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**Directed overexpression of insulin in Leydig cells causes a progressive loss
of germ cells**

**Katayoon Shirneshan^a, Stefan Binder^a, Detlef Böhm^a, Stephan Wolf^a, Ulrich Sancken^a,
Andreas Meinhardt^b, Michael Schmid^c, Wolfgang Engel^a, Ibrahim M. Adham^{a,*}**

^aInstitute of Human Genetics, University of Göttingen, D-37073 Göttingen, Germany;

^bDepartment of Anatomy and Cell Biology, University of Giessen, D-35385 Giessen,
Germany; ^cInstitute of Human Genetics, University of Würzburg, D-97074 Würzburg,
Germany.

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Corresponding author at: Institute of Human Genetics, University of Göttingen, Heinrich-
Düker-Weg 12, D-37073 Göttingen, Germany. Tel.: +49 551 397522; fax: +49 551 399303.

E-mail address: iadham@gwdg.de (I. Adham)

Abstract

The primary goal of this study was to determine the 5' region of the *Insl3* gene that specifically targets the expression of human insulin to Leydig cells, and to explore whether the testicular proinsulin is efficiently processed to insulin that is able to rescue the diabetes in different mouse models of diabetes. We show here that the sequence between nucleotides -690 to +4 of mouse *Insl3* promoter is sufficient to direct the Leydig cell-specific expression of the human insulin transgene (*Insl3-hIns*). We also found that the 3' untranslated region (3'UTR) of *Insl3* was effective in enhancing transgene expression of the insulin *in vivo*. Expression analysis revealed that the temporal expression pattern of the *hIns* transgene in Leydig cells of transgenic testes is roughly the same as that of the endogenous *Insl3*. Despite the Leydig cells translate human proinsulin and secrete a significant level of free C-peptide into the serum, the Leydig cell-derived insulin is not able to overcome the diabetes in different mouse models of diabetes, suggesting a lack of glucose sensing mechanisms in the Leydig cells. A consequence of overexpression of the human proinsulin in Leydig cells was the decrease of fertility of transgenic males at older ages. Germ cells in transgenic males were able to initiate and complete spermatogenesis. However, there was a progressive and age-dependent degeneration of the germ cells that lead to male infertility with increasing age.

1. Introduction

Diabetes mellitus is a group of diseases characterised by an absolute or relative insulin deficiency and by poor glucose control (hyperglycemia) in the blood. In type I diabetes, also known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus, there is an absolute lack of insulin due to the progressive loss of pancreatic β -cells in the islets of Langerhans. Despite intensive research efforts, daily insulin injection remains the only therapy. This unsatisfactory treatment strongly affects the life style of the patients and cannot achieve the same degree of glycemic control as provided by endogenous insulin produced from the pancreatic β -cells. Therefore, transplantation of insulin-producing tissues and gene therapy are potential therapeutically strategies to overcome type I diabetes. Large-scale application of islet cell implantations to type I diabetic patients is hindered by limited availability of insulin-producing tissues and the subsequent need for life-long immunosuppressants (Remuzzi et al., 1994). An alternative approach is the ectopic expression of insulin in other tissues using gene therapy. The goal is to restore and to control insulin production in another tissue, thereby reducing the elevated blood glucose levels to a normal range without causing hypoglycemia. For successful therapy, at least three crucial issues must be adequately addressed. The ectopic tissues expressing engineered insulin must have constitutive and regulated secretory pathways and express proprotein convertases PC2 and PC1/3 that are required for proinsulin processing (Steiner, 1998). These cells must have glucose sensing mechanisms for adequate regulation of insulin secretion according to blood glucose levels.

Several approaches, which have been taken in mind these crucial issues, have addressed the feasibility of *in vivo* gene therapy for the delivery of insulin to diabetic patients. Target tissues tested include liver, muscle, pituitary, exocrine pancreas and intestinal K-cells (Valera et al., 1994; Kolodka et al., 1995; Lipes et al., 1996; Goldfine et al., 1997; Bartlett et al.,

1997; Abai et al., 1999; Bochan et al., 1999; Cheung et al., 2000; Lu et al., 2005). The results are rather disappointing.

