

Sex-steroid milieu determines diabetes rescue in *pttg*-null mice

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Abstract

Male mice that are *pttg*-null develop sexually dimorphic diabetes with hypoinsulinemia secondary to reduced post-natal β -cell proliferation and an inability to expand islet cell mass with aging. We therefore examined the effects of sex-steroid manipulation on diabetes development in *pttg*^{-/-} male mice. Surgical gonadectomy was followed by implantation of 90-day slow-release pellets releasing 17 β -estradiol (0.36 mg/pellet), placebo or dihydrotestosterone (DHT; 12.5 mg/pellet). Mean fasting blood sugars at the end of the study were 414 \pm 54 mg/dl for *pttg*^{-/-} controls and 371 \pm 14 mg/dl for *pttg*^{-/-} mice gonadectomized and treated with DHT compared with 124 \pm 40 and 85 \pm 12 mg/dl in gonadectomized *pttg*^{-/-} males treated with placebo or estradiol, respectively ($P < 0.01$ compared with control *pttg*^{-/-}). Gonadectomy with and without estradiol treatment did not increase the very low circulating insulin levels in *pttg*-null males

(fasting insulin 0.44 \pm 0.04 ng/ml in *pttg*^{-/-} controls, 0.47 \pm 0.07 and 0.4 ng/ml in *pttg*^{-/-} gonadectomized males treated with placebo or estradiol, respectively). Gonadectomy increased serum adiponectin levels (4.9 \pm 0.08 μ g/ml in *pttg*^{-/-} controls versus 13 \pm 0.08 and 7.5 \pm 0.6 μ g/ml in *pttg*^{-/-} gonadectomized males treated with placebo or estradiol, respectively; $P < 0.001$ and $P < 0.05$), accompanied by increased insulin sensitivity. The results show that gonadectomy delayed, and gonadectomy with additional estradiol treatment prevented, diabetes development in *pttg*^{-/-} males, possibly through increased insulin sensitivity mediated by elevated serum adiponectin levels. Male-selective effects of disrupted β -cell proliferation in the absence of *pttg* are restored by sex-steroid effects on peripheral insulin sensitivity.

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Introduction

pttg is the functional mammalian homolog of yeast securin (Pei & Melmed 1997, Zou *et al.* 1999), which facilitates sister-chromatid separation during the mitotic transition from metaphase to anaphase (Pei & Melmed 1997). *pttg*-null male mice develop hyperglycemia secondary to hypoinsulinemia starting at 6 months of age (Wang *et al.* 2003). By 1 year, >80% of male *pttg*^{-/-} mice are diabetic with hypoinsulinemia secondary to reduced post-natal β -cell proliferation and an inability to expand islet cell mass with aging. Diabetes onset is accompanied by loss of fat tissue. In contrast, *pttg*^{-/-} female mice rarely develop diabetes before 1 year of age and the incidence of diabetes in older *pttg*-null females (more than 1 year) is increased. Moreover, ovariectomy causes earlier onset of diabetes at 6 months of age in female *pttg*^{-/-} mice (Wang *et al.* 2003).

Several animal models exhibit sexually dimorphic diabetic phenotypes (Rossini *et al.* 1978, Paik *et al.* 1982, Kava *et al.* 1989, Leiter 1989, Efrat 1991, Shi *et al.* 1994, Kim *et al.* 2001, Thomas *et al.* 2001, Weksler-Zangen *et al.* 2001, Geisler *et al.* 2002, Li *et al.* 2003, Iglesias *et al.* 2004). In some, male gonadectomy had no effect, or accentuated the diabetic phenotype (Leiter 1981, 1989, Leiter *et al.*

1989, Efrat 1991, Kava *et al.* 1992), while in others gonadectomy protected against development of diabetes (Rossini *et al.* 1978, Maclaren *et al.* 1980, Kava *et al.* 1992, Shi *et al.* 1994, Weksler-Zangen *et al.* 2001). Removal of testosterone from the sex-steroid milieu of the male animal improved insulin sensitivity in most studies, and thus was protective against development of hyperglycemia. The protective role of estrogen in the pathogenesis of diabetes has also been demonstrated. Estrogen positively affects insulin sensitivity and increases insulin production (Bailey & Ahmed-Sorour 1980), and ovariectomy of animals with a genetic predisposition to develop diabetes triggered diabetes onset in some female animals (Puah & Bailey 1985, Efrat 1991, Shi *et al.* 1994). Moreover, animals in which estrogen receptor α or the aromatase genes have been disrupted have increased insulin resistance and impaired glucose tolerance (Heine *et al.* 2000, Jones *et al.* 2000).

To elucidate the role of sex steroids in the pathogenesis of diabetes in *pttg*-null animals, males were surgically gonadectomized at the age of 4 weeks and implanted with 90-day slow-release pellets releasing 17 β -estradiol, placebo or dihydrotestosterone (DHT). Fasting blood glucose levels were monitored, and glucose- and

insulin-tolerance tests were performed to assess the effects of these manipulations on diabetes development in this model.

Materials and Methods

Animals

pttg^{-/-} mice (Wang *et al.* 2001) were kept in a hybrid background derived from C57/BL6 and 129 SvJ mouse strains. Animals were housed with a 12-h light:12-h darkness cycle and fed standard chow *ad libitum*. Experiments were approved by the Institutional Animal Care and Use Committee of Cedars-Sinai Research Institute, Los Angeles, CA, USA.

Four-week-old male *pttg*^{+/+} and *pttg*^{-/-} mice were surgically gonadectomized (or sham operated) under iso-flurane anesthesia and implanted every 90 days with estradiol (0.36 mg/pellet; Innovative Research of America, Sarasota, FL, USA) or placebo (Saba *et al.* 2002, van Eickels *et al.* 2003). Controls included *pttg*^{+/+} and *pttg*^{-/-} males with no intervention, or gonadectomized and treated with DHT (12.5 mg/pellet; same time points as other pellets).

Blood assays

Fasting blood glucose was measured twice a month starting at 4 weeks of age from tail blood samples after an overnight fast, using OneTouch Ultra glucometer (Lifescan; Johnson and Johnson, Milpitas, CA, USA). Animals with blood-glucose measurements above 150 mg/dl were considered as having diabetes.

Blood collected from the tail vein after an overnight fast was allowed to clot and then separated by ultracentrifugation. The following serum analytes were measured: insulin concentrations were measured in samples collected during glucose loading using an ultra-sensitive rat ELISA kit (CrystalChem, Downers Grove, IL, USA); fasting concentrations of adiponectin were measured using a mouse adiponectin RIA kit (Linco Research, St Charles, MO, USA); and C-peptide levels were measured by RIA using a rat C-peptide RIA kit (Linco Research).

Leptin and insulin were measured using the Lincoplex Adipokine panel (Linco Diagnostics, St Charles, MO, USA). Estradiol levels were measured by RIA using a 3rd Generation Estradiol RIA kit (Diagnostic Systems Laboratories, Webster, TX, USA).

Intraperitoneal insulin and glucose-tolerance test

For glucose-tolerance testing (GTT), mice were fasted 16 h before i.p. glucose injection (1 g/kg body weight) and tail-vein blood collected at the indicated times. For insulin-tolerance testing (ITT), mice were fasted 6 h

before i.p. insulin injection (1 unit/kg body weight), and glucose measured at specific time points.

