Transplantation of PC1/3-Expressing α-cells Improves Glucose Handling and Cold Tolerance in Leptin-resistant Mice

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Type 2 diabetes (T2D) is characterized by elevated blood glucose levels owing to insufficient secretion and/or activity of the glucose-lowering hormone insulin. Glucagon-like peptide-1 (GLP-1) has received much attention as a new treatment for diabetes because of its multiple blood glucose–lowering effects, including glucose-dependent enhancement of insulin secretion, inhibition of gastric emptying, and promotion of the survival and growth of insulin-producing β-cells. GLP-1, along with GLP-2 and oxyntomodulin, is produced in the intestinal L-cell via processing of proglucagon by prohormone convertase 1/3 (PC1/3), while in the pancreatic α-cell, coexpression of proglucagon and the alternate enzyme PC2 typically results in differential processing of proglucagon to yield glucagon. We used alginate-encapsulated α-cells as a model to evaluate continuous delivery of PC1/3- or PC2-derived proglucagon products. In high fat–fed and *db/db* mice, PC1/3-, but not PC2-expressing α-cells improved glucose handling and transiently lowered fasting glucose levels, suggesting that continuous delivery of PC1/3-derived proglucagon products via cell therapy may be useful for diabetes treatment. In addition, we show that long-term treatment with PC1/3-expressing, but not PC2-expressing, α-cells improved cold-induced thermogenesis in *db/db* mice, demonstrating a previously unappreciated effect of one or more PC1/3-derived α-cell products.

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INTRODUCTION

Proglucagon, which is expressed in pancreatic α-cells, intestinal L-cells, and in specific neurons in the central nervous system, is differentially processed to yield numerous peptides belonging to the glucagon superfamily of hormones.[1] Although members of this superfamily share significant peptide sequence homology, they have diverse and sometimes opposing regulatory functions. In the pancreatic α-cells, prohormone convertase (PC2) is the predominant processing enzyme, with the result that the major bioactive proglucagon-derived peptide (PGDP) arising in these cells is glucagon,[2,3] a glucose-raising hormone opposing insulin action. While glucagon activity is crucial for preventing hypoglycemia under normal conditions, hyperglucagonemia accompanies and may exacerbate type 2 diabetes (T2D).[4]

In contrast to the α-cell, in the enteroendocrine L-cell and the brain, PC1/3 is the predominant processing enzyme acting on proglucagon, liberating glucagon-like peptide-1 (GLP-1), GLP-2, and oxyntomodulin.[5,6] GLP-1 has garnered significant therapeutic interest for the treatment of diabetes because of its pleiotropic blood glucose–lowering effects, including enhancement of glucose-stimulated insulin secretion, inhibition of gastric emptying and glucagon secretion, and promotion of β-cell proliferation and survival.[1,7] GLP-2 and oxyntomodulin also tend to lower blood glucose levels by promoting satiety and inhibiting gastric emptying.[8,9] Thus proglucagon, depending on whether it is processed by PC2 or PC1/3, gives rise to products that tend to have either glucose-raising or glucose-lowering effects.

We have previously shown that expression of PC1/3 rather than PC2 in α-cells induces GLP-1 production and converts the α-cell from a hyperglycemia-promoting cell to one that lowers blood glucose levels and promotes islet survival in rodent models of type 1 diabetes.[10,11] Here we have examined the metabolic effects of transplanting encapsulated PC1/3-expressing α-cells in rodent models of T2D, and demonstrate that cell therapy with PC1/3-expressing α-cells improves glucose handling and improves cold thermogenesis in leptin-resistant mice.

RESULTS

We and others have previously reported that αTCΔPC2 cells express PC1/3, but no functional PC2, and produce GLP-1.[10,12] We found that compared to PC2-expressing αTC-1 cells, αTCΔPC2 cells secrete higher levels of immunoreactive GLP-2 (255.8 ± 20.6 ng/ml versus 129.2 ± 17.0 ng/ml; $n = 2$) and oxyntomodulin (50.0 ± 5.7 ng/ml versus 27.7 ± 1.5 ng/ml; $P < 0.01$; $n = 4$; data not shown). In this study, we used encapsulated αTCΔPC2 cells to assess the efficacy of cell therapy with PC1/3-expressing α-cells in mouse models of T2D. We transplanted 40×10^6 encapsulated

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cells to the intraperitoneal (IP) cavity of 16-week-old C57BL/6 mice that had been fed a 60% kcal from fat diet from 4 weeks of age. Cell transplantation had no effect on body weight (**Figure 1a**), and we could not detect any changes in food intake (data not shown). However, αTCΔPC2 recipients had lower fasted blood glucose levels than sham-operated controls at two posttransplant time points (**Figure 1b**; 8.8 ± 0.3 mmol/l versus 10.4 ± 0.5 mmol/l at day 4, $P < 0.05$; 9.8 ± 0.3 mmol/l versus 11.0 ± 0.3 mmol/l at day 13, $P < 0.05$). An IP glucose tolerance test performed 7 days after transplant revealed that αTCΔPC2 recipients had better glucose tolerance than controls, as indicated by a more rapid lowering of blood glucose levels (**Figure 1c**; $P < 0.001$ at 60 minutes and $P < 0.05$ at 120 minutes) and an overall decrease in glycemic excursion (**Figure 1c** inset; $P < 0.01$). Plasma insulin levels were not significantly different before glucose injection (1.10 ± 0.12 µg/l for cell transplant recipients versus 0.52 ± 0.10 µg/l for sham-operated controls at time 0; $P = 0.12$; $n = 5–6$) but cell transplant recipients tended to have higher plasma insulin levels 7 minutes after glucose injection (1.47 ± 0.30 µg/l versus 0.78 ± 0.13 µg/l for sham; $P = 0.08$; $n = 5–6$).