INSL3, a member of the insulin superfamily, is synthesized as preprotein in pre- and postnatal Leydig cells and play an essential role in growth of gubernaculum ligament during transabdominal descent of the testis (Zimmermann et al., 1997; 1999; Balvers et al., 1998; Nef and Parada, 1999; Adham et al., 2002; Koskimies et al., 2003). Although the structure of circulating INSL3 is only known in bovine (Büllesbach and Schwabe, 2002), the presence of certain conserved amino acids of the A and B chain at the N and C termini of the pro-INSL3 suggests that the mode of in vivo processing of pro-INSL3 and resulting structure of INSL3 is similar to that of insulin and relaxin (Adham et al., 1993; Hombach-Klonisch et al., 2004). It is known that the processing of the proinsulin occurs in the trans-Golgi by a regulated secretory pathway (Kelly, 1985; Rhodes and Halban, 1987). Similar to islet β cells, several endocrine cells such as intermediate lobe pituitary cells, and gastrointestinal G and K cells efficiently process proinsulin to mature, biologically active insulin via a regulated pathway of protein secretion (Lipes et al. 1996, Cheung et al., 2000; Lu et al., 2005), whereas in hepatocytes, which have only a constitutive pathway of protein secretion, proinsulin processing to insulin is extremely inefficient (Valera et al., 1994; Kolodka et al., 1995).

To assess the efficiency of the endocrine Leydig cells in processing the proinsulin to the biological active insulin and to determine the ability of the testicular insulin to overcome the diabetes in mice, we overexpressed insulin in the Leydig cells during pre- and postnatal development. The rationale for this study was underpinned by our previous study showing that ectopic expression of *Insl3* in β -cells rescue the cryptorchidism in *Insl3*-null males. These results reveal that the β -cells efficiently processed the *Insl3* gene product to the functional hormone. To achieve our goal, the insulin was targeted using the upstream regulated sequence of murine *Insl3* gene, and transgenic mice were generated. To determine whether the

testicular insulin would rescue diabetes observed in the Pax-4 deficient mice, we introduced the transgenic allele into the *Pax-4*^{-/-} mice.

2. Materials and Methods

2.1. Generation of transgenic mice

The 0.7-kb *HindIII/XbaI* fragment containing the 5' flanking region of mouse *Ins13* was isolated from the p690-CAT plasmid (Zimmermann et al., 1998) and subcloned into pBluescript IKS+ (Stratagen) to produce pIns13. To generate the Ins13-hIns/1 construct, a 1.7-kb genomic fragment containing exons 1-3 of human insulin gene (*hIns*) was amplified using the primers Hins2F: 5'- GCC GCT CTA GAC CTG AGC CCT CCA GGA CAG -3' and Hins2R: 5'- GCC GCG CGG CCG CGG GAG GGG CTC ACA ACA GTG C -3'. The 1.7-kb amplified fragment with an artificial XbaI site at 5'-end and a NotI site at 3'-end was inserted into the XbaI/NotI-digested pIns13 to create Ins13-hIns/1 construct. A 1.3-kb *XbaI/NotI* fragment containing the genomic sequence of *hIns* without the 3' untranslated region was amplified using the primers Hins2F and Hins11R: 5'- GCC GCG CGG CCG CCC TGC AGG CTG CGT CTA GTT -3' and subcloned into pIns13 to create the pIns13-hInsΔ3'. Finally, a 250-bp *NotI/SstII* genomic fragment containing the 3' untranslated region of mouse *Ins13* was amplified using the primers Mins11F: 5'- GCC GCG CGG CCG CTG AAG GAG CAC AGG GCT CAG G -3' and Mins11R: 5'- GCC GCC CGC GGC GGT CTG GTC CGT GTG TGA CC -3', and inserted into pIns13-INS to generate the construct Ins13-INS/2. The nucleotide sequence of the amplified fragments was confirmed by DNA sequencing.

The fragments containing mouse *Ins13* promoter-human insulin hybrid gene (Ins13-hIns) were separated from the constructs Ins13-hIns/1 and Ins13-hIns/2 by *HindIII/NotI*, purified by agarose gel electrophoresis, and microinjected into fertilized FVB/N eggs to generate transgenic mice Ins13-INS/1 and Ins13-INS/2.

Mice were genotyped for the presence of the transgene by PCR designed to amplify a region spanning the junction between the mouse *Ins13* promoter and the human insulin gene.