Histological, immunohistological and morphometric analysis

Pancreata were isolated immediately after CO₂ euthanasia and fixed overnight at 4 °C in 10% buffered formalin, followed by processing and paraffin embedding. Blocks were sectioned (3–5 µM) and stained with hematoxylin and eosin. For immunostaining following antigen retrieval with citrate buffer, guinea-pig anti-insulin and rabbit anti-glucagon (both from Dako-cytomation, Carpinteria, CA, USA) were used. Primary antibodies were visualized using rhodamine-conjugated goat anti-guinea-pig antibody (Jackson ImmunoResearch, West Grove, PA, USA) and fluorescein-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) using an Olympus fluorescence microscope and digital camera. For morphometric analysis, 1× images of the hematoxylin and eosin sections were used to calculate pancreatic surface area using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Consecutive sections from the same block were immunostained for insulin and glucagon. Using ImageJ software the numbers of islets (defined as a minimum of five insulin-positive cells) in the same section were counted and the surface area of insulin-positive and glucagon-positive cells measured and averaged for all islets in the section. β-Cell area in the section was calculated by summing the individual insulin-positive cells area expressed as a percentage of the total pancreatic area observed.

Statistical analysis

Insulin resistance was assessed by the homeostasis model assessment-insulin resistance (HOMA-IR) index (Matthews *et al.* 1985), which was calculated as [fasting blood glucose (mg/dl) × fasting insulin (mg/ml)]/405 (Umeda *et al.* 2003).

Statistical comparisons were performed using the one tailed unpaired *t*-test with Welch's correction, unless otherwise stated. One-way ANOVA was used when appropriate and is stated in the text. Data were analyzed using Prism software (Prism 4; Graphpad Software Inc, San Diego, CA, USA). Data in graphs are depicted as means ± s.e.m. Circulating leptin concentrations were log-transformed to normalize the distribution.

Results

*Diabetes is prevented in gonadectomized *pttg*^{-/-} males treated with estradiol, and delayed in gonadectomized *pttg*^{-/-} males*

Eighty percent of sham-operated males and all males with no intervention (control) *pttg*^{-/-} mice developed

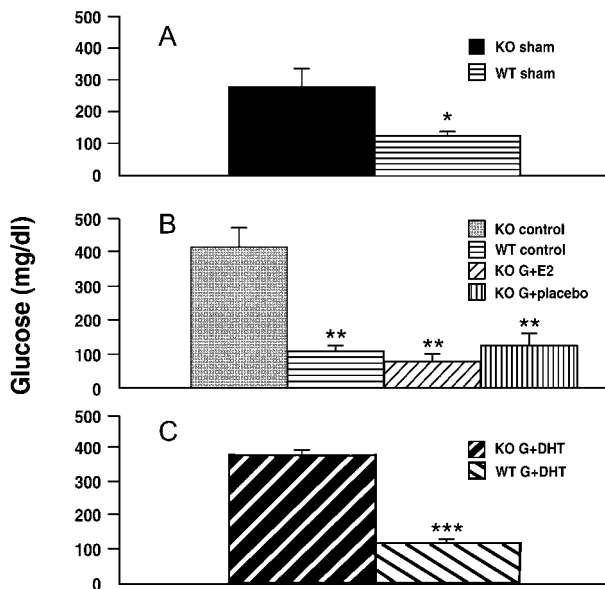


Figure 1 Fasting blood glucose in *pttg*^{-/-} (KO) and *pttg*^{+/+} (WT) male mice after sex-steroid manipulation. (A) Fasting blood glucose in 10-month-old sham-operated *pttg*^{-/-} (mean 276 ± 57 mg/dl) and *pttg*^{+/+} (123 ± 7 mg/dl) males; **P*<0.05. (B) Fasting blood glucose in 10-month-old male *pttg*^{-/-} controls (414 ± 54 mg/dl) and male *pttg*^{+/+} controls (104 ± 7 mg/dl), 13-month-old *pttg*^{-/-} gonadectomized males treated with estradiol (KO G+E2; 85 ± 12 mg/dl) and gonadectomized *pttg*^{-/-} males treated with placebo (KO G+placebo; 124 ± 40 mg/dl); ***P*<0.01 compared with *pttg*^{-/-} control males. (C) Fasting blood glucose in 9-month-old gonadectomized *pttg*^{-/-} (KO G+DHT; 371 ± 14 mg/dl) and *pttg*^{+/+} (WT G+DHT; 108 ± 16 mg/dl) males treated with DHT; ****P*<0.001.

hyperglycemia by 10 months (mean fasting blood glucose: sham *pttg*^{-/-} 276 ± 57 mg/dl versus *pttg*^{+/+} 115 ± 28 mg/dl, and control *pttg*^{-/-} 414 ± 54 mg/dl versus *pttg*^{+/+} 104 ± 7 mg/dl; *P*<0.05 and *P*<0.01 respectively, *n*=4–5 animals per group; Fig. 1).

Gonadectomized *pttg*^{-/-} males treated with estradiol (*n*=4) had normal fasting blood glucose levels for up to 13 months (mean fasting blood glucose: 85 ± 12 mg/dl compared with control *pttg*^{-/-} males at 10 months of age; *P*<0.01; Fig. 1B). Mean serum estradiol levels in treated *pttg*^{-/-} and *pttg*^{+/+} mice were higher than in control *pttg*^{-/-} mice (253 ± 97, 230 ± 56 and 13 ± 1 pg/ml respectively; *P*<0.01 by one-way ANOVA with Kruskal–Wallis test). GTT (1 g glucose/kg body weight) was performed serially on mice gonadectomized and treated with estradiol (*n*=4) at 3, 6, 9 and 12 months, and these values remained normal (data not shown).

Gonadectomized *pttg*^{-/-} male mice treated with placebo (*n*=5) were protected from developing diabetes mellitus (mean fasting blood glucose at 13 months: 124 ± 40 mg/dl versus *pttg*^{-/-} controls 414 ± 54 mg/dl; *P*<0.01; Fig. 1B), while a single gonadectomized *pttg*^{-/-} male developed overt fasting hyperglycemia

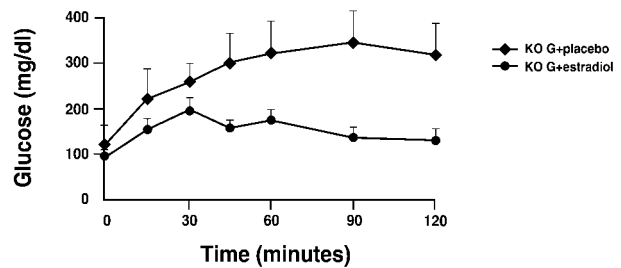


Figure 2 GTT in 12-month-old *pttg*^{-/-} (KO) and *pttg*^{+/+} (WT) male mice after sex-steroid manipulation. Glucose (1 g/kg body weight) was injected intraperitoneally and blood glucose measured after 15, 30, 45, 60, 90 and 120 min. Glucose values are from GTT of gonadectomized *pttg*^{-/-} males treated with estradiol (KO G+estradiol) and placebo (KO G+placebo); *n*=4–5 in each group.