As αTCΔPC2 cells improved glycemia in the high fat–fed model of glucose intolerance, we hypothesized that transplantation of αTCΔPC2, but not αTC-1 cells might delay the onset of diabetes in the *db/db* mouse model of T2D. We did not detect any impact of cell transplant on either body weight or food intake (data not shown). Although αTCΔPC2 cell transplant initially lowered fasting blood glucose levels after transplant (6.6 ± 0.2 mmol/l versus 7.7 ± 0.3 mmol/l at 2 days after transplant; $P < 0.01$), blood glucose levels were not different thereafter and both groups of animals developed diabetes at a similar rate (**Figure 2a**). In contrast, αTC-1 recipients had elevated blood glucose levels compared to sham-operated controls at several time points (**Figure 2b**; 18.7 ± 1.8 mmol/l versus 12.7 ± 1.9 mmol/l at day 2, $P < 0.05$; 27.1 ± 0.9 mmol/l versus 20.1 ± 2.5 mmol/l at day 20, $P < 0.05$). To determine whether αTCΔPC2 cell transplant influenced body composition in *db/db* mice, we performed nuclear magnetic resonance imaging 17 days after transplant. αTCΔPC2 recipients were found to have a slight but statistically significant increase in the ratio of lean:lipid body mass (**Figure 2c**; $P < 0.05$) without any change in total body mass (data not shown), indicating that a product from the αTCΔPC2 cell transplants decreased fat mass and/or increased lean mass in these mice.

Despite their only transient lowering of fasted glucose levels, αTCΔPC2 cells profoundly improved glucose tolerance 7 days after transplant, with cell-treated mice having lower blood glucose levels at each time point (**Figure 3a**; $P < 0.05$ at 0 minutes, $P < 0.01$ at 7, 60, and 120 minutes, and $P < 0.001$ at 15 minutes) and a 40% reduction in total glycemic excursion compared to sham-operated controls (**Figure 3a** inset; $P < 0.01$). In contrast, at 4 days after transplant, αTC-1 recipients had no improvement in glucose tolerance compared to sham-operated controls (**Figure 3c**). Indeed blood glucose levels were actually increased at several time points (11.5 ± 2.2 mmol/l versus 5.8 ± 1.0 mmol/l at

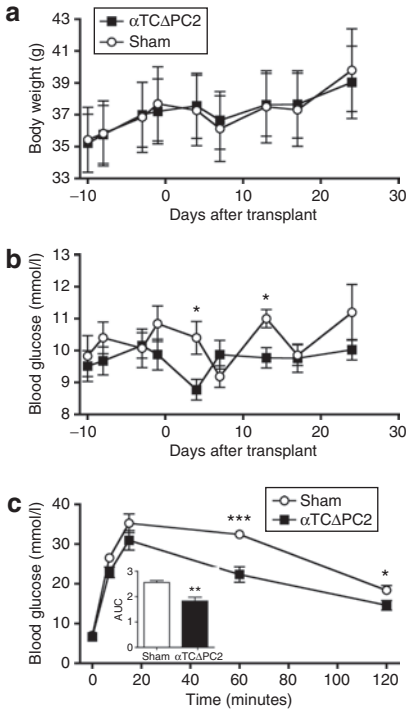

Figure 1 Transplantation of encapsulated prohormone convertase 1/3–expressing α-cells improves glucose homeostasis in a mouse model of diet-induced obesity and hyperglycemia. Sixteen-week-old male mice fed a 60% fat diet from 4 weeks of age received sham surgery or cell transplant on day 0 ($n = 8$ per condition). (**a**) Body weight and (**b**) blood glucose were monitored after a 4-hour morning fast. (**c**) An intraperitoneal glucose tolerance test (2 g/kg) was performed 7 days after transplant. AUC, area under the curve. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to sham.

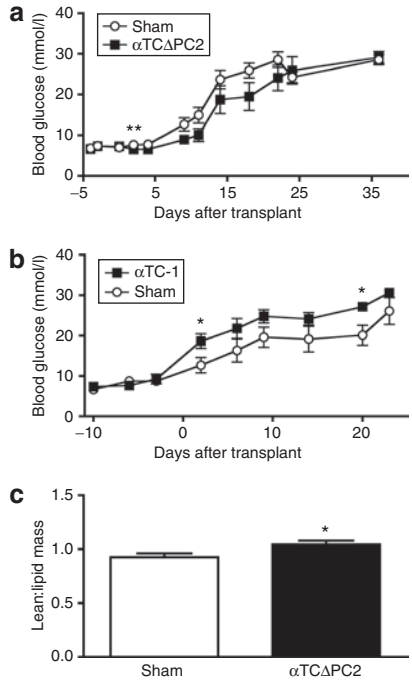

Figure 2 Metabolic parameters in *db/db* mice receiving prohormone convertase 1/3 (PC1/3)-expressing or PC2-expressing α-cell therapy. Blood glucose was measured followed a 4-hour morning fast in mice receiving (**a**) αTCΔPC2 or (**b**) αTC-1 cell transplants or sham surgery. (**c**) Lean-to-lipid body mass ratio was determined by nuclear magnetic resonance imaging 17 days after transplant. *$P < 0.05$, **$P < 0.01$ compared to sham; $n = 5–6$ per group.