For genotyping the transgenic *Ins13-INS/1* animals, PCR assays with primers Lyk2, 5'-GCCGCAAGCTTCCGCACCTGGGAGAGG-3' and HinsR3, 5'-GGCAGAAGGACAGTGATCTGG-3' were performed. Thermal cycling was carried out for 35 cycles, denaturation at 94°C for 30 sec, annealing at 54°C for 30 sec, and extension at 72°C for 45 sec. Primers Lyk2 and HinsR5: 5'-AAAAAAGTGACACCTGACCCCCTGC-3' were used for genotyping the transgenic *Ins13-INS/2* mice. Copy number and number of integration sites of transgenic founders were determined by a quantitative real-time PCR approach as described previously (Boehm et al., 2004).

Transgenic lines were maintained on the genetic background of FVB/N. All animal experiments were carried out using protocols approved by the Medical Faculty of University of Göttingen and Research Advisory Committee.

2.2. Generation of *Ins13-hIns: Pax4^{-/-}* double mutant mice

Mating *Ins13-hIns* hemizygous males with *Pax4^{+/-}* females (Sosa-Pineda et al. 1997) produced *Ins13-hIns: Pax4^{+/-}* mice, which were then crossed to produce mice homozygous for the disrupted *Pax4* gene and hemizygous for the *Ins13-hIns* transgene (*Ins13-hIns: Pax4^{-/-}*). The following primers were used to discriminate between wild-type and mutant *Pax4* alleles. Primers Pax 4F: 5' ATG GCT GTG TGA GCA AGA TCC -3' and primer Pax 4R: 5' TCC AGC TTC CCT TAA CTC CAG -3' were designed to amplify a 250-bp fragment of wild-type allele. The primer LacZ-F. 5' TTT GCG TAA GTG AAG CGA C -3' was used with the primer LacZ-R: 5' AGC GGC TGA TGT TGA ACT G -3' to amplify a 500-bp fragment of mutant *Pax4* allele.

2.3. RNA analysis

Different tissues were dissected out from 3-month-old wild-type and transgenic mice or from testes of 1-, 3-, 5-, 6-, 8-, 10-, 15-, 20-, and 25-d-old males. Total RNA was prepared using total RNA Isolation Reagent (Biomol, Hamburg), electrophoresed on 1.2% agarose gels

containing 2.2 M formaldehyde and transferred onto a nylon membrane. The membrane was hybridized with ^{32}P -labelled human insulin cDNA fragment. RNA integrity was checked by rehybridization of blots with a cDNA probe for human elongation factor-2.

RT-PCR assays were performed using 2 μg of total RNA and a One Step RT-PCR kit (QIAGEN). Primers to amplify the *PC1* cDNA fragment were 5'- TCA ACC AGA GCA CAT GAA GC -3' and 5'- GCA GCA GCC TGT CAT CTC TA -3', and those to amplify the *PC2* transcript were 5'- ATG GAG GGC GGT TGT GGA TC -3' and 5'- CAG GTA CCA TTG CTT TGT AAA GAG A -3'.

2.4. Immunohistochemistry

Testis and pancreas from wild-type and *Ins13-hIns* transgenic mice were fixed in Bouin's fixative and transferred to 70% ethanol before paraffin embedding. Sections (5 μm) were cut from paraffin blocks with a rotary microtome and mounted on SuperFrost Plus glass slides (Fisher Scientific, Nepean, Canada). Sections were deparaffinized in xylene, rehydrated in a descending alcohol. Thereafter, tissue sections were preincubated for 1 h with 10% normal horse serum in 2% Tween/PBS and incubated overnight at 4°C with 1:500 mouse monoclonal anti-insulin antibody (Sigma, St. Luis, USA), washed in PBS, and then incubated with CY3-conjugated goat anti-mouse antibody (1:500) (Sigma) for 30 min at room temperature. Slides were then washed in PBS, stained with DAPI (Vector), and examined with a BX60 microscope (Olympus, Hamburg, Germany) with fluorescence equipment and an analysis software program (Soft Imaging System, Münster, Germany).