(260–370 mg/dl). By 12 months, gonadectomized *pttg*^{-/-} males treated with placebo were glucose-intolerant compared with gonadectomized males with additional estradiol treatment. The mean area under the curve of GTT values was lower in gonadectomized *pttg*^{-/-} animals treated with estradiol when compared with gonadectomy alone (18.5 ± 2.3 versus 35.2 ± 7.4 arbitrary units; *P*<0.05; Fig. 2).

Gonadectomized *pttg*^{-/-} male mice treated with DHT (*n*=4) developed diabetes as expected (mean fasting blood glucose at 9 months: 371 ± 14 versus 108 ± 16 mg/dl in *pttg*^{+/+} similarly treated animals; *n*=5 *P*<0.001; Fig. 1C), and were also glucose intolerant, similar to control *pttg*^{-/-} males.

Diabetes onset was accompanied by weight loss in *pttg*^{-/-} male controls and in sham-operated and gonadectomized animals with supplemental DHT. *pttg*^{-/-} gonadectomized males with and without estradiol treatment had lower body weights at an early age, irrespective of diabetes onset, when compared with *pttg*^{-/-} and *pttg*^{+/+} control mice (data not shown).

Gonadectomy with or without additional estradiol therapy fails to elicit fasting and post-challenge insulin responses in pttg^{-/-} males

Insulin levels were measured to determine whether the observed protection from diabetes conferred by sex-steroid manipulation was due to increased circulating insulin concentrations. Fasting serum insulin levels in control *pttg*^{+/+} males rose gradually with age with no similar increase noted in *pttg*^{-/-} males (1.95 ± 0.6 versus 0.44 ± 0.04 ng/ml at 10 months; *P*<0.05; Fig. 3A). Interestingly, although gonadectomized *pttg*^{-/-} males treated with estradiol were protected from developing hyperglycemia, they did not exhibit increased fasting insulin levels (Fig. 3A). Measurement of C-peptide levels in the same animals confirmed this observation (data not shown). Eight-month-old gonadectomized *pttg*^{-/-} males had

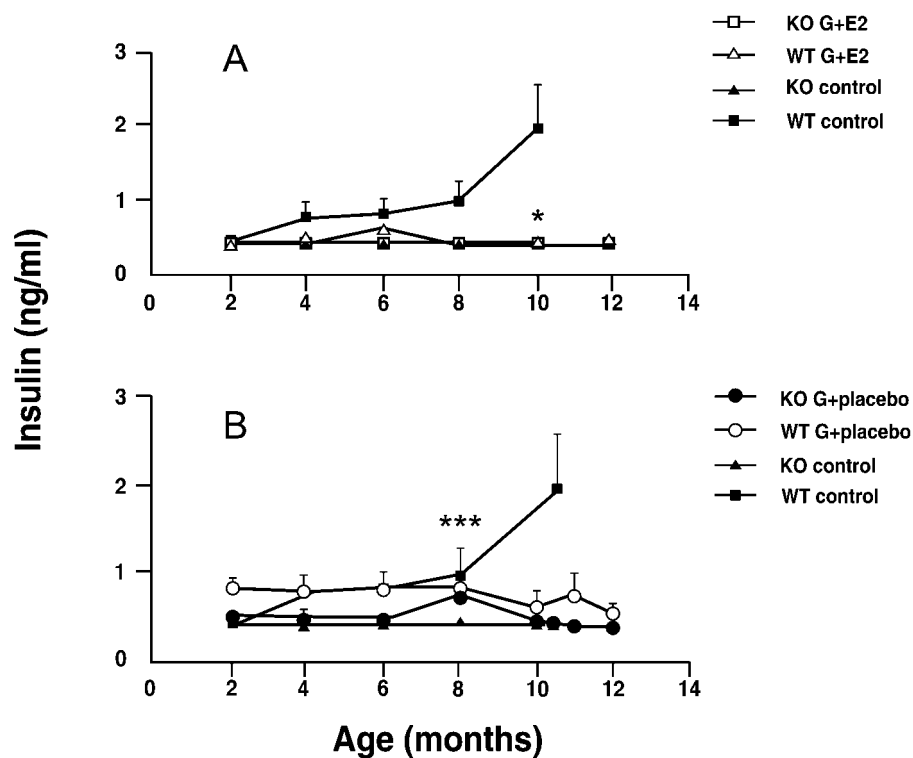


Figure 3 Fasting serum insulin levels in *pttg*^{-/-} (KO) and *pttg*^{+/+} (WT) male mice after sex-steroid manipulation, measured at 2, 4, 6, 8 and 10 months. (A) *pttg*^{-/-} control males (KO control; ▲), *pttg*^{+/+} control males (WT control; ■), gonadectomized *pttg*^{-/-} males treated with estradiol (KO G+E2; □) and gonadectomized *pttg*^{+/+} males treated with estradiol (WT G+E2; △); **P*<0.05 for *pttg*^{+/+} male controls versus *pttg*^{-/-} male controls at 10 months. (B) *pttg*^{-/-} (●) and *pttg*^{+/+} (○) male controls, *pttg*^{-/-} (▲) and gonadectomized *pttg*^{+/+} (■) males treated with placebo; ****P*<0.001 for gonadectomized *pttg*^{-/-} males treated with placebo versus *pttg*^{-/-} control males at 8 months.

modestly increased fasting insulin levels compared with control *pttg*^{-/-} animals (0.734 ± 0.06 versus 0.43 ± 0.03 ng/ml; *P*<0.001 by one-way ANOVA; Fig. 3B), an increase that was not evident at other experimental time points and was not confirmed by C-peptide levels. Most circulating fasting insulin measurements in *pttg*^{-/-} control males were below the detection sensitivity of the Lincoplex kit. It is therefore possible that insulin levels in gonadectomized *pttg*^{-/-} males exceeded those of controls, but the assay was not sufficiently sensitive to detect differences. Thus it remains unclear as to whether gonadectomy alone resulted in unambiguously elevated insulin levels in *pttg*^{-/-} males. Serial insulin levels in gonadectomized *pttg*^{-/-} and *pttg*^{+/+} males treated with DHT were in accord with those of control animals (data not shown).

The appropriate wild-type insulin response to a glucose challenge (GTT) was markedly attenuated in *pttg*^{-/-} males (for the 0, 30, 60 min time points (*P*<0.05) and for the 120 min time point (*P*<0.02); Fig. 4A). Moreover, gonadectomy of *pttg*^{-/-} males with or without estradiol

treatment did not normalize insulin levels following a glucose challenge, compared with age-matched control *pttg*^{-/-} males (0, 30, 60 and 120 min time points; *P*>0.05; Fig. 4). Gonadectomized *pttg*^{-/-} males treated with DHT also failed to raise insulin levels following a glucose load when compared with *pttg*^{+/+} mice that exhibited a normal response to glucose (data not shown).

These results indicate that gonadectomy, followed by additional estradiol or no added treatment, protected male *pttg*^{-/-} mice from developing diabetes in both the fasted and the post-glucose-challenged state without appreciably increasing insulin levels.

