied by beta cells, divided by the cross-sectional area of total tissue. Beta cell mass per pancreas was estimated as product of relative cross-sectional area of beta cells per total tissue, and weight of the pancreas. Beta cell mass was calculated by examining pancreata from 10 animals for each group.

Statistical analysis

All values depicted as mean $\pm$ standard error from 10 individuals, are considered and significant if $P < 0.05$. All data were statistically analysed using one-way ANOVA with Bonferroni correction.

Results

Generation of beta cell-specific TBR2-depleted mice

RIP-CreERT (4) and TBR2fx/fx (26) mice are established mouse strains and have been widely used in previous studies. Here, we bred RIP-CreERT mice with TBR2fx/fx mice to generate RIP-CreERT/TBR2fx/fx animals. Cre-negative TBR2fx/fx littermates were used

as controls. RIP-CreERT/TBR2fx/fx mice then received tamoxifen to induce depletion of TBR2 in beta cells at 8 weeks of age.

Two weeks later (at 10 weeks of age, or time for PPx), islets were isolated from these animals to examine depletion of TBR2 in beta cells. The TBR2fx/fx mouse has LoxP sites flanking exon 4, the functional domain of TBR2. When tamoxifen is given, Cre recombinase is translocated to nuclei to remove exon 4 of TBR2, thus inactivating TBR2 (Fig. 1a). We used a primer pair that amplifies a product of 1195 bp from intact genomic DNA, but not from genomic DNA of induced removal of exon 4 of TBR2.

Conventional PCR was performed on genomic DNA (Fig. 1b) or protein (Fig. 1c) from isolated islets from both TBR2fx/fx and RIP-CreERT/TBR2fx/fx mice, with or without tamoxifen treatment, showing much weaker signals specifically from tamoxifen-treated RIP-CreERT/TBR2fx/fx mouse islets. This weak signal may derive from non beta cells in the islets, including alpha cells, delta cells, PP cells, endothelial cells and others (Fig. 1b, c). Besides genomic DNA, we also examined protein levels of TBR2 knock-down, as some receptors may have a fairly long half-life, resulting in presence of proteins of receptor after gene depletion. Our data suggest that TBR2 of the beta cells had been substantially depleted by 2 weeks after, thus confirming beta cell-specific TBR2 depletion in RIP-CreERT/TBR2fx/fx mice after tamoxifen treatment.

Beta cell-specific TBR2 depletion slightly increased beta cell proliferation in the short term after PPx

At this stage we performed 50% PPx on these mice, 2 weeks after tamoxifen treatment. We examined beta cell-proliferation and quantified beta cell mass 1, 4, 8

Figure 1. Generation of beta cell-specific TBR2-depleted mice. (a) RIP-CreERT and TBR2fx/fx mice were crossed to generate RIP-CreERT/TBR2fx/fx mice. Cre-negative TBR2fx/fx littermates were used as controls. (b, c) RIP-CreERT/TBR2fx/fx mice then received tamoxifen to induce depletion of TBR2 in beta cells at 8 weeks of age. Two weeks later, islets were isolated from these mice to examine depletion of TBR2 in beta cells by PCR, using genomic DNA as template and a primer pair that amplifies a product of 1195 bp from intact genomic DNA, but not from genomic DNA of induced removal of exon 4 of TBR2 (b), or by western blotting (c). GAPDH was used as a control.

and 12 weeks after PPx. Blood glucose levels of mice remained normal during the experiment (Fig. 2a). We found that beta cell mass in RIP-CreERT/TBR2fx/fx mice was slightly higher than that in control TBR2fx/fx animals 1 week after PPx (Fig. 2b). Moreover, adaptation of beta cell mass was further confirmed by pancreatic insulin content (Fig. 2c), and seemed to result from higher beta cell proliferation (Fig. 2d, e). These data are consistent with previous reports (23,24).

Beta cell-specific TBR2 depletion resulted in reduction in beta cell mass by 12 weeks after PPx

We then evaluated beta cell mass and beta cell proliferation over the long term after PPx. While no differences in beta cell mass or pancreatic insulin content were detected at 4 and 8 weeks after PPx, we found a significant reduction in beta cell mass or pancreatic insulin content by 12 weeks after PPx in RIP-CreERT/TBR2fx/fx animals (versus 8 weeks, Fig. 2b, c). Interestingly, no significant difference in beta cell proliferation was detected after 4 weeks post PPx in RIP-CreERT/TBR2fx/fx mice compared to TBR2fx/fx (Fig. 2d). These data suggest that

beta cell-specific TBR2 depletion resulted in decrease in beta cell mass over the long term after PPx, which was unrelated to beta cell proliferation.

Beta cell-specific TBR2 depletion resulted in long term increased beta cell death after PPx

We thus examined whether beta cell apoptosis could have been the cause of reduction in long term beta cell mass after PPx in RIP-CreERT/TBR2fx/fx mice. We performed TUNEL staining at different time points after PPx, and essentially detected significant increases in beta cell apoptosis directly after 8 weeks post PPx (Fig. 3a, b). These data suggest that beta cell-specific TBR2 depletion increased beta cell death over the long term after PPx, resulting in reduction of beta cell mass.