2.5. Measurement of proinsulin and C-peptide level in protein extracts and serum

To determine pancreatic and testicular proinsulin content, whole pancreas and testis was removed from the mice, weighted, and homogenized in 20 vol of cold acidic ethanol (75% ethanol, 1.5% concentrated HCl) followed by 48 h of agitation at 4°C. Thereafter, insulin was quantified in the supernatants of the samples by time-resolved fluoroimmunoassay. Insulin

concentrations in testis and pancreas extracts were determined by Auto DELFIA Insulin Assay following the supplier's protocol (Perkin-Elmer Life Sciences, Wallac Oy, Finland). Serum C-peptide was determined by radioimmunoassay (RIA) (Linco Research, St. Charles, USA) following the manufacturer's instructions, using specific ^{125}I -labelled human C-peptide as tracer and a human C-peptide antibody. Antibody was 100% crossreactive with human C-peptide and < 4% crossreactive with human proinsulin (Sensitivity of 0.1 ng/ml).

2.5. Generation of diabetic mice

To induce insulin-dependent diabetes, 2-month-old male mice were injected intraperitoneally with streptozotocin (STZ) (Sigma, Munich, Germany) (33.3 mg/kg body weight), dissolved in 50 mmol/l citrate buffer (pH 4.5) for 5 consecutive days. Blood glucose was determined four times a week in blood obtained from the tail vein using a Heamogluco-Test glucometer (Roche Molecular Biochemicals, Mannheim, Germany). Mice were considered diabetic after two consecutive blood glucose values of more than 250 mg/dl.

For glucose tolerance tests, STZ-treated transgenic and non-treated wild-type mice were fasted overnight with free access to water and thereafter injected intraperitoneally with glucose (1 mg/kg body weight). Blood samples were obtained from the tail vein before the glucose injection and at 20, 40, and 60 min after injection, and the glucose concentration was measured as indicated above.

2.6. Fertility test and sperm parameters

To investigate the fertility of the transgenic males on a FVB/N background, 3-month-old male mice were mated each with two wild-type FVB/N females. Females were checked for the presence of vaginal plug and/or pregnancy. Pregnant females were removed to holding cages to give birth. Litter size sired by each group of males was determined after 4-, 8- and 12-months of breeding.

The epididymides from transgenic male mice were collected and dissected in IVF medium. Sperm number in the cauda epididymis was determined using a Neubauer counting cell chamber. Motility was analysed by a CEROS computer-assisted semen analysis system (version 10; Hamilton Thorne Research, Beverly, Mass).

2.7. Statistical analysis

All results are presented as the means \pm SD (SEM). The significance of differences was assessed using the Student's *t* test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Generation of *Insl3-hIns* transgenic mice

Analyses of the mouse *Insl3* promoter *in vitro* predicted that the proximal region of *Insl3* promoter might be sufficient for targeted expression of reporter gene in Leydig cells (Zimmermann et al., 1998; Koskimies et al., 2002). To determine the regulatory sequences of *Insl3*, which are required for Leydig cell-specific expression of reporter gene *in vivo* and for stability of transgenic RNA in Leydig cells, two transgenic constructs were designed using the 5' flanking region of *Insl3* (-690 to +4) fused to the human insulin gene (*hIns*). In the first construct (*Insl3-hIns/1*), the three exons of *hIns* including the 3'untranslated region were ligated downstream of 5'regulatory sequence of *Insl3*. In the second construct (*Insl3-hIns/2*), the 3'untranslated sequence of *hIns* was replaced with that of mouse *Insl3* (Fig. 1A). Both fusion constructs were microinjected into the pronuclei of fertilized FVB/N mouse eggs. Four and five transgenic founders were found to carry the *Insl3-hIns/1* and the *Insl3-hIns/2* fusion allele, respectively, as detected by PCR assay using specific primers (data not shown). Transgenic mice were maintained as hemizygotes and mated to wild-type FVB/N for further analyses.

