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Generation of mouse models for type 1 diabetes by selective depletion of pancreatic beta cells using toxin receptor-mediated cell knockout



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ABSTRACT

By using the toxin receptor-mediated cell knockout (TRECK) method, we have generated two transgenic (Tg) murine lines that model type 1 (insulin-dependent) diabetes. The first strain, C.B-17/Icr-Prkdc^{scid}/Prkdc^{scid}-INS-TRECK-Tg, carries the diphtheria toxin receptor (hDTR) driven by the human insulin gene promoter, while the other strain, C57BL/6-*ins2*(BAC)-TRECK-Tg, expresses hDTR cDNA under the control of the mouse insulin II gene promoter. With regard to the C.B-17/Icr-Prkdc^{scid}/Prkdc^{scid}-INS-TRECK-Tg strain, only one of three Tg strains exhibited proper expression of hDTR in pancreatic β cells. By contrast, hDTR was expressed in the pancreatic β cells of all four of the generated C57BL/6-*ins2*(BAC)-TRECK-Tg strains. Hyperglycemia, severe ablation of pancreatic β cells and depletion of serum insulin were observed within 3 days after the administration of diphtheria toxin (DT) in these Tg mice. Subcutaneous injection of a suitable dosage of insulin was sufficient for recovery from hyperglycemia in all of the examined strains. Using the C.B-17/Icr-Prkdc^{scid}/Prkdc^{scid}-INS-TRECK-Tg model, we tried to perform regenerative therapeutic approaches: allogeneic transplantation of pancreatic islet cells from C57BL/6 and xenogeneic transplantation of CD34⁺ human umbilical cord blood cells. Both approaches successfully rescued C.B-17/Icr-Prkdc^{scid}/Prkdc^{scid}-INS-TRECK-Tg mice from hyperglycemia caused by DT administration. The high specificity with which DT causes depletion in pancreatic β cells of these Tg mice is highly useful for diabetogenic research.

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1. Introduction

Conditional *in vivo* depletion of lineage-specific cells can be achieved through administration of diphtheria toxin (DT) to TRECK-Tg mice that carry a human diphtheria toxin receptor (hDTR) cDNA transgene driven by a tissue-specific promoter. Unlike conditional knockout mice, cell depletion is initiated by DT administration; therefore, the effects of the depletion can be observed in the same Tg strain by comparing cohorts pre- and post-administration of DT [1]. Moreover, the administration of

DT to TRECK-Tg mice causes depletion in hDTR expressing cells and therefore specifically eliminates these cells without eliciting an inflammatory response. We succeeded in generating the first TRECK-Tg mouse model for the specific ablation of hepatocytes in the mouse liver under the control of the albumin promoter [2]. This technique has been successfully applied to generate several models for human diseases [2–4] and has revealed several previously unknown *in vivo* cellular functions [5,6].

Type 1 diabetes (T1D) is a major disease that has garnered considerable attention for both children and young adults. T1D is a severe autoimmune disease, and its onset is known to be caused by an immunological destruction of pancreatic β cells, the insulin-producing islet cells [7]. In humans and mice, the etiology of T1D, including the natural diabetogenic agents [8,9] and pathogenic processes that result in T1D, has not been well characterized [10–12]. By contrast, artificial diabetogenic procedures in mice, e.g., alloxan- or streptozotocin-induced T1D models [13,14], are well established, and these models have been widely used for developing drugs for T1D and for pathogenic studies of diabetes-related complications. However, the onset incidences in both

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models vary with different mouse strains, sex, administration dose, breeding, etc. In particular, sex- [15] and/or strain differences [16–19] strongly influence the incidence of onset. Therefore, it is difficult to establish optimal conditions for the onset of the alloxan- or streptozotocin-induced T1D. Furthermore, side effects of the drugs, including alloxan- or streptozotocin-induced nephropathy [20] and streptozotocin-induced hepatopathy [21,22], are severe and make model generation difficult.