0 minutes; 17.8 ± 2.3 mmol/l versus 10.8 ± 2.0 mmol/l at 120 minutes; $P < 0.05$; $n = 6$) and there was no change in overall glycemic excursion (**Figure 3c** inset). As has been reported previously,[13] *db/db* mice were hyperinsulinemic even after an overnight fast and had defective glucose-stimulated insulin secretion in the IP glucose tolerance test. However, αTCΔPC2 cell recipients displayed lower fasting insulin levels (2.13 ± 0.28 μg/l versus 3.47 ± 0.43 μg/l for sham-operated controls; $P < 0.05$) and a re-establishment of insulin secretion in response to glucose injection (**Figure 3b**; $P < 0.05$ at 7 and 15 minutes, $P < 0.01$ at 60 minutes). By 28 days after transplant the beneficial impact of αTCΔPC2 cells on glucose tolerance was no longer evident, except for a slight lowering of blood glucose levels at the 15-minute time point (**Figure 3d**; $P < 0.05$ at 7 minutes). Plasma GLP-1 levels were assessed in a blood sample taken 14 days after transplant, and although GLP-1 was below the level of detection (~20 pmol/l) in all but one sham-operated mouse, αTCΔPC2 recipients had plasma GLP-1 levels of 71.3 ± 9.2 pmol/l ($n = 6$).

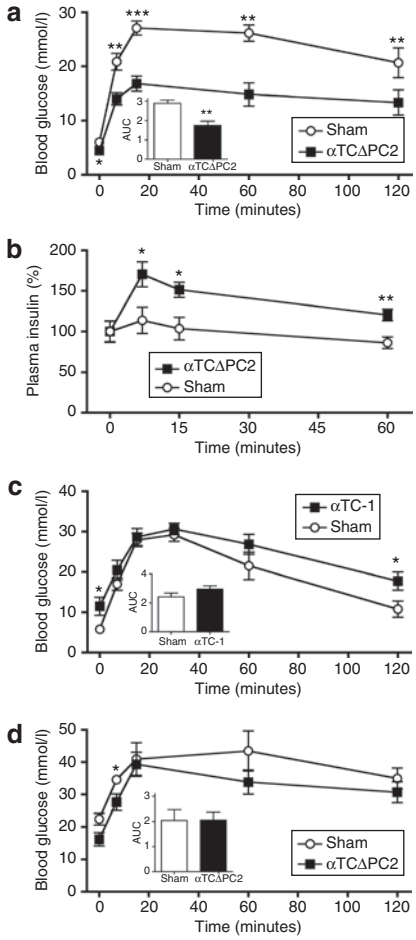

Figure 3 Glucose homeostasis in *db/db* mice receiving prohormone convertase 1/3 (PC1/3)-expressing or PC2-expressing α-cell therapy. Intraperitoneal glucose tolerance tests (IPGTTs) (2 g/kg) were performed following an overnight fast 7 days (**a**, **b**), 4 days (**c**), or 28 days (**d**) after transplantation of αTCΔPC2 (**a**, **b**, **d**) or αTC-1 (**c**) cells. AUC, area under the curve. For the day 7 IPGTT, plasma was collected at the indicated time points and assayed for insulin (**b**), expressed as % increase compared to basal. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared to sham; $n = 5$–6 per group.

Mice with genetic defects in the leptin receptor signaling pathway (*e.g.*, *db/db* mice) are known to have lower body temperature in both the light and dark periods, as well as defective cold-induced thermogenesis, compared to littermate controls.[14] To assess whether α-cell products could improve body temperature regulation in this model, we implanted subcutaneous temperature sensors in *db/db* mice that had received αTCΔPC2 or αTC-1 cell transplant, or sham surgery. At 29 days after transplant, αTCΔPC2 recipients had a remarkable improvement in their ability to maintain their body temperature during acute housing in a 4 °C environment, with higher body temperature at almost every time point (**Figure 4a**; $P < 0.05$ at 20, 75, 90, and 105 minutes; $P < 0.01$ at 45, 60, and 120 minutes). In contrast, αTC-1 recipients had no improvement in their ability to maintain their body temperature compared to control animals at 30 days after transplant (**Figure 4b**).