3.2. Expression analysis of transgenic insulin in Leydig cells

To determine whether the *Insl3*-INS transgenic alleles are transcriptional active, Northern blots containing total RNA extracted from testes of F1-animals of four transgenic *Insl3*-INS/1 and five transgenic *Insl3*-hIns/2 founders were hybridized with the *hIns* cDNA probe. As shown in figure 1B, the expression of *Insl3*-hIns/1 transgenic allele was only detectable at low level in testis of line #4. In contrast, high levels of *hIns* mRNA were found in testes of four *Insl3*-hIns/2 transgenic lines (Line #5, #7, #8 and #9). Using the sensitive RT-PCR assay, we were able to detect the *hIns* transcripts in testes of transgenic lines #1, #2 and #3, but not in line #6. Breeding of transgenic mice from line #6 with wild-type females revealed that the transgenic allele in this line is only transmitted to male offspring indicating the integration of fusion construct in the Y-chromosome. Using real time PCR, we have estimated the number of copies and integration sites of transgenic allele in genome of transgenic lines #4, #5, #7, #8 and #9. PCR analysis of founders and their progeny showed that the *Insl3*-hIns transgenic DNA was only inherited with constant copy numbers in lines #4, #5 and #9 suggesting the presence of one integration site in genome of these lines (data not shown). Higher levels of the *Insl3*-hIns/2 expression in testes of transgenic lines #5 and #9 with 3 and 16 copies of transgenic allele, respectively, than that of *Insl3*-hIns/1 allele in transgenic line #4 with 9 copies suggested that the 3'untranslated sequence of *Insl3* enhances transgene expression *in vivo*. Due to single integration site of the transgene in genome and the robust transgenic expression level in testes of transgenic lines #5 and #9, these lines were used for further analyses. Transgenic mice of both lines were healthy and had a normal lifespan.

To determine the extent of transgene expression, we performed Northern blot analysis of various tissues from hemizygous offspring of line #5 using a *hIns* cDNA probe. A transcript of the expected size (0.7-kb) was observed in testis of transgene, but not in other tissues (Fig. 1C).

The temporal regulation of the *hIns*-transgene expression was analyzed by Northern blot with testicular RNA from sequential time points of postnatal development. During the first three weeks of testis development, equal levels of the transgene transcript were observed. The *Ins13* expression was also determined from the same blot using an *Ins13* cDNA probe. After three weeks of postnatal testis development, the *hIns* mRNA level gradually increased, showing an expression pattern roughly similar to that of the endogenous *Ins13* mRNA (Fig. 1D).

The distribution of hINS protein in the testis was determined by using monoclonal antibody to human insulin. In testis sections of transgenic mice, insulin immunoreactivity was detected in Leydig cells, confirming that human insulin protein production was specifically targeted to Leydig cells (Fig. 1E). Results of these expression experiments provide evidence that the sequence -690 to +4 of the 5' flanking region of *Ins13* directs *in vivo* the transcription of reporter gene in Leydig cells but not in other tissues, indicating that the cis-acting elements of Leydig cell-specific expression reside within this region.

3.3. Leydig cells translate and process proinsulin

To determine the translation rate of the transgene, proinsulin content was measured in testes and pancreas of fasted wild-type and transgenic males (> 8-week-old) by a fluoroimmunoassay, which specifically recognizes the human and mouse form of proinsulin. As shown in figure 2A, proinsulin levels in testicular extracts of transgenic mice ($10.75 \pm 0.99 \mu\text{U}/\text{mg}$, $n = 13$) were not significantly different from that in pancreas of wild-type ($11.6 \pm 1.34 \mu\text{U}/\text{mg}$, $n = 6$) and transgenic pancreas ($12.23 \pm 1.08 \mu\text{U}/\text{mg}$, $n = 13$). Similar concentrations of proinsulin were found in transgenic and control pancreas. In contrast, only basal level of proinsulin was detected in wild-type testis (Fig. 2A). These results suggest that the human proinsulin produced by transgenic allele do not alter the expression of endogenous insulin production in the pancreas.

To determine the testicular expression of prohormone convertases PC2 and PC1/3 that process the proinsulin to insulin, we performed RT-PCR analysis. This analysis revealed the expression of PC1 and PC1/3 in testicular Leydig cells (data not shown).

Whether testicular proinsulin is processed to insulin which is secreted into serum, free human C-peptide in serum of fasted wild-type and transgenic males was measured with C-peptide-specific immunoassay that has no cross-reactivity with mouse insulin or human proinsulin. The levels of human C-peptide in samples collected from individual mice of transgenic line #5 and #9 was 0.51 ± 0.45 ng/ml ($n = 11$) and 0.43 ± 0.41 ng/ml ($n = 11$), respectively, whereas serum C-peptide levels in wild-type males were below the detection limits of radioimmunoassay (Fig. 2B). However, the C-peptide levels in serum of both transgenic lines were lower than the normal level of C-peptide in human serum (0.5-4.0 ng/ml). The presence of human C-peptide in serum of transgenic males indicates that the Leydig cell-produced proinsulin is processed to insulin, and secreted into the serum. However, transgenic mice display normal blood glucose levels whether fed or fasted (Fig. 2C). The body weights of transgenic males at the age of 3- and 6-months were indistinguishable from those of their control littermates (Fig. 2D).