To overcome the drawbacks of drug-induced T1D models, we generated the Tg mice expressing the hDTR under control of the human *INS* and mouse *ins2* promoters by the TRECK method [2]. hDTR is expressed exclusively on the surface of target cells in the TRECK-Tg mice. Without administration of DT, the Tg mice maintain a healthy condition. Once DT is administered to the Tg mice, the lineage of cells expressing hDTR is severely depleted. We have successfully generated two lines of *ins*-TRECK-Tg mice with different vectors or strains. These lines include C.B-17/lcr-*Prkdc*^{scid}/*Prkdc*^{scid} (SCID)-*INS*-TRECK-Tg mice and C57BL/6 (B6)-*ins2*(BAC)-TRECK-Tg mice. All of the Tg mice were hyperglycemic 3 days after DT administration. Immunohistochemical analysis revealed that hyperglycemia was caused by the depletion of pancreatic β cells. Furthermore, hyperglycemia was cured by the administration of insulin. Ultimately, our evidence supports that these TRECK-Tg mice may be used to model T1D.

2. Materials and methods

An expanded Section 2 is in the online data [Supplementary material](#)

2.1. Construction of transgenes

2.1.1. Plasmid vector

Transgene was constructed, as shown previously [2], with the 1.9 kbp human insulin promoter region, rabbit β -globin intron, human HB-EGF L148S/P149T mutant cDNA [23] and the

polyadenylation signals of rabbit β -globin and SV40 (Fig. 1A). The 3.8 kbp DNA fragment was excised by double digestion with *Sph*I and *Xho*I and purified using the QIAquick gel extraction kit (QIAGEN, Valencia, CA, USA) and the Wizard DNA Clean-Up System (Promega, Madison, WI, USA).

2.1.2. BAC vector

A recombinant BAC clone was generated using a recombineering method with *galK* selection [24]. A mouse BAC clone that contained *ins2* (RP23-92L23) was purchased from BACPAC Resources Center, Children's Hospital Oakland Research Institute (Oakland, USA). To perform the first replacement, the *ins2* coding region in RP23-92L23 was replaced by *galK* as previously described to generate BAC/*galK*. Then, the *galK* insert was replaced by the hDTR cDNA to generate BAC/hDTR (Fig. 1B).

2.2. Transplantation of pancreatic islets from C57BL/6 mice

Langerhans islets were prepared from the C57BL/6 pancreas. Briefly, intraductal perfusion of pancreases was performed using HBSS containing 1.5 mg/ml collagenase P (Roche, Indianapolis, USA). Langerhans islets were separated by centrifugation using Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) and isolated by hand picking under a dissecting microscope. Two hundred islets were transplanted beneath the renal capsule.

2.3. Transplantation of human umbilical cord blood-derived cells

We have been approved the ethics committee of The Tokyo Metropolitan Institute of Medical Science (approved no. 17-2-3) and the Tokyo Cord Blood Bank (07-11-02) to use human umbilical cord blood. Mononuclear cells in the cord blood were isolated on Ficoll Paque (GE healthcare, Pittsburgh, USA) and processed further to isolate CD34⁺ cells by using a magnetic cell sorter direct CD34 progenitor cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Seven days