To further characterize the beneficial effects of αTCΔPC2 cell transplant on body temperature regulation, additional cell transplants were performed in *db/db* mice and sham-operated wild-type C57BLKS/J controls. As in our previous study, transplantation of αTCΔPC2 cells did not delay diabetes onset in *db/db* mice, although transplant recipients displayed improved glucose tolerance 7 days after transplant, with lower blood glucose levels at several time points and a 45% decrease in area under the curve compared to sham-operated *db/db* mice (data not shown). In this second cohort of mice, we performed an initial cold tolerance test 5 days after transplant, and found no difference between sham-operated and αTCΔPC2-transplanted *db/db* mice, though both groups had an impaired ability to maintain their body temperature compared to wild-type controls (**Figure 5a**). However, by 26 days after transplant, *db/db* αTCΔPC2 cell recipients were better able to maintain their body temperature during cold exposure

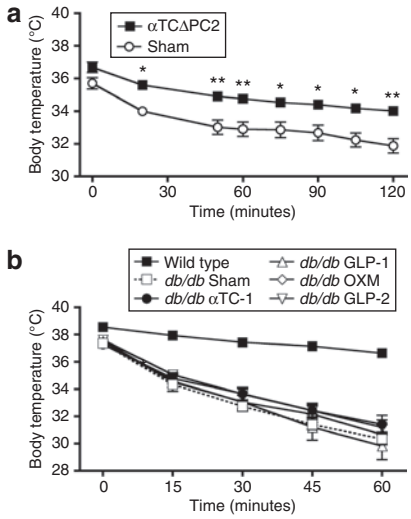

Figure 4 Cold-induced thermogenesis in *db/db* mice receiving prohormone convertase 1/3 (PC1/3)-expressing or PC2-expressing α-cell therapy or continuous infusion of a proglucagon-derived peptide. Cold tolerance tests were performed 29 days after transplantation of αTΔPC2 cells (**a**) or 30 days after transplantation of αTC-1 cells or implantation of a miniosmotic pump loaded with glucagon-like peptide-1 (GLP-1), GLP-2, or oxyntomodulin (OXM; **b**). Mice were housed at 4 °C and body temperature was monitored using a hand-held transponder ($n = 5$–6 per group). *$P < 0.05$, **$P < 0.01$ compared to sham.

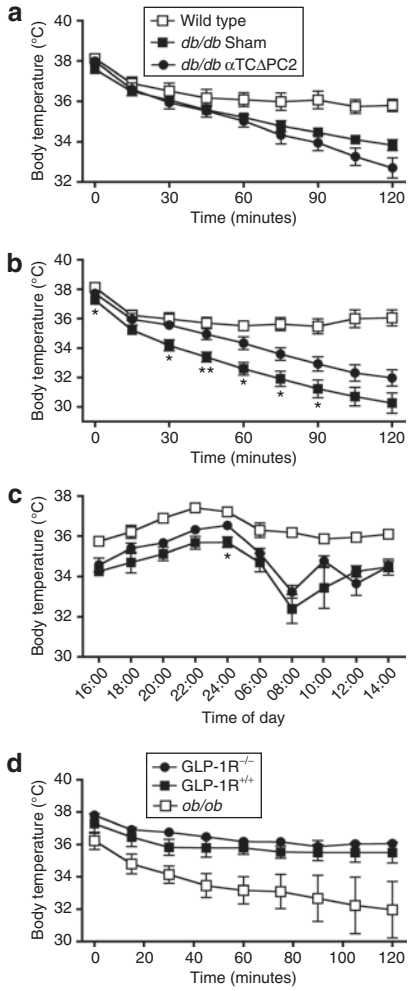

Figure 5 Cold tolerance and diurnal body temperature in *db/db* mice receiving prohormone convertase 1/3 (PC1/3)-expressing α-cell therapy. Mice were individually housed for 2 hours at 4 °C 5 days (**a**) and 26 days (**b**) after transplant. (**c**) Diurnal body temperature was measured in mice housed individually at room temperature. (**d**) Cold tolerance was assessed in GLP-1R$^{+/+}$ and GLP-1R$^{-/-}$ mice. *P < 0.05, **P < 0.01 for *db/db* sham versus *db/db* αTCΔPC2 transplant; n = 7–8 (**a**–**c**) or n = 3–5 (**d**) per group. GLP-1, glucagon-like peptide-1.

than *db/db* controls (Figure 5b; P < 0.05 at 0, 30, 60, 75, and 90 minutes; P < 0.01 at 45 minutes), though they still had lower body temperatures than wild-type controls toward the end of the cold exposure period. We monitored changes in body temperature over a 24-hour period in a cohort of singly housed mice, and found that at all time points, both groups of *db/db* mice had lower body temperature than wild-type controls. However, αTCΔPC2 recipients tended to have higher body temperatures than sham-operated *db/db* mice, particularly during the dark phase (Figure 5c; P < 0.05 at 24:00 hours; P = 0.011 for entire 24-hour period using paired Student's t-test).

Our observation that αTCΔPC2 cells improved body temperature regulation in *db/db* mice led us to hypothesize that one or more of the PC1/3-derived proglucagon products might have a role in body temperature regulation. We reasoned that if GLP-1 were involved in maintenance of body temperature, then mice with ablation of the GLP-1R might have impaired cold tolerance. However, a 2-hour cold tolerance test revealed no difference in body temperature between GLP-1R$^{-/-}$ and GLP-1R$^{+/+}$ mice (aged 10–12 weeks), and both groups were better able to thermoregulate than *ob/ob* control mice (Figure 5d). We also implanted IP miniosmotic pumps infusing GLP-1$_{7\text{-}36NH2}$, GLP-2, or oxyntomodulin into *db/db* mice, and tested cold thermogenesis 30 days after implantation. We detected no difference in the cold thermogenic ability of mice receiving any of the peptides compared to control mice (Figure 4b).