3.4. Testicular insulin can not rescue the diabetic Pax-4 deficient mice

We have then investigated the efficiency of Leydig cell-derived human insulin to rescue the diabetes in Pax4-deficient mice, in which the development of β - and σ -cells of the pancreas is impaired (Sosa-Pineda et al., 1997). We have introduced the *Ins13-hIns/2* allele into the genome of *Pax4*^{-/-} mice by breeding the line # 5 with the *Pax4*^{+/-} mice and analyzed the glucose level and lifespan of the *Pax4*^{-/-}: *Ins13-hIns/2* double mutant mice in F2 generation. The resulting double-mutant progeny from heterozygous intercrosses were born as expected from Mendelian ratio, but died within 7 days after birth. Blood glucose levels of the double mutant neonates were not significantly different from those in *Pax4*^{-/-} littermates

(220.3 ± 12.2 versus 242 ± 9.3 , $n = 6$ in each group), but were significantly higher than that in wild-type littermates (120.3 ± 4.6 , $n = 6$). These results indicate that testicular INS was not able to overcome the diabetes resulting from the impaired development of pancreatic β -cells in *Pax4*^{-/-} mice.

3.5. Leydig cells lack the glucose sensing mechanism

The insufficiency of testicular insulin to rescue the *Pax4*^{-/-} neonates from diabetes may be due to impairment of Leydig cells to secrete high levels of human insulin in response to increase of blood glucose levels. Therefore, we examined whether insulin production from Leydig cells was able to maintain glucose tolerance in the transgenic animals. Streptozotocin (STZ), a β -cell toxin, was administered to transgenic and wild-type mice. In both transgenic and wild-type animals, STZ-treatment resulted in hyperglycemia (Fig. 2E) indicating the development of diabetes. To ensure that the STZ treatment did not destroy the Leydig cells in these experimental animals, expression of the *Insl3* was determined in testes of controls and STZ-treated transgenic animals. Results of the RNA analysis revealed that the expression of *Insl3* is not affected from STZ-treatment, indicating that the Leydig cells escape the STZ treatment (data not shown). Fasted STZ-treated transgenic and untreated wild-type animals were then intraperitoneally injected with glucose. Blood glucose levels were dramatically decreased in control animals at 40 and 60 min after injection. In contrast, the human insulin produced from Leydig cells of transgenic mice was not sufficient to restore normal glucose tolerance (Fig. 2F).

3.6. Progressive loss of germ cells in the *Insl3*-hIns-transgenic testis

To identify and establish homozygous transgenic mice, 10 transgenic males and females from F2 generation were crossed with wild-type mice. Genotyping of offspring from these breeding revealed that all homozygous transgenic females of line #5 and #9 are fertile. In contrast, all fertile males of both transgenic lines are hemizygous for transgene. Using real

time PCR, we have estimated the copy number of transgenic allele in the genomes of 4 infertile males and their fertile littermates. PCR analysis revealed that infertile males from each line have double copy numbers of transgenic allele as compared to hemizygous males suggesting that increased expression of transgenic allele impairs the fertility of homozygous animals.

Breeding of hemizygous transgenic males with wild-type mice revealed that the fertility of transgenic males at 4-months-old did not significantly differ from that of controls. However, the litter size produced by transgenic males declined with advancing age (Table 1). We noticed a large variability in the age on onset of infertility among transgenic mice. Some mice were initially fertile but developed infertility later on.

To investigate the underlying cause of reproductive defects in transgenic males, sperm parameters were analyzed in great detail. As shown in Table 1, significant reduction in the mean number of spermatozoa and the proportion of motile and progressively motile sperm collected from the cauda epididymides of 8- and 12-months-old transgenic mice ($p < 0.001$).