*Increased insulin sensitivity accompanied by elevated serum adiponectin and leptin levels in gonadectomized *pttg*^{-/-} males*

To assess whether diabetes rescue occurred as a result of increased insulin sensitivity, ITTs were performed on mice aged 8–11 months (*n*=3 animals per group). Control *pttg*^{+/+} and *pttg*^{-/-} males were assessed at 8 months

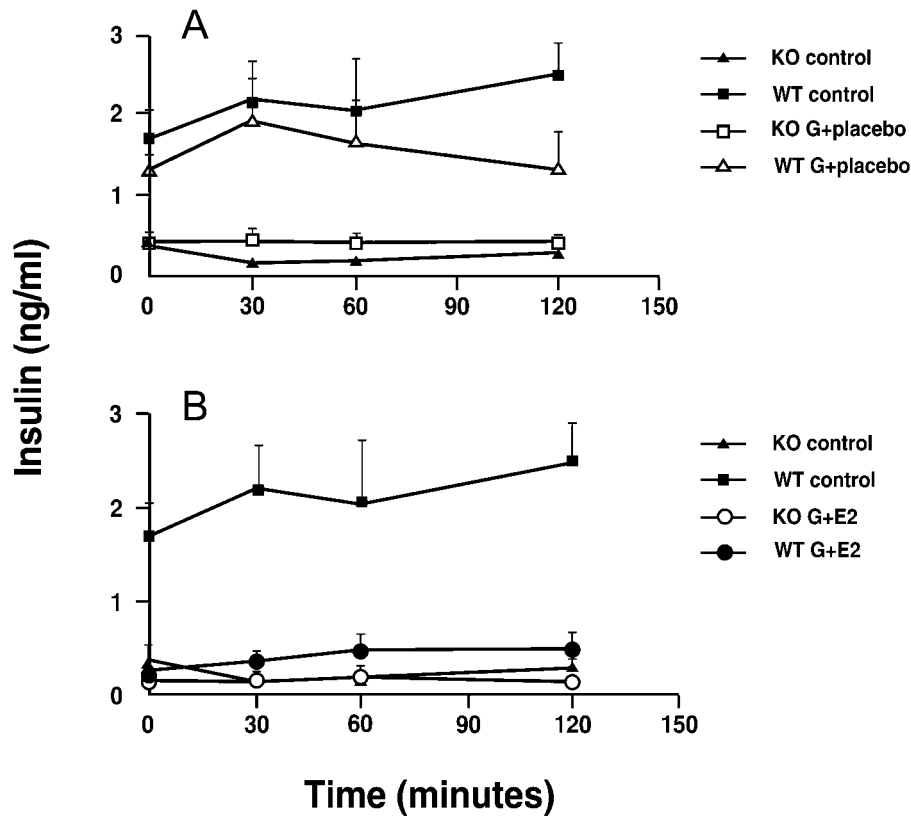


Figure 4 Insulin levels during GTT of 9-month-old male *pttg*^{-/-} (KO) and *pttg*^{+/+} (WT) mice after sex-steroid manipulation. Blood was collected for insulin measurement at baseline (0) and after 30, 60 and 120 min during IPGTT. (A) *pttg*^{-/-} controls (▲) failed to elicit a normal insulin response when compared with *pttg*^{+/+} controls (■) for the 0, 30 and 60 min time points ($P < 0.05$) and at 120 min ($P < 0.02$). Gonadectomy (*pttg*^{-/-}, □; *pttg*^{+/+}, △) did not rescue insulin production during GTT in *pttg*^{-/-} males. (B) Gonadectomy with additional estradiol therapy (*pttg*^{-/-}, ○; *pttg*^{+/+}, ●) did not rescue insulin production during GTT in *pttg*^{-/-} males ($n = 3-5$ in each group).

(mean fasting glucose: 107 ± 21 and 204 ± 81 mg/dl respectively), gonadectomized *pttg*^{+/+} and *pttg*^{-/-} males treated with DHT at 9 months (mean fasting glucose: 108 ± 16 and 371 ± 14 mg/dl respectively), and gonadectomized *pttg*^{+/+} and *pttg*^{-/-} males treated with placebo or estradiol were assessed at 10 and 11 months respectively (all were normoglycemic).

A modest increase in insulin sensitivity was observed in gonadectomized *pttg*^{+/+} and *pttg*^{-/-} males both with and without estradiol treatment. The time required to attenuate basal glucose levels by 50% was ~ 60 min in control *pttg*^{-/-} males versus 30 and 40 min for gonadectomized *pttg*^{-/-} males receiving placebo or estradiol respectively. Gonadectomized *pttg*^{-/-} males treated with DHT did not improve insulin sensitivity.

The HOMA-IR (Homeostasis Model Assessment) index (Matthews *et al.* 1985, Umeda *et al.* 2003), a physiologic marker for insulin resistance, was calculated for each group of animals at 2 month intervals starting at the age of 2 months. A higher HOMA index score is

an estimated reflection of increased insulin resistance. Gonadectomized *pttg*^{-/-} males with and without added estradiol treatment had a lower HOMA index at 10 months when compared with control *pttg*^{-/-} and *pttg*^{+/+} males, indicating that gonadectomy with or without estradiol treatment likely enhanced insulin sensitivity (0.096 ± 0.02 in gonadectomized *pttg*^{-/-} males treated with placebo, 0.108 ± 0.18 in gonadectomized *pttg*^{-/-} males treated with estradiol and 0.368 ± 0.08 in control *pttg*^{-/-}; $P < 0.025$ comparing *pttg*^{-/-} gonadectomized with and without additional estradiol with control *pttg*^{-/-}). These observations were confirmed by correlating fasting glucose and insulin levels (data not shown). *pttg*^{+/+} animals have low glucose levels in the face of high insulin levels, while *pttg*^{-/-} animals have high glucose levels in the face of low insulin levels. Gonadectomy with and without additional estradiol treatment resulted in changing *pttg*^{-/-} mice to the area on the correlation plot associated with low insulin and low glucose levels, thus implying increased insulin sensitivity.