Wild-type mice and αTCΔPC2-transplanted or sham-operated *db/db* mice were killed 34 days after transplant and tissues were collected for analysis. As expected, *db/db* mice had greater epididymal white adipose tissue (WAT) mass (Figure 6a; P < 0.001 for both groups versus wild type) and intrascapular brown adipose tissue (BAT) mass (Figure 6b; P < 0.001 for sham and P < 0.05 for αTCΔPC2 recipients versus wild type) than did wild-type controls, though the mass of these fat depots did not differ between sham-operated and cell-transplanted *db/db* mice. Although both groups of *db/db* mice had larger adipocytes in the WAT depot and larger lipid droplets in the adipocytes in the BAT depot compared to wild-type controls, we were not able to detect any difference between the groups of *db/db* mice (Figure 6c and data not shown). Using western blot analysis, we could not detect any difference in uncoupling protein 1 (UCP-1) protein levels in the BAT depot of sham- versus αTCΔPC2-treated *db/db* mice (Figure 6d,e).

DISCUSSION

In this study, we sought to use cell therapy to evaluate the consequences of continuous delivery of either PC1/3- or PC2-derived α-cell products in mouse models of T2D. Unlike prototypical α-cells which express PC2, αTCΔPC2 cells are derived from mice lacking bioactive PC2 and therefore the capacity for glucagon production, but express high levels of PC1/3.[10,12] We have previously shown that αTCΔPC2 cells improve glucose handling in normal mice, and prevent streptozotocin-induced hyperglycemia, effects that appeared to be primarily attributable to the secretion of GLP-1 from the transplanted cells.[10] Here we selected αTCΔPC2 cells and αTC-1 cells to evaluate continuous cell therapy with either PC1/3-expressing or PC2-expressing α-cells in T2D.

Alginate encapsulation protects transplanted cells from immune attack and has been used for long-term maintenance of transplanted islets in models of diabetes.[15,16] We used encapsulation to evaluate long-term therapy with PC1/3- or PC2-expressing α-cells in mice without the need for immunosuppression or use of immunocompromised recipients. Capsules remained interspersed throughout the IP cavity of recipients, though beyond ~3–4 weeks after transplant capsules occasionally hardened and clumped together. However, it has been previously demonstrated that plasma GLP-1 levels remain elevated in C57BL6 mice 30 days after transplantation of encapsulated αTCΔPC2 cells,[10] and in the current study plasma glucagon levels remained elevated 36 days after transplantation of encapsulated αTC-1 cells in *db/db* mice (data not shown). Taken together these data suggest that the encapsulated cells remained functional throughout the duration of our studies.

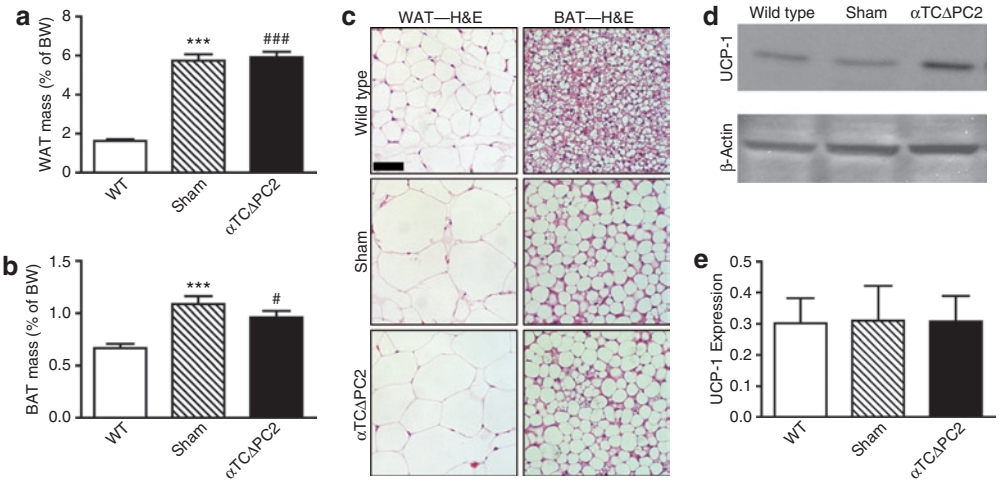

Figure 6 Brown adipose tissue (BAT) and white adipose tissue (WAT) morphology in *db/db* mice receiving prohormone convertase 1/3 (PC1/3)-expressing α-cell therapy. Intrascapular BAT and epididymal WAT were collected 34 days after transplant. (**a**,**b**) WAT and BAT mass as a % of body weight. (**c**) Representative hematoxylin and eosin–stained images of WAT and BAT (scale bar = 120 µm). (**d**,**e**) Western blot showing uncoupling protein 1 (UCP-1) and β-actin levels. Blots are representative of three to five experiments. UCP-1 expression is expressed as pixel intensity and is normalized for blot background and for β-actin expression. ***,###$P < 0.001$ versus wild type; #$P < 0.05$ versus wild type; $n \geq 4$ per group.