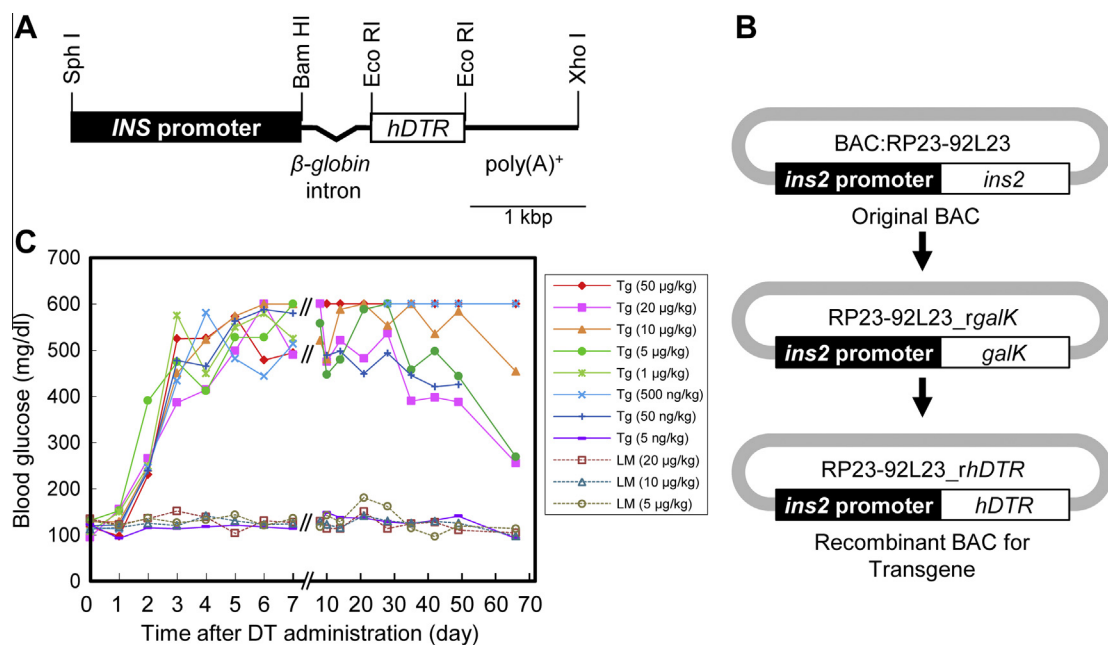


Fig. 1. Generation of a transgene for *ins*-TRECK-Tg mice. (A) Construct of a plasmid-based transgene. The *alb* promoter of the original TRECK cassette was replaced with a *Sph*I/*Bam*HI fragment of the human insulin (*INS*) promoter [2]. (B) Generation of a BAC-based transgene. The DNA fragment from the first exon to the poly(A) signal of a BAC clone carrying the complete mouse *ins2* gene (RP23-92L23) was replaced by the *galK* cassette. The resultant recombinant BAC (RP23-92L23_*galK*) was replaced again by the hDTR cassette to finally obtain a recombinant hDTR BAC. Only the scaffold structures of the BAC clones are shown. (C) Induction of hyperglycemia in SCID-*INS*-TRECK-Tg mice by DT administration. Mice were intraperitoneally administered the indicated doses of DT at day 0. Then, blood glucose levels were measured. Tg and LM stand for transgenic mice and their littermates, respectively. Values over 600 mg/dl, which is the upper limit of the blood glucose meters, were indicated as 600 mg/dl.

after the injection of 1 $\mu\text{g}/\text{kg}$ DT, SCID-INS-TRECK-Tg mice were injected intravenously with a total of $1\text{--}4 \times 10^5$ human cells and were given 50 μl of anti-asialo GM1 antiserum (Wako Pure Chemical Industries, Osaka, Japan) every 11 days to deplete NK cells.

3. Results

3.1. DT-induced hyperglycemia

We obtained three Tg strains by direct injection of the plasmid based TRECK-Tg cassettes into the pronucleus-eggs of SCID mice. Only one of these Tg strains showed the desired phenotype of DT-induced hyperglycemia. Therefore, we carried out further experiments using the SCID-INS-TRECK-Tg #70 strain that displayed a phenotype. By contrast, all of the B6-*ins2*(BAC)-TRECK-Tg strains displayed DT-induced hyperglycemia, although the sensitivity to DT varied among strains. We used #5 strain for further experiments (Table 1). The Tg strains used here will be deposited and available from RIKEN BioResource Center (Tsukuba, Japan).

Dosages of 5 ng/kg, 50 ng/kg, 500 ng/kg, 5 $\mu\text{g}/\text{kg}$, 10 $\mu\text{g}/\text{kg}$, 20 $\mu\text{g}/\text{kg}$, and 50 $\mu\text{g}/\text{kg}$ of DT was administered to SCID-INS-TRECK-Tg mice, and their littermates (non-Tg mice) were given 5 $\mu\text{g}/\text{kg}$, 10 $\mu\text{g}/\text{kg}$, and 20 $\mu\text{g}/\text{kg}$ of DT. Hyperglycemia was induced in Tg mice administered over 50 ng/kg of DT. Non-Tg mice did not display any elevation of blood glucose levels. Blood glucose was elevated 2 days after DT administration and reached a higher level (>600 mg/dl) than the upper limit of the blood glucose meters by 7 days. The elevation of blood glucose over the threshold for hyperglycemia (200 mg/dl) was maintained for 65 days after DT administration; however, little dose-dependency was observed (Fig. 1C). All Tg mice administered over 50 ng/kg of DT were hyperglycemic with blood glucose levels >200 mg/dl by 2 days after DT administration, and their blood glucose levels were maintained at >400 mg/dl for 50 days after DT administration.