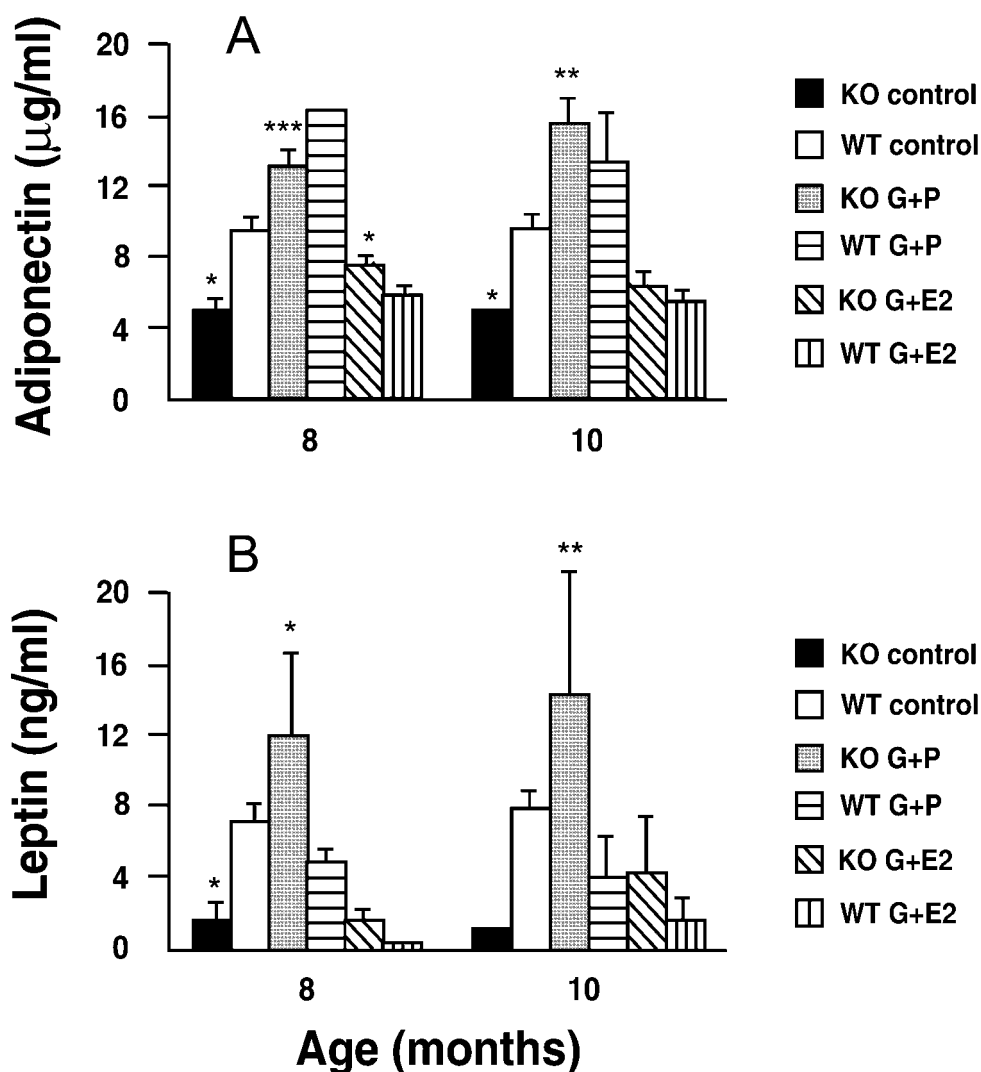


Figure 5 Serum adipokine levels in male *pttg*^{-/-} (KO) and *pttg*^{+/+} (WT) mice after sex-steroid manipulation. (A) Serum adiponectin levels. (B) Serum leptin levels. Control *pttg*^{-/-} males (black bars) had lower adiponectin and leptin levels when compared with age-matched *pttg*^{+/+} control males (white bars); **P*<0.05; ***P*<0.01. Gonadectomized *pttg*^{-/-} males (gray bars) had elevated adiponectin (A) and leptin levels (B) at 8 and 10 months when compared with age-matched control *pttg*^{-/-} males (black bars); **P*<0.05; ***P*<0.01; ****P*<0.001. Gonadectomized *pttg*^{-/-} males treated with estradiol (diagonally striped bar) have elevated adiponectin (A) but not leptin (B) levels at 8 months compared with age-matched control *pttg*^{-/-} males (black bars); **P*<0.05 (*n*=3–5 in each group). G, gonadectomy; P, placebo; E2, estradiol.

To determine potential mechanisms for the observed enhanced insulin sensitivity, fasted serum adipokine levels were measured (*n*=3–5 in each group; Fig. 5). *pttg*^{-/-} males exhibit a gradual decrease in adiponectin and leptin concentrations with age, as compared with *pttg*^{+/+} males (for adiponectin at 8 months: 4.9 ± 0.8 versus 9.4 ± 0.9 µg/ml (*P*<0.05) and at 10 months: 3.9 ± 1 versus 9.6 ± 0.9 µg/ml (*P*<0.01); for leptin at 8 months: 1.9 ± 1.4 versus 8.6 ± 1.5 ng/ml (*P*<0.05) and at 10 months: 1 ± 0.5 versus 9.2 ± 1.8 ng/ml (*P*<0.01) in control *pttg*^{-/-}

and *pttg*^{+/+} respectively; all by one-way ANOVA). Similar observations were made in gonadectomized *pttg*^{-/-} males treated with DHT, as compared with wild-type counterparts (data not shown). In contrast, gonadectomized *pttg*^{-/-} males have elevated levels of both adipokines as compared with age-matched control *pttg*^{-/-} males. Adiponectin at 8 months (Fig. 5A) was 13 ± 0.8 µg/ml in gonadectomized *pttg*^{-/-} versus 4.9 ± 0.8 µg/ml in control *pttg*^{-/-} (*P*<0.001), and at 10 months 15 ± 1.6 µg/ml in gonadectomized *pttg*^{-/-} versus 3.9 ± 1 µg/ml in

control *pttg*^{-/-} ($P < 0.01$; both by one-way ANOVA). Leptin levels at 8 months (Fig. 5B) were 14.3 ± 5.7 ng/ml in gonadectomized *pttg*^{-/-} mice versus 1.9 ± 1.3 ng/ml in control *pttg*^{-/-} ($P < 0.05$), and at 10 months they were 17.3 ± 8.4 ng/ml in gonadectomized *pttg*^{-/-} versus 1 ± 0.5 ng/ml in control *pttg*^{-/-} mice ($P < 0.01$). Serum adiponectin but not leptin levels were also elevated in 8-month-old gonadectomized *pttg*^{-/-} mice treated with estradiol (Fig 5A; 7.5 ± 0.6 μ g/ml in gonadectomized *pttg*^{-/-} mice treated with estradiol versus 3.9 ± 1 μ g/ml in control *pttg*^{-/-} mice; $P < 0.05$).

*Pancreatic β -cell area is not increased in gonadectomized *pttg*^{-/-} males with or without estradiol therapy*

The percentage β -cell area occupying total pancreatic sections was markedly reduced in *pttg*^{-/-} control males when compared with *pttg*^{+/+} controls (0.08 ± 0.07 versus $7.9 \pm 2.5\%$; $P < 0.001$ by ANOVA and Bonferroni group analysis). Although gonadectomy followed by estradiol or no added therapy increased the percentage β -cell area in *pttg*^{-/-} males (0.27 ± 0.24 and 0.18 ± 0.05 versus $0.08 \pm 0.07\%$ respectively) this difference was not significant when assessed by ANOVA and Bonferroni group analysis. The average islet size and the number of islets per μm^2 was also unaffected by gonadectomy in *pttg*^{-/-} males with and without added estradiol therapy, compared with *pttg*^{-/-} control male mice (data not shown).