In both high fat–fed mice and *db/db* mice, αTCΔPC2 cells improved glucose tolerance and modestly, if transiently, reduced fasting blood glucose, while treatment of *db/db* mice with αTC-1 cells increased fasted glucose levels and worsened glucose tolerance. In high fat–fed mice, αTCΔPC2 cells increased insulin output in response to glucose, consistent with the known insulinotropic effect of GLP-1.[17] Moreover, in *db/db* mice αTCΔPC2 cells reduced fasting insulin levels and restored glucose-stimulated insulin secretion. However, αTCΔPC2 cell transplant was unable to delay the onset of overt diabetes in *db/db* mice, and had minimal impact on glucose tolerance by 1 month after transplant. We initially suspected that chronically elevated blood glucose levels in *db/db* mice might have limited secretion from the α-cell transplants. However, in contrast to the native α-cell, which increases secretion of glucagon in response to low circulating glucose concentrations,[18] *in vitro* experiments showed that αTCΔPC2 cells were unresponsive to changes in media glucose concentration (data not shown). This suggests that hyperglycemia itself probably did not limit the release of GLP-1 and other PC1/3-derived proglucagon products from the cell transplants. In rodents, however, chronic hyperglycemia may induce downregulation of the GLP-1R.[19] It is therefore possible that as blood glucose control worsened, *db/db* mice gradually became insensitive to the effects of transplant-derived GLP-1.

Interestingly, despite the known anorectic effects of GLP-1[20,21] and oxyntomodulin,[22] we were unable to detect any changes in food intake or body weight in *db/db* mice receiving αTCΔPC2 cell transplant compared to sham surgery. It is possible that the sensitivity of our methods was insufficient to observe changes in food intake. An alternative possibility is that the pathways mediating these effects were desensitized because of chronic receptor stimulation, although there is little evidence to support the notion that chronic GLP-1R agonism induces desensitization in rodents[23] or humans.[24] Leptin normally acts via the long form of the leptin receptor (ObRb) to indicate the status of adipose tissue stores to the central nervous system. In *db/db* mice, the ablation of ObRb and loss of this signaling axis renders these mice in a perceived state of negative energy balance, resulting in massive hyperphagia and obesity, decreased energy expenditure, and suppression of energy-intensive activities such as reproduction and growth.[25,26] It is likely that αTCΔPC2 cell transplant was simply unable to overcome this "energy conservation" phenotype in *db/db* mice for more than a short period.

Loss of leptin receptor signaling in mice leads to abnormal thermoregulation and decreased capacity for adaptive thermogenesis.[14] This is thought to relate to decreased activation of leptin-sensitive sympathetic nerves innervating BAT.[27,28] In accordance with previous observations,[14] in our study *db/db* mice displayed lower body temperatures than wild-type mice, although the amplitude and phase of their diurnal body temperature fluctuations mimicked those of wild-type mice. Unexpectedly, we observed that short-term exposure to 4 °C unmasked an improvement in the thermogenic ability of *db/db* mice bearing an αTCΔPC2 cell transplant, although this was evident only after several weeks of exposure to transplanted αTCΔPC2 cells. This is consistent with the idea that increases in adaptive thermogenic capacity develop over the course of weeks, rather than days.[27,29] While acute exposure to a subthermoneutral temperature mainly activates shivering as a mechanism for maintaining body temperature, longer-term exposure activates nonshivering (or adaptive) thermogenesis. The key mediator of this process is thought to be UCP-1, which uncouples cellular respiration from adenosine triphosphate synthesis such that heat is generated.[27,29,30] We were unable to detect any differences in UCP-1 protein levels between sham-operated and cell-treated *db/db* mice; however, it remains possible that cell transplants altered the degree of activation of UCP-1, which contributes to functional UCP-1 levels.[29] Alternatively, cell transplantation may have increased shivering thermogenesis, a possibility that seems plausible given our observation that αTCΔPC2 recipients had a slight increase in lean:lipid body mass ratio compared to sham-operated controls. The mechanism driving the αTCΔPC2 cell–induced increases in body temperature of *db/db* mice therefore remains unclear, as does the specific PGDP mediating this effect.