Beta cell-specific TBR2 depletion did not significantly alter beta cell mass

To exclude the possibility that beta cell proliferation and apoptosis were naturally different in TBR2-ablated beta cells, we also analysed sham-treated mice after

Figure 2. Changes in beta cell mass and beta cell proliferation in mice after PPx. We performed 50% PPx on these mice at 2 weeks after tamoxifen treatment. BrdU was labelled for 24 h. (a) Blood glucose. (b–d) Beta cell mass (b), pancreatic insulin content (c) and beta cell proliferation (d) were quantified at 1, 4, 8 and 12 weeks after PPx. (e) Representative images of BrdU and insulin double immunostaining. DAPI, nuclear staining. * $P < 0.05$. Scale bar is 20 μ m.

Figure 3. Changes in beta cell apoptosis in mice after PPx. TUNEL staining was performed at different time points after PPx. (a, b) TUNEL + insulin + cells were evaluated, shown by quantification (a), and by representative images (b). * $P < 0.05$. Scale bar is 20 μm.

tamoxifen treatment. We found that specific TBR2-depletion in beta cells did not alter beta cell mass (Fig. 4a), beta cell proliferation (Fig. 4b) and beta cell apoptosis (Fig. 4c) over the 12 week duration of the experiment. These data suggest that beta cell apoptosis following proliferation in TBR2-depleted beta cells after PPx did not seem to result from natural TBR2 ablation in beta cells.

Discussion

Previous studies have highlighted an essential role for TGF- β receptor signalling in stimulated beta cell

expansion in the adult (23–25,30). However, these studies have only focused on short-term effects of inhibition of TGF- β receptor signalling on beta cell proliferation and beta cell mass adaption in response to stimulants. As TGF- β receptor signalling is essential for maintaining the functional phenotype of beta cells (31), and as proliferating beta cells are proposed to undergo some degree of dedifferentiation (23), we were prompted to examine whether loss of TGF- β receptor signalling could affect beta cells that had proliferated.

RIP-CreERT/TBR2fx/fx mouse model is an ideal beta cell-specific TBR2-depleted model. Compared to

Figure 4. Beta cell mass, pancreatic insulin and beta cell proliferation in sham-treated mice. (a–c) Sham-treated mice were analysed after tamoxifen treatment, by beta cell mass (a), beta cell proliferation (b) and beta cell apoptosis (c) at the time points for analysing PPx-treated mice.

previously used Pdx1-CreER (23), or Ptf1a-Cre (24) for induction of depletion of TBR2 in beta cells, RIP-CreERT is more specific, as Pdx1 is expressed in both beta cells and delta cells in the adult pancreas, and Ptf1a-cre targets to most pancreatic cell types. These notions were confirmed by our examination of islet genomic DNA from the RIP-CreERT/TBR2fx/fx mouse, showing very efficient knock-out of TBR2 in beta cells. As we have previously discussed, very weak signalling of wild-type TBR2 band should be derived from non-beta cells of the islet fraction. In addition, our results are consistent with the fact that beta cells constitute 80–85% of all endocrine cells, or about 80% of all the cell types in the islet (32–34).

After PPx, we detected a short increase in beta cell mass at day 7, resulting from higher levels of beta cell proliferation in RIP-CreERT/TBR2fx/fx mice. These data are consistent with one previous report (24), and may result from attenuated cell-cycle inhibitory effects by TGF- β signalling on PPx-induced beta cell proliferation through abolition of TGF- β receptor signalling in beta cells. However, beta cell mass significantly decreased in these mice by 12 weeks after PPx, suggesting that beta cell loss had occurred. As there was no difference in beta cell proliferation between RIP-CreERT/TBR2fx/fx mice and control mice by 4 weeks after PPx, beta cell proliferation should contribute minimally to alterations in beta cell mass by 4 weeks after PPx. Thus, we hypothesized that the reduction in beta cell mass could result from beta cell death. As beta cell necrosis is unlikely to occur in this long-term model, beta cell apoptosis was thus examined. Our results confirmed our hypothesis, showing that beta cell-specific TBR2 depletion induced beta cell apoptosis over the long term after PPx. As beta cell mass at 12 weeks after PPx decreased to the level before PPx, it may be expected that apoptotic beta cells could be those that had just completed proliferation. Loss of TGF- β receptor signalling in beta cells may render these cells susceptible to apoptosis, as TGF- β receptor signalling has a well-known role in protecting cell phenotype, specifically in beta cells (31).

Taken together, our study suggests an additional role of TGF- β receptor signalling during beta cell proliferation, which should be taken into consideration when designing strategies for increasing functional beta cell mass to treat diabetes.

Conflict of interest

None disclosed.

Author contributions

Conceived and designed the experiments: CL. Performed the experiments and analysed the data: CL, XZ, YP, YM, XL, ML and JZ. Wrote the paper: CL.

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