Discussion

In *pttg*-null mice, sexually dimorphic diabetes is associated with β -cell hypoplasia, likely a consequence of disrupted securin function. Failure of differentiated islets to proliferate to maturation results in hypoinsulinemia, hyperglycemia and severe lipodystrophy at 6 months of age (Wang *et al.* 2003). It is unclear why β -cells are apparently selectively affected by securin deficiency. β -Cells undergo a proliferation phase before the end of the first postnatal month, when the pancreas is subject to dynamic changes in response to variations in insulin demand. Securin loss and resultant abnormal cell-cycle progression directly affect β -cell mass expansion, resulting in diabetes. It is likely that loss of fat tissue and reduced body weight are secondary to hypoinsulinemia. *pttg*^{-/-} animals also exhibit selective pituitary, splenic and testicular hypoplasia, with no apparent functional deficits (Wang *et al.* 2001, 2003).

The results shown here demonstrate that gonadectomy alters the course of diabetes in *pttg*^{-/-} males, with four of five males being free of diabetes for up to 13.5 months, and one male animal developing diabetes at 12 months. As a group these gonadectomized males showed glucose intolerance for the first time at 12 months of age. Sexually dimorphic hyperglycemia has been described in several

rodent diabetes models in which female sex conferred complete or partial protection from diabetes development (Rossini *et al.* 1978, Paik *et al.* 1982, Kava *et al.* 1989, 1992, Leiter 1989, Efrat 1991, Shi *et al.* 1994, Kim *et al.* 2001, Thomas *et al.* 2001, Weksler-Zangen *et al.* 2001, Geisler *et al.* 2002). Several of the models cited demonstrated that male gonadectomy is protective for diabetes development. These include streptozotocin-induced diabetes, in which gonadectomy ameliorated (Rossini *et al.* 1978) or attenuated (Maclaren *et al.* 1980) hyperglycemia, the obese Zucker rat (Kava *et al.* 1992), the Cohen diabetic rat (Weksler-Zangen *et al.* 2001) and the OLET fatty rat (Shi *et al.* 1994). In most of these studies, removal of testosterone from the sex-steroid milieu of the male animal improved insulin sensitivity, while in others, like the streptozotocin model, the mechanism for the negative effect of testosterone on blood sugar levels is unclear (Maclaren *et al.* 1980, Kromann *et al.* 1982, Paik *et al.* 1982). The negative effects of testosterone have been shown in a study where neonatal therapy of female rats with testosterone caused fetal imprinting followed by subsequent development of insulin resistance at an older age (Nilsson *et al.* 1998). In humans male hypogonadism is associated with glucose intolerance, decreased lean body mass and increased fat mass, mostly through alterations in insulin-sensitivity markers (Fukui *et al.* 2000, Stellato *et al.* 2000, Oh *et al.* 2002, Bhasin 2003). Gonadectomy of *pttg*^{-/-} male mice further lowered body weight which might account for protective effects of gonadectomy on diabetes development in *pttg*-null mice.

In the work shown here, we demonstrate that the delay and attenuation of diabetes development in gonadectomized *pttg*^{-/-} males was not caused by a consistent rise in insulin levels but rather through a decrease in insulin resistance, as reflected in the ITT and the HOMA-IR index. Insulin sensitivity as assessed by the ITT measures mainly muscle glucose clearance, while insulin sensitivity of fat and liver are not appreciably reflected by this test (Goren *et al.* 2004). This might explain the lack of a more robust increase in insulin sensitivity in gonadectomized *pttg*^{-/-} male mice with or without estradiol therapy, as shown by the ITT. Tests directly assessing fat or liver insulin sensitivity may point to greater differences between these groups. For technical reasons, we performed ITT on control mice at 8 months of age and compared results with those of older gonadectomized mice treated with estradiol or placebo. Control *pttg*^{-/-} and *pttg*^{+/+} male mice would have been expected to show more insulin resistance if assayed at an older age, so the finding that gonadectomy increases insulin sensitivity would have likely been magnified, rather than diminished, if the ITT was performed at similar ages.

Increased insulin sensitivity was associated with a dramatic rise in serum adiponectin levels following gonadectomy of both *pttg*^{-/-} and *pttg*^{+/+} males. Adiponectin has been identified as an insulin-sensitizing peptide. Low

adiponectin levels are a feature of insulin resistance in humans and rodents with insulin resistance, whether accompanied by either lipodystrophy or obesity (Yamauchi *et al.* 2001, Berg *et al.* 2002, Haque *et al.* 2002), and exogenous adiponectin administration reverses insulin resistance in rodents (Haque *et al.* 2002). In insulinopenic rodent models for diabetes like the non-obese diabetic (NOD) mouse and streptozotocin-induced diabetes, treatment with adiponectin normalized glucose levels, without a rise in insulin (Berg *et al.* 2001). Given these results, adiponectin is a strong candidate for mediating the observed improvement in diabetes onset in gonadectomized *pttg*^{-/-} male mice. Decreased adiponectin levels seen in age-matched *pttg*^{-/-} control male mice when compared with *pttg*^{+/+} control animals is likely secondary to the *pttg*-null lipodystrophy, which is caused primarily by the low insulin levels. Several studies in humans and rodents have shown sexual dimorphism in plasma levels of adiponectin, with males having lower levels than females (Berg *et al.* 2002, Nishizawa *et al.* 2002). Furthermore, hypogonadal males with low testosterone levels have higher levels of adiponectin when compared with eugonadal males, and testosterone treatment attenuates adiponectin levels (Lanfranco *et al.* 2004). In rodents, male but not female gonadectomy was followed by increased adiponectin levels and improved insulin sensitivity (Nishizawa *et al.* 2002). Increased adiponectin levels in females is likely related to the low-testosterone state (Nishizawa *et al.* 2002).

Serum leptin levels also increased in gonadectomized *pttg*^{-/-} male mice when compared with control *pttg*^{-/-} males. Leptin, secreted from fat tissue, is a body-weight regulator through its control of feeding and energy expenditure. The association between leptin levels and insulin resistance are not yet fully delineated (Ceddia *et al.* 2002). The observed increase in serum leptin levels in gonadectomized *pttg*^{-/-} males was not as marked as the increased adiponectin levels, and therefore leptin is not a major candidate for diabetes protection in our model.

Compared with gonadectomy alone, estradiol treatment of gonadectomized *pttg*^{-/-} male mice conferred additional protection from diabetes development. These animals had normal fasting blood glucose levels for up to 13.5 months and a normal response to IPGTT for up to 12 months. These changes were accompanied by increased indices of insulin sensitivity and higher levels of adiponectin, but not leptin.

Several studies have shown that estrogen exerts protective effects on male animals with a genetic predisposition to develop diabetes. In *db/db* mice on the C57BL/KsJ background chronic low-dose estradiol therapy effectively ameliorated the severity of diabetes and obesity typical of this model, reduced body mass and restored functional pancreatic islet cytoarchitecture (hypercytolipidemia, cytohypertrophy and atrophy). These changes were accompanied by increased pancreatic and serum insulin levels

(Garris & Garris 2005). In obese hIAPP (human islet amyloid polypeptide) males, diabetes was prevented when estradiol therapy was started at a young age, and diabetes controlled when estradiol therapy was initiated at an older age. These protective effects were mediated by increased insulin sensitivity secondary to estrogen-induced weight loss. Estradiol therapy in these mice also prevented β -cell degeneration and amyloid deposition (Geisler *et al.* 2002).