3.2. Histological and immunohistological analysis of pancreatic islets

Little or no morphological changes were observed with the staining pancreatic islets from Tg mice with HE in comparison to non-Tg mice (Fig. 2Aa and Ab). However, hDTR signals were observed in the Tg mice when the pancreas of the Tg and non-Tg mice were immunostained with anti-hDTR, the molecular nature and *in vivo* function of which is the heparin-binding epidermal growth factor (HB-EGF) (Fig. 2Ac and Ad). Pancreatic islets from Tg mice were also immunostained with anti-insulin and anti-glucagon antibodies (Fig. 2B). In the pancreatic islets of Tg mice without DT administration, insulin-producing β cells preferentially occupied in the islets. By contrast, administration of DT caused a drastic decrease in the number of β cells, although the DT-induced depletion of β cells was not complete. These results suggest that the

Table 1
Summary of T1D phenotypes in C57BL/6-*ins2*(BAC)-TRECK-Tg mouse strains.

Strain no.	5	14	17	33
DT dose ($\mu\text{g}/\text{kg}$)	1	1	100	1
Plasma glucose (mg/dl)	>600	>600	400–500	>600
Glycosuria	+++	+++	++	+++
Ketonuria	+++	+++	+	+++
Insulin dependency	+++	+++	+++	+
Blood insulin level	Low	ND	ND	ND
Islet lesion	+++	+++	++	+++

ND, not done.

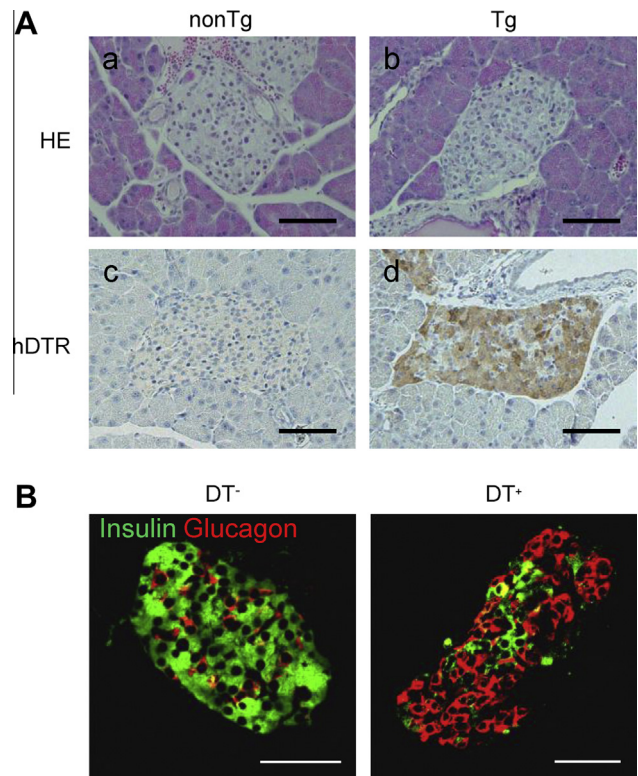


Fig. 2. Immunohistochemical study of the islets of SCID-INS-TRECK-Tg and WT mice. (A) Hematoxylin-eosin staining (a and b) and immunostaining with an anti-hDTR antibody (c and d). Panels a and c are pancreases of non-Tg mice and b and d are those of Tg mice. Scale bars represent 100 μm . (B) Immunohistochemical staining of pancreatic islets. The left panel is an islet from Tg mice that was not administered DT and the right panel is after 7 days of administration of 5 $\mu\text{g}/\text{kg}$ of DT. The sections of pancreas were stained by anti-insulin (green) and anti-glucagon (red) antibodies, respectively. Scale bars represent 50 μm .

DT-induced hyperglycemia is caused by depletion of pancreatic β cells and consequently the deprivation of plasma insulin.