It is intriguing to note that for some time, glucagon has been proposed to have thermogenic properties, although only when administered at ~1,000× circulating levels.[31] A more recent report found no thermogenic effect of purified glucagon and suggested that the earlier observations may have arisen from contamination of the glucagon preparation with another PGDP.[32] Indeed we also observed no impact of glucagon-producing αTC-1 cell transplants on cold thermogenesis in *db/db* mice. There is evidence that GLP-1 can modulate core body temperature, but results from various studies have been somewhat contradictory. Several studies in rats have reported that GLP-1 administered ICV or intravenously increases body temperature,[33,34] whereas in the Japanese quail GLP-1 has been reported to decrease body temperature when administered ICV or intravenously.[35] However, a role for GLP-1 in cold-induced thermogenesis has not, to our knowledge, been described. Nevertheless, the notion that GLP-1 arising from transplanted αTCΔPC2 cells may have improved thermogenesis in *db/db* mice is consistent with recent studies showing that peripherally delivered GLP-1 can activate autonomic control centers in the central nervous system and thereby influence peripheral sympathetic functions such as blood pressure and heart rate.[36,37] We found that GLP-1R$^{-/-}$ mice do not have a defective ability to thermoregulate in the cold, suggesting that GLP-1R signaling is not essential for cold thermogenesis. This does not, however, preclude any involvement of GLP-1R signaling in body temperature regulation, because any deleterious effect of GLP-1R ablation on temperature regulation may simply be masked by other redundant mechanisms controlling this key metabolic parameter. Whether other PC1/3-derived PGDPs (*i.e.*, GLP-2, oxyntomodulin) might contribute to the observed effects also remains possible. However, at the doses tested we were unable to detect an improvement in cold thermogenesis in response to long-term continuous infusion of GLP-1, GLP-2, or oxyntomodulin in isolation. It is also possible that another as yet unidentified non-PGDP secreted from αTCΔPC2 cells may mediate this effect.

In summary, this study provides a model for evaluating long-term delivery of PC1/3-derived PGDPs via cell therapy. Our studies demonstrate that transplantation of αTCΔPC2 cells improves glucose handling in mouse models of T2D, and indicate that one or more products of αTCΔPC2, but not αTC-1 cells, may have a previously unappreciated effect on thermogenesis and body temperature regulation. This work provides additional evidence to support the notion that manipulation of the processing enzyme profile of the α-cell can alter its overall characteristics by changing the balance of peptides arising from it.

MATERIALS AND METHODS

Tissue culture media, antibiotics, and fetal bovine serum were obtained from Invitrogen Canada (Burlington, ON, Canada). αTC-1 (clone 9) cells were obtained from the American Type Culture Collection. Total GLP-1 RIA and GLP-2 ELISA kits were from Linco Research (St. Charles, MO); ultrasensitive insulin ELISA kits were from Alpco Diagnostics (Salem, NH); and oxyntomodulin RIA kits were from Phoenix Pharmaceuticals (Burlingame, CA). Western blotting reagents were from GE Healthcare (Buckinghamshire, UK), and the BCA kits for protein quantification were from Pierce (Rockford, IL). Imaging and quantification was performed using an Axiovert 200 microscope (Carl Zeiss, Toronto, ON, Canada) connected to a digital camera (Retiga 2000R; QImaging, Burnaby, BC, Canada) controlled with Openlab 5.0 software (Improvision, Lexington, MA).

Animals. All experiments were approved by the University of British Columbia Animal Care Committee. *db/db* mice and wild-type C57BLKS/J mice (BKS.Cg-m $^{+/+}$ Leprdb/J, stock #000642 and stock #000662, respectively; received at 4-week age), and diet-induced obese C57BL/6 mice (fed a 60% kcal from fat diet from 6-week age and obtained at 16-week age) were obtained from Jackson Laboratories (Bar Harbor, ME). GLP-1R$^{-/-}$ mice,[38] provided by Dr D. Drucker (University of Toronto), were maintained on a C57BL/6 background and genotyped as described elsewhere.[10] Mice were maintained on a 12-hour light/dark cycle and received a standard chow diet (#5015; LabDiet, St. Louis, MO; contains 19.8% kcal from protein, 54.9% kcal from carbohydrates, and 25.3% kcal from fat, 60% of which is soy- or lecithin-derived and 40.0% of which is from lard) except for diet-induced obese mice, which were maintained on diet D12492 from Research Diets (New Brunswick, NJ; contains 20.0% kcal from protein, 20.0% kcal from carbohydrate, and 60% kcal from fat, comprised of 9.3% soybean oil and 90.7% lard). Blood glucose and body weight were monitored two to three times weekly following a 4-hour morning fast. Blood glucose monitoring and blood sampling were carried out on restrained, unanesthetized mice via the saphenous vein using heparinized microcapillary tubes. Plasma was stored at −20 °C until assay. Glucose tolerance tests were performed by IP delivery of glucose (2 g/kg) to recipient mice following an overnight fast. Blood glucose was monitored for 2 hours after glucose delivery and insulin was measured using an ultrasensitive mouse insulin ELISA. Food intake was measured by providing a known mass of food and weighing food remaining in the hopper 24 hours later for ≥4 days.

Cell culture and transplantation of encapsulated cells. αTC-1 and αTCΔPC2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were maintained at 37 °C/5% CO_2 and passaged as necessary using 0.25% trypsin/1 mmol/l EDTA with media refreshment every 2–4 days. Medium was collected after a 24-hour static incubation and assayed for immunoreactive GLP-2 and oxyntomodulin.