Estradiol has been shown both *in vivo* and *in vitro* to have positive effects on the structure, size, number and function of pancreatic islet β -cells (Bailey & Ahmed-Sorour 1980, Puah & Bailey 1985, Zhu *et al.* 1998, Choi *et al.* 2005). Recent evidence supports the presence of a novel non-nuclear estrogen receptor which exerts rapid actions in the endocrine pancreas, enabling calcium fluxes that favor insulin secretion (Ropero *et al.* 2002, Sutter-Dub 2002). In our experiments serum insulin levels were not increased following gonadectomy and estradiol therapy; moreover, we did not observe beneficial trophic effects of estradiol on islet morphology. The results favor increased insulin sensitivity induced by estradiol in *pttg*-null mice. Mechanisms by which estradiol conferred almost total protection against diabetes development in *pttg*^{-/-} males are still undetermined.

Estrogen has a key role in body fat composition. Lack of estrogen, as seen in the postmenopausal state, is characterized by increased body fat and changes in body fat distribution shifting from peripheral to central adiposity concomitant with increased insulin resistance (Gambacciani *et al.* 2001, Wu *et al.* 2001, Liu *et al.* 2004). Male mice lacking estrogen receptors have increased adipose tissue, increased insulin resistance and impaired glucose tolerance (Heine *et al.* 2000).

The additional protection conferred by estradiol in our model could be due to decreased adipose tissue or its mobilization from central (android) to peripheral (gynoid) depots, which indirectly increases insulin sensitivity. Estradiol treatment in gonadectomized *pttg*^{-/-} and *pttg*^{+/+} male mice was accompanied with weight loss (data not shown), most probably through decreased fat mass, but we did not perform quantitative assessment of fat tissue and/or its distribution.

Taken together, the results suggest that gonadectomy with and without estradiol therapy confers diabetes protection in *pttg*^{-/-} male mice by increased insulin sensitivity associated with increased serum adiponectin levels and elevated leptin levels. Delayed diabetes onset in *pttg*^{-/-} females may also in part occur secondarily to higher levels of adiponectin and leptin when compared with age-matched *pttg*^{-/-} control males. Supporting these conclusions is the fact that the percent β -cell area, average islet size and the number of pancreatic islets per μm^2 were not increased in *pttg*^{-/-} males following gonadectomy, either with or without estradiol therapy. This conclusion is also supported by the unaltered fasted or challenged insulin and C-peptide levels following these

sex-steroid interventions. These results show that, in the absence of *pttg*, effects of male-selective β -cell hypoplasia are restored by sex-steroid environmental changes and their effect on peripheral insulin sensitivity.

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References

- Bailey CJ & Ahmed-Sorour H 1980 Role of ovarian hormones in the long-term control of glucose homeostasis. Effects of insulin secretion. *Diabetologia* **19** 475–481.
- Berg AH, Combs TP, Du X, Brownlee M & Scherer PE 2001 The adipocyte-secreted protein Acrp30 enhances hepatic insulin action. *Nature Medicine* **7** 947–953.
- Berg AH, Combs TP & Scherer PE 2002 ACRP30/adiponectin: an adipokine regulating glucose and lipid metabolism. *Trends in Endocrinology and Metabolism* **13** 84–89.
- Bhasin S 2003 Effects of testosterone administration on fat distribution, insulin sensitivity, and atherosclerosis progression. *Clinical Infectious Diseases* **37** Suppl 2 S142–S149.
- Ceddia RB, Koistinen HA, Zierath JR & Sweeney G 2002 Analysis of paradoxical observations on the association between leptin and insulin resistance. *FASEB Journal* **16** 1163–1176.
- Choi SB, Jang JS & Park S 2005 Estrogen and exercise may enhance beta-cell function and mass via insulin receptor substrate 2 induction in ovariectomized diabetic rats. *Endocrinology* **146** 4786–4794.
- Efrat S 1991 Sexual dimorphism of pancreatic beta-cell degeneration in transgenic mice expressing an insulin-ras hybrid gene. *Endocrinology* **128** 897–901.
- Fukui M, Koyama M, Nakagawa Y, Itoh Y, Nakamura N & Kondo M 2000 Castration and diabetes. *Diabetes Care* **23** 1032–1033.
- Gambacciani M, Ciaponi M, Cappagli B, De Simone L, Orlandi R & Genazzani AR 2001 Prospective evaluation of body weight and body fat distribution in early postmenopausal women with and without hormonal replacement therapy. *Maturitas* **39** 125–132.
- Garris DR & Garris BL 2005 Estrogenic restoration of functional pancreatic islet cytoarchitecture in diabetes (db/db) mutant C57BL/KsJ mice: relationship to estradiol localization, systemic glycemia, and persistent hyperinsulinemia. *Cell Tissue Research* **319** 231–242.
- Geisler JG, Zawalich W, Zawalich K, Lakey JR, Stukenbrok H, Milici AJ & Soeller WC 2002 Estrogen can prevent or reverse obesity and diabetes in mice expressing human islet amyloid polypeptide. *Diabetes* **51** 2158–2169.
- Goren HJ, Kulkarni RN & Kahn CR 2004 Glucose homeostasis and tissue transcript content of insulin signaling intermediates in four inbred strains of mice: C57BL/6, C57 BLKS/6, DBA/2, and 129X1. *Endocrinology* **145** 3307–3323.
- Haque WA, Shimomura I, Matsuzawa Y & Garg A 2002 Serum adiponectin and leptin levels in patients with lipodystrophies. *Journal of Clinical Endocrinology and Metabolism* **87** 2395–2398.
- Heine PA, Taylor JA, Iwamoto GA, Lubahn DB & Cooke PS 2000 Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *PNAS* **97** 12729–12734.
- Iglesias A, Murga M, Laresgoiti U, Skoudy A, Bernales I, Fullaondo A, Moreno B, Lloreta J, Field SJ, Real FX *et al.* 2004 Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice. *Journal of Clinical Investigations* **113** 1398–1407.
- Jones ME, Thorburn AW, Britt KL, Hewitt KN, Wreford NG, Proietto J, Oz OK, Leury BJ, Robertson KM, Yao S *et al.* 2000 Aromatase-deficient (ArKO) mice have a phenotype of increased adiposity. *PNAS* **97** 12735–12740.
- Kava RA, West DB, Lukasik VA & Greenwood MR 1989 Sexual dimorphism of hyperglycemia and glucose tolerance in Wistar fatty rats. *Diabetes* **38** 159–163.
- Kava RA, West DB, Lukasik VA, Wypijewski C, Wojnar Z, Johnson PR & Greenwood MR 1992 The effects of gonadectomy on glucose tolerance of genetically obese (fa/fa) rats: influence of sex and genetic background. *International Journal of Obesity Related Metabolism Disorders* **16** 103–111.
- Kim JH, Sen S, Avery CS, Simpson E, Chandler P, Nishina PM, Churchill GA & Naggert JK 2001 Genetic analysis of a new mouse model for non-insulin-dependent diabetes. *Genomics* **74** 273–286.
- Kromann H, Christy M, Lernmark A, Nedergaard M & Nerup J 1982 The low dose streptozotocin murine model of type 1 (insulin-dependent) diabetes mellitus: studies in vivo and in vitro of the modulating effect of sex hormones. *Diabetologia* **22** 194–198.
- Lanfranco F, Zitzmann M, Simoni M & Nieschlag E 2004 Serum adiponectin levels in hypogonadal males: influence of testosterone replacement therapy. *Clinical Endocrinology (Oxford)* **60** 500–507.
- Leiter EH 1981 The influence of genetic background on the expression of mutations at the diabetes locus in the mouse IV. Male lethal syndrome in CBA/Lt mice. *Diabetes* **30** 1035–1044.
- Leiter EH 1989 The genetics of diabetes susceptibility in mice. *FASEB Journal* **3** 2231–2241.
- Leiter EH, Chapman HD & Coleman DL 1989 The influence of genetic background on the expression of mutations at the diabetes locus in the mouse. V. Interaction between the db gene and hepatic sex steroid sulfotransferases correlates with gender-dependent susceptibility to hyperglycemia. *Endocrinology* **124** 912–922.
- Li FX, Zhu JW, Tessem JS, Beilke J, Varella-Garcia M, Jensen J, Hogan CJ & DeGregori J 2003 The development of diabetes in E2f1/E2f2 mutant mice reveals important roles for bone marrow-derived cells in preventing islet cell loss. *PNAS* **100** 12935–12940.
- Liu ML, Xu X, Rang WQ, Li YJ & Song HP 2004 Influence of ovariectomy and 17 beta-estradiol treatment on insulin sensitivity, lipid metabolism and post-ischemic cardiac function. *International Journal of Cardiology* **97** 485–493.
- Maclaren NK, Neufeld M, McLaughlin JV & Taylor G 1980 Androgen sensitization of streptozotocin-induced diabetes in mice. *Diabetes* **29** 710–716.
- Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF & Turner RC 1985 Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28** 412–419.
- Nilsson C, Niklasson M, Eriksson E, Bjorntorp P & Holmang A 1998 Imprinting of female offspring with testosterone results in insulin resistance and changes in body fat distribution at adult age in rats. *Journal of Clinical Investigations* **101** 74–78.
- Nishizawa H, Shimomura I, Kishida K, Maeda N, Kuriyama H, Nagaretani H, Matsuda M, Kondo H, Furuyama N, Kihara S *et al.* 2002 Androgens decrease plasma adiponectin, an insulin-sensitizing adipocyte-derived protein. *Diabetes* **51** 2734–2741.
- Oh JY, Barrett-Connor E, Wedick NM & Wingard DL 2002 Endogenous sex hormones and the development of type 2 diabetes in older men and women: the Rancho Bernardo study. *Diabetes Care* **25** 55–60.