The morphology of pancreatic islets was not changed as indicated by HE staining (Fig. 2A). However, morphological abnormality was observed when the islets were immunostained with anti-insulin and anti-glucagon antibodies (Fig. 2B). Although β cells are surrounded by α cells in the islets of non-Tg mice (data not shown), this structure was irregularly arranged in Tg mice. This morphological abnormality was also observed in all *INS*-TRECK-Tg mice and *ins2*(BAC)-TRECK-Tg mice. The reason for this abnormality is currently unknown.

3.3. Decrease of plasma insulin and absence of glucose-induced recovery of plasma insulin levels

The level of plasma insulin was markedly decreased by 5 days after the administration of DT to the Tg mice. The serum insulin level was less than 2 ng/ml in Tg mice without DT administration and non-Tg with/without DT administration. The insulin level of Tg mice reached 0.26 ng/ml at 5 days after DT administration. This level was not increased when 2 g/kg glucose was orally administered. By contrast, DT administration did not affect the serum insulin level of non-Tg mice, and the non-Tg mice demonstrated a remarkably increased plasma insulin level of approximately 9 ng/ml 10 min after glucose challenge (Fig. 3A). This result showed that pancreatic β cells were functionally depleted by DT administration, which is consistent with the morphological evidence that β cells were depleted by the administration of DT to Tg mice (Fig. 2B).

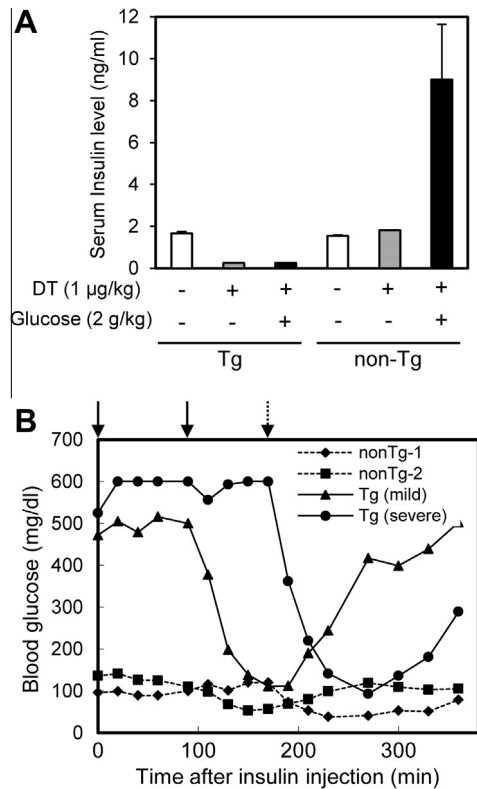


Fig. 3. Insulin-dependent hyperglycemia in SCID-INS-TRECK-Tg mice. (A) SCID-INS-TRECK-Tg mice and non-Tg littermates were administered 1 µg/kg of DT or PBS. After 5 days, mice were orally administered 2 g/kg glucose. Serum insulin levels were measured before and after 10 min of glucose treatment. (B) Tg mice and non-Tg littermates were administered 5 µg/kg DT. After 7 days, mice were injected with super short-acting insulin at doses of 400 mU/kg at 0 min and 4 U/kg at 100 min. Mouse with severe hyperglycemia was injected with an additional 40 U/kg at 180 min. Arrows with a straight line indicate the time of injection and the arrow with a dotted line shows the third injection time point for Tg mice with severe hyperglycemia.

3.4. Hyperglycemia is cured by insulin injection

The blood glucose levels of Tg mice with DT-induced hyperglycemia were temporally decreased with injection of super

short-acting insulin; however, the efficacy of the insulin action was dependent on the severity of hyperglycemia. The first injection of 400 mU/kg insulin at 0 min did not cause any effect in both mild and severe cases. The second injection of 4 U/kg at 100 min caused a clear decrease of blood glucose in mice with mild hyperglycemia within 30 min of the injection but did not cause a decrease blood glucose levels in mice with severe hyperglycemia. The third injection of 40 U/kg at 180 min was sufficient to decrease blood glucose levels within 30 min of the injection. The reversion of hyperglycemia started approximately 60 min after the insulin injection in both mild and severe cases of hyperglycemia. In both cases, blood glucose levels were rapidly elevated to an abnormal level (Fig. 3B). This result confirmed that the hyperglycemia was induced by deprivation of plasma insulin caused by β cell depletion (see Fig. 2B).