Cells were grown to ~80% confluence before encapsulation. For all transplants, 1.5–2 ml of capsules (~4.0×10^7 cells/mouse) were transplanted in <3 ml total volume. Experiments were performed using passages 20–35 for αTCΔPC2 and p8-12 for αTC-1. After trypsinization, pelleted cells were resuspended in phosphate-buffered saline without $CaCl_2$ and a cell count was performed using a hematocytometer. Cells were resuspended in a mixture of 1.5% sodium alginate (IE-1010; Inotech Biosystems International; Rockville, MD; molecular weight 475 kd; G/M ratio 65–75%/25–35%) and morpholinepropanesulfonic acid and transferred to a sterile encapsulator (Inotech Biosystems International). Encapsulation was performed according to the manufacturer's instructions using a 250-μm nozzle and encapsulation settings as follows: electrical charge ~1.0 kV, vibration frequency ~1,200 Hz, pump speed ~600. Capsules (500–700 μm) were washed in phosphate-buffered saline without $CaCl_2$ and loaded to sterile syringes attached to 18-G catheters. Recipient mice were anesthetized using isoflurane, and capsules were injected to the IP cavity as described elsewhere.[10] Sham-operated mice received an equal volume of sterile saline under identical conditions.

Implantation of miniosmotic pumps. Rat GLP-2, human GLP-$1_{7\text{-}36NH2}$, and oxyntomodulin were from American Peptide (Sunnyvale, CA) and were reconstituted in sterile saline. Miniosmotic pumps (28 days infusion, #1004; Alzet, Cupertino, CA) were loaded with peptide (7.5 pmol/kg·min) or saline and preequilibrated overnight at 37 °C. The following day pumps were implanted to the peritoneal cavity of isoflurane-anesthetized *db/db* mice (age ~6 weeks).

Cold tolerance testing and body temperature analysis. Mice were implanted subcutaneously with sterile temperature transponders (IPTT-300; Bio Medic Data Systems, Seaford, DE) through a ~3-mm incision in the intrascapular region at the same time of cell transplant/pump implant.

Transponders were implanted longitudinally in the subcutaneous space, parallel but to one side of the spine so as not to interfere with the animal's movement. The incision was closed with a wound clip before the animal was allowed to recover from anesthesia. GLP-1R$^{-/-}$ and *ob/ob* mice were implanted with transponders 2 days before cold tolerance testing.

Twenty-four-hour body temperature profiles were assessed using a hand-held Pocket Scanner (DAS-5007; Bio Medic Data Systems, Seaford, DE) to read the temperature transponders in singly housed mice. For cold tolerance testing, mice were singly housed in cages without bedding for the duration of the experiment. Mice were housed at 4 °C and body temperature was measured every 15 minutes for 1–2 hours using the Pocket Scanner. Any mouse that lost ≥7 °C from basal body temperature was returned to ambient temperature, warmed using a heating pad, and excluded from the study.

Body composition analysis. Measurements were performed using a Bruker Biospec 70/30 7 Tesla MRI scanner (Bruker Biospin, Ettlingen, Germany). Nuclear magnetic resonance signal from the body was acquired using a quadrature volume RF coil tuned to 300 MHz. The "free" water component corresponding to body fluids (*e.g.*, urine and CSF) was typically <5% of the total signal. The ratio of lean/fat tissue (weight/weight) was calculated as described elsewhere.[39]

Plasma and tissue collection. At the indicated time, mice were anesthetized with isoflurane, and a cardiac puncture was performed to collect plasma before cervical dislocation. Pancreas, intrascapular BAT, and epididymal WAT were removed, rinsed in phosphate-buffered saline, fixed in 4% paraformaldehyde and paraffin sectioned. A portion of intrascapular BAT was collected, rinsed in phosphate-buffered saline, and flash frozen in liquid nitrogen. Plasma GLP-1 was measured using a total GLP-1 RIA kit.

BAT and WAT analysis. BAT and epididymal WAT sections were stained with hematoxylin and eosin and imaged using brightfield microscopy. Lipid droplet size was measured using an inbuilt algorithm. Other sections were stained with a rabbit UCP-1 antibody (10983; 1:1,000; Abcam, Cambridge, MA) and horseradish peroxidase–conjugated secondary antisera. Intrascapular BAT was ground using a prechilled mortar and pestle and transferred to a microcentrifuge tube with 100 µl of lysis buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 10 mmol/l EDTA, 10 mmol/l $Na_4P_2O_7$, 100 mmol/l NaF, with 1 mmol/l phenylmethanesulphonylfluoride, 2 mmol/l Na_3VO_4, 0.4% Triton-X, and 1% protease inhibitor cocktail added immediately before use). Samples were vortexed and incubated on ice for 30 minutes. The protein-containing supernatant was removed to a fresh set of tubes. Equal amounts of protein were electrophoresed on a 10% acrylamide gel using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a 0.2-µm polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). After blocking, the membrane was incubated with rabbit anti-UCP-1 antibody (1:4,000;[40]) overnight at 4 °C. The membrane was incubated with alkaline phosphatase–linked polyclonal secondary antibody (1:5,000) and developed using an enhanced chemiluminescence kit (GE Healthcare; Buckinghamshire, UK). The membrane was washed, reblocked, and immunoblotted with an alkaline phosphatase–linked β-actin antibody, and UCP-1 expression was normalized for β-actin levels.

Data analysis. Data analysis was carried out using Prism 4.0 (GraphPad, San Diego, CA). Data are presented as mean ± SEM and were analyzed using a two-tailed Student's *t*-test except where noted. Statistical significance was set at 5% and the number of experiments performed are shown in figure legends. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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