- Paik SG, Michelis MA, Kim YT & Shin S 1982 Induction of insulin-dependent diabetes by streptozotocin. Inhibition by estrogens and potentiation by androgens. *Diabetes* **31** 724–729.
- Pei L & Melmed S 1997 Isolation and characterization of a pituitary tumor-transforming gene (PTTG). *Molecular Endocrinology* **11** 433–441.
- Puah JA & Bailey CJ 1985 Insulinotropic effect of ovarian steroid hormones in streptozotocin diabetic female mice. *Hormone Metabolism Research* **17** 216–218.
- Ropero AB, Soria B & Nadal A 2002 A nonclassical estrogen membrane receptor triggers rapid differential actions in the endocrine pancreas. *Molecular Endocrinology* **16** 497–505.
- Rossini AA, Williams RM, Appel MC & Like AA 1978 Sex differences in the multiple-dose streptozotocin model of diabetes. *Endocrinology* **103** 1518–1520.
- Saba S, Zhu W, Aronovitz MJ, Estes NA 3rd, Wang PJ, Mendelsohn ME & Karas RH 2002 Effects of estrogen on cardiac electrophysiology in female mice. *Journal of Cardiovascular Electrophysiology* **13** 276–280.
- Shi K, Mizuno A, Sano T, Ishida K & Shima K 1994 Sexual difference in the incidence of diabetes mellitus in Otsuka-Long-Evans-Tokushima-Fatty rats: effects of castration and sex hormone replacement on its incidence. *Metabolism* **43** 1214–1220.
- Stellato RK, Feldman HA, Hamdy O, Horton ES & McKinlay JB 2000 Testosterone, sex hormone-binding globulin, and the development of type 2 diabetes in middle-aged men: prospective results from the Massachusetts male aging study. *Diabetes Care* **23** 490–494.
- Sutter-Dub MT 2002 Rapid non-genomic and genomic responses to progestogens, estrogens, and glucocorticoids in the endocrine pancreatic B cell, the adipocyte and other cell types. *Steroids* **67** 77–93.
- Thomas MK, Devon ON, Lee JH, Peter A, Schlosser DA, Tenser MS & Habener JF 2001 Development of diabetes mellitus in aging transgenic mice following suppression of pancreatic homeoprotein IDX-1. *Journal of Clinical Investigations* **108** 319–329.
- Umeda M, Kanda T & Murakami M 2003 Effects of angiotensin II receptor antagonists on insulin resistance syndrome and leptin in sucrose-fed spontaneously hypertensive rats. *Hypertension Research* **26** 485–492.
- van Eickels M, Patten RD, Aronovitz MJ, Alsheikh-Ali A, Gostyla K, Celestin F, Grohe C, Mendelsohn ME & Karas RH 2003 17-beta-estradiol increases cardiac remodeling and mortality in mice with myocardial infarction. *Journal of the American College of Cardiology* **41** 2084–2092.
- Wang Z, Yu R & Melmed S 2001 Mice lacking pituitary tumor transforming gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division. *Molecular Endocrinology* **15** 1870–1879.
- Wang Z, Moro E, Kovacs K, Yu R & Melmed S 2003 Pituitary tumor transforming gene-null male mice exhibit impaired pancreatic beta cell proliferation and diabetes. *PNAS* **100** 3428–3432.
- Weksler-Zangen S, Yagil C, Zangen DH, Ornoy A, Jacob HJ & Yagil Y 2001 The newly inbred cohen diabetic rat: a nonobese normolipidemic genetic model of diet-induced type 2 diabetes expressing sex differences. *Diabetes* **50** 2521–2529.
- Wu SI, Chou P & Tsai ST 2001 The impact of years since menopause on the development of impaired glucose tolerance. *Journal of Clinical Epidemiology* **54** 117–120.
- Yamauchi T, Kamon J, Waki H, Terauchi Y, Kubota N, Hara K, Mori Y, Ide T, Murakami K, Tsuboyama-Kasaoka N *et al.* 2001 The fat-derived hormone adiponectin reverses insulin resistance associated with both lipoatrophy and obesity. *Nature Medicine* **7** 941–946.
- Zhu M, Mizuno A, Kuwajima M, Ogino T, Murakami T, Noma Y, Sano T & Shima K 1998 Ovarian hormone-induced beta-cell hypertrophy contributes to the homeostatic control of beta-cell mass in OLETF female rat, a model of Type II diabetes. *Diabetologia* **41** 799–805.
- Zou H, McGarry TJ, Bernal T & Kirschner MW 1999 Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. *Science* **285** 418–422.

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