3.5. Hyperglycemia is cured by allogeneic transplantation of mouse pancreatic islets and by xenogeneic transplantation of human umbilical cord blood-derived cells

The DT-induced SCID-INS-TRECK-Tg hyperglycemic mice were given the pancreatic islets of C57BL/6 via transplant beneath the renal capsule. The blood glucose levels of these mice decreased and reached normal levels (<200 mg/dl) 5 weeks after DT-administration (Fig. 4A). This normal blood glucose level was maintained for more than 15 weeks. DT-treated Tg mice that did not receive islet-transplantation maintained high glucose levels (>600 mg/dl). This result indicated that the islet transplantation restored β cell function in DT-treated Tg mice. Consistently, immunohistochemical analysis clearly showed that β cells within the transplanted tissues generated insulin (Fig. 4B).

Then, we performed xenogeneic transplantation of human umbilical cord blood-derived cells to the SCID-INS-TRECK-Tg mice treated with DT. Human umbilical cord blood-derived cells were divided into two groups: CD34⁺ and CD34⁻ cells. CD34⁺ cells contain stem cells that replenish pancreatic β cells [25,26]. Our preliminary results demonstrated that the transplantation of CD34⁺ cells recovered blood glucose to normal levels 70 days after transplantation while the administration of CD34⁻ cells did not cause such an effect (Fig. 4C). This result indicated that the transplanted CD34⁺ cells were able to differentiate into pancreatic β cells that secreted insulin or that some factor(s) produced by the CD34⁺ cells stimulated pancreatic β cell differentiation from its stem cells. This should be clarified by further experiments.

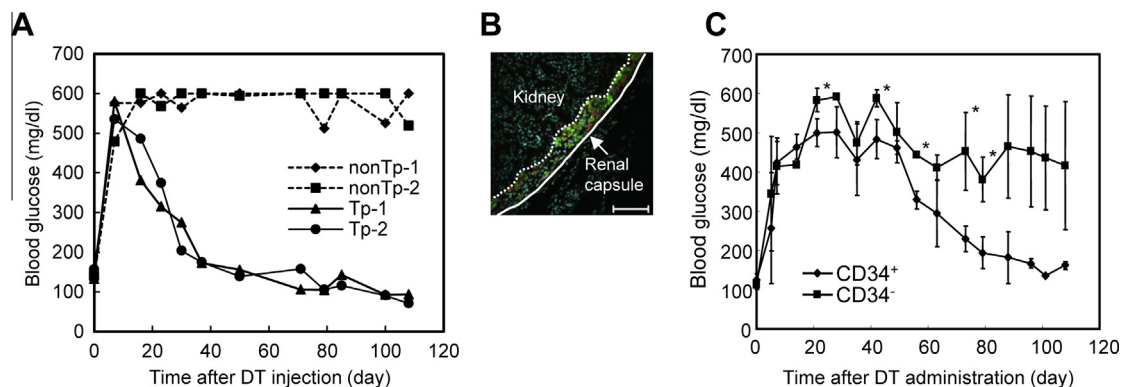


Fig. 4. Hyperglycemia is cured by allogeneic and xenogeneic transplantation therapy. (A) Mice were transplanted with 200 islets from non-diabetic C57BL/6 inbred mice 7 days after administration of 5 µg/kg of DT. The blood glucose levels of mice with transplanted islets (solid lines) and control mice that did not receive transplants (dashed lines) were measured at the indicated time points. Values over 600 mg/dl, which is the upper limit of the blood glucose meters, were indicated as 600 mg/dl. (B) The kidney section was stained with antibodies against insulin (green) and glucagon (red) and counterstained with DAPI (blue). Scale bars represent 100 µm. (C) CD34⁺ or CD34⁻ cells fractionated from human umbilical cord blood cells were transferred intravenously into Tg mice 7 days after DT administration. Blood glucose levels were measured at the indicated time points. Data have been expressed as mean \pm SD. Statistical significance was determined by Student's *t* test. Asterisks represent statistical significance ($p < 0.05$).