Next, we investigated potential pathologic alterations in testes of 4-, 8- and 12-months-old transgenic mice. At 4-months-old, there were no gross morphological differences between wild-type and transgenic seminiferous tubules (Fig. 3A, B). Transgenic testes from 8-month-old revealed a mixed atrophy type of damage showing seminiferous tubules with apparently normal spermatogenesis, tubules with focally degenerated germ cells or tubules with almost complete loss of spermatogenic cells. Often damaged tubules contained vacuoles. Overall, a large variability in the degree of testicular degeneration was observed within one tubule cross-section, amongst neighbouring tubules and amongst testes of different transgenic mice at this age (Fig. 3C). During the next months, the phenotype becomes more pronounced. In testes of 12-months-old transgenic mice, about 85% of all seminiferous tubules displayed severe impairment of spermatogenesis with severe vacuolization of most affected tubules (Fig. 3D). To determine whether the germ cell loss in transgenic testis resulted from increased germ cell

apoptosis, we used TUNEL assay to detect apoptotic cells in 4- and 8-months-old wild-type and transgenic testis. In transgenic testis, we observed only a few apoptotic cells, and the number and position of these cells were similar to those of wild-type, indicating that the degeneration of spermatogenic cells is not due to increased germ cell apoptosis (data not shown). Therefore, the observed phenotype may be caused by impairment of early germ cell proliferation or differentiation rather than by a specific arrest during spermatogenesis.

4. Discussion

Previous cell culture experiments have shown that the sequence from -690 to +4 of *Ins13* gene was able to direct expression of reporter constructs in Leydig cell lines (Zimmermann et al., 1998; Koskimies et al., 2002). In order to determine whether this part of the promoter is also sufficient in vivo to direct tissue- and cell-specific expression, transgenic mice were generated. The transgene *Ins13-hIns/1* contains the sequence between -690 to +4 of *Ins13* promoter cloned upstream of the human insulin gene as reporter. The data show that this transgenic allele is expressed at low levels in all four generated lines. The results of many studies showed that the 3'-untranslated region of a gene influences the stability of its mRNA (Ross, 1995; Kakoki et al., 2004). Based on these results, we replaced the 3'-untranslated region of human insulin gene with that of mouse *Ins13* in the second construct *Ins13-hIns/2*. Four of five *Ins13-hIns/2* transgenic lines showed significantly higher transgene expression than those in testes of *Ins13-hIns/1* transgenic lines, despite the presence of comparable copy number of both transgenic alleles. These results suggest that the 3'-untranslated region of *Ins13* contains elements, which are responsible for stability of transgenic transcripts in Leydig cells. Just as endogenous *Ins13* expression, expression of transgenic *Ins13-hIns/2* allele is restricted to Leydig cells and expression level is constant through the first three weeks of postnatal testis development and increases gradually thereafter. Thus, the promoter and the

3' untranslated region of *Ins13*, which we have characterized here, will provide a useful tool to direct the specific expression of genes to Leydig cells *in vivo*.

We next explored whether the testicular proinsulin is efficiently processed to insulin which is able to rescue the diabetes in Pax-4^{-/-} mice. The *in vivo* data revealed that proinsulin content of transgenic testes was not significantly different from that of wild-type pancreas. The presence of a significant amount of immunoreactive free human C-peptide in serum of transgenic mice suggests that the ectopically produced insulin is secreted in the circulation. However, the testis-derived insulin is not able to reverse diabetic symptoms in Pax4-deficient mice. Endogenous insulin expression and secretion in pancreatic β - cells is regulated by glucose, so that insulin will be produced and released in response to rise in blood glucose levels and is suppressed as blood glucose concentration declines. Results of glucose tolerance experiments revealed that a major limitation of this current gene delivery system is that the insulin secretion in the Leydig cells is not glucose regulated. A number of studies have engineered a variety of cells and tissues to produce insulin in transgenic mice. Lack of the glucose sensing mechanisms in target tissues, including liver, pituitary, muscle and exocrine pancreas, limit the clinical application of these approaches (Kolodka et al., 1995; Lipes et al., 1996; Goldfine et al., 1997; Bartlett et al., 1997; Abai et al., 1999; Valera et al., 1994; Bochan et al., 1999). In contrast, transgenic mice producing human proinsulin under the control of the K-cell specific *GIP* promoter have been shown to produce mature bioactive insulin in a glucose concentration-dependent manner (Cheung et al., 2000).