4. Discussion

We have generated two co-isogenic lines of *ins*-TRECK-Tg mice. All of the lines developed hyperglycemia within 2 days of DT administration (Fig. 1C). It is evident that hyperglycemia is caused by the destruction of pancreatic β cells, which results in the severe depletion of serum insulin (Figs. 2B and 3A). Furthermore, subcutaneous injection of recombinant insulin can cure hyperglycemia when administered at sufficient doses (Fig. 3B). An evident difference between Tg and non-Tg mice is their pancreatic morphology. Namely, normal pancreatic islets consisted of approximately 80% β cells surrounded by 20% of other cells, including α cells, while the islets of Tg mice did not show such a systematic morphology; rather, the α and β cells were randomly mixed and constructed (Fig. 2B). This abnormality was observed in other *ins*-TRECK-Tg mice, and we speculate that the growth hormone-like activity of hDTR affects the pancreatic morphology of Tg mice. This possibility will be proven by the use of mutated hDTR that lacks growth hormone-like activity [23].

The decrease of blood glucose levels observed in SCID-*INS*-TRECK-Tg mice 30 days after DT administration may be caused by transdifferentiation of β cells according to the findings of Thorel et al., who showed that β cells were transdifferentiated from α cells after 1 month of DT administration when β cells were severely ablated [4].

SCID mice can be transplanted with allograft and xenograft, and thus are suitable for the development of regenerative therapies. To establish this therapy for T1D, we performed allograft and xenograft transplantation for DT-treated SCID-*INS*-TRECK-Tg mice to cure hyperglycemia. When pancreatic islet cells were transplanted beneath the renal capsule, the hyperglycemia of SCID-*INS*-TRECK-Tg mice recovered 4–5 weeks after transplantation (Fig. 4A) while SCID-*INS*-TRECK-Tg mice without transplantation maintained hyperglycemia for 15 weeks. We confirmed that the transplants were engrafted in the subrenal capsule and contained functional pancreatic β cells (Fig. 4B). This result clearly demonstrated that engrafted β cells produced sufficient insulin to recover hyperglycemia in SCID-*INS*-TRECK-Tg mice and strongly suggests that blood insulin levels were elevated.

Furthermore, we succeeded in curing hyperglycemia in SCID-*INS*-TRECK-Tg mice through transplantation of CD34⁺ human umbilical cord blood cells, which may contain stem cells for pancreatic β cells [27,28]. We speculate that engrafted CD34⁺ human umbilical cord blood cells differentiated into pancreatic β cells in the SCID-*INS*-TRECK-Tg mice and then secreted insulin into the blood or that some factor(s) produced by the CD34⁺ human umbilical cord blood cells stimulated to the differentiation of its stem cell population into pancreatic β cells or the transdifferentiation of α cells to β cells. The direct detection of human insulin in the mouse serum will prove which of these processes are at play, but this will be difficult because the amino acid sequence of insulin has little differences between humans and mice [29,30].

The reliance of BAC vector was, as expected, much better than that of the plasmid vector. For example, one out of three of the SCID-*INS*-TRECK-Tg mouse strains correctly expressed hDTR in pancreatic β cells, while the other two strains ectopically expressed the receptor and consequently died within 2 days after DT administration. By contrast, all four B6-*ins*2(BAC)-TRECK-Tg mouse strains correctly expressed hDTR in β cells, although sensitivity to DT differed among the strains. To carry out BAC-transgenesis, we directly injected a supercoiled BAC clone into pronucleus-stage eggs, and circular BAC clones were randomly cut and integrated into nuclear genomes. However, the cut position seems hardly to affect with reliance of transgene expression. Thus, it is evident that BAC-transgenesis is superior to plasmid-transgenesis (Table 1).

As mentioned in Figs. 1C and 4A,C SCID-*INS*-TRECK-Tg mice lived for more than two months without insulin treatment. The long survival of SCID-*INS*-TRECK-Tg mice has a great advantage for research concerning the regeneration or recovery of β cell functions.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.05.114>.

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