Insulin signalling is important for promoting and regulating of growth, differentiation, and metabolism. Disruption of components of the insulin-signaling pathway can result in infertility (Rane et al., 2000; Brüning et al., 2000). Reduced secretion of LH and FSH in diabetic men and in conditional knockout mice, which lack the insulin receptor (IR) in brain, revealed that insulin plays a central role in regulation of hypothalamic pituitary testicular axis (Brüning et al., 2000; Baccetti et al., 2002). Impaired spermatogenesis of diabetic men was

similar to those in IR-conditional knockout mice and characterized by increased germ cell depletion and Sertoli cell vacuolization. A notable phenotype in Ins13-hIns transgenic males is a progressive loss of germ cells. Normal glucose level in blood of transgenic males leads to exclude the idea that the disrupted spermatogenesis in aged mice is a result of diabetes. Therefore, there are two explanations for impaired fertility of aged mice. The ectopic expression of transgenic insulin gene in Leydig cells is not regulated by glucose level as it is the case for endogenous gene in β -cells. The overexpression may lead to overload of Leydig cells with proinsulin and induce endoplasmic reticulum (ER) stress. Such chronic ER stress caused by excessive demand of insulin in type II diabetes is responsible for β -cell exhaustion and decrease its secretory capacity (Oyadomari et al., 2002). Therefore, we suggest that the overload of Leydig cells with proinsulin may be deleterious for Leydig cell function. Dysregulation of steroidogenesis in transgenic males could be perturbed and that could explain the disruption observed on spermatogenesis yield and sperm motility. Alternatively, the Leydig cell-producing insulin has autocrine effects that negatively influence germ and Sertoli cell survival.

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Figure legends

Fig. 1. Generation and expression analysis of the *Insl3-hIns* transgenic alleles. A, Schematic representation of the pInsl3-hIns/1 and pInsl3-hIns/2 transgenic construct. The pInsl3-hIns/1 transgenic construct consists of the 0.7-kb of the *Insl3* promoter (solid box) and the entire human insulin gene (*hIns*). In the pInsl3-hIns/2, the *SstI/NotI* genomic fragment containing the 3' untranslated region of the human insulin gene was replaced with that of the mouse *Insl3* (square box). Numbered boxes indicate location of exons of the *hIns*; thin lines show intron sequence. Relevant restriction sites used for generation of the transgenic vectors are indicated. H, *HindIII*; N, *NotI*; S, *SstI*; X, *XbaI*. B, Expression of the *Insl3-hIns/1* and *Insl3-hIns/2* transgenic allele in adult testes of different transgenic (L) lines and wild-type mice (WT) was determined by Northern blot hybridization with the *hIns* cDNA. Integrity of the RNA samples was confirmed by rehybridization of the blot with the elongation factor-2 cDNA (EF-2). C, Hybridization of Northern blot with total RNA from different tissues of transgenic line # 5 reveals that the expression of the *Insl3-hIns/2* transgenic allele is restricted in the testis. D, Expression pattern of *Insl3-hIns/2* transgenic allele during the postnatal development of testis was determined by hybridization of Northern blot with testicular RNA isolated from transgenic mice of line # 5 at different postnatal days (P). Blot was subsequently hybridized with the *hIns*-, *Insl3*- and *EF-2*-cDNA probes. D, Immunohistochemistry on sections of wild-type (WT) and transgenic testis (Tg). Insulin is highly accumulated in the Leydig cells of *Insl3-hIns* transgenic mice.

Fig. 2. Proinsulin content in testis and pancreas, C-peptide level in serum and regulation of testicular insulin release. A, Insulin and proinsulin immunoreactivity in testis and pancreas. RIA analysis was performed in acid extracts of testis and pancreas obtained from fasted wild-type (WT) and transgenic mice (Tg). B, Level of human C-peptide in serum of fasted wild-

type and transgenic mice of line #5 and #9. C, Tail vein blood glucose measured in ad libitum-fed or 16-hour-fasted wild-type and transgenic males at the age of 3 and 6 months (M). D, Body weight of male wild-type and transgenic mice is given at the age of 3 and 6 months (M). E, Hyperglycemia development in wild-type and transgenic mice after STZ-treatment. Glucose level was determined at the indicated times after STZ-treatment. F, Intraperitoneal insulin tolerance test. Thirty days after STZ treatment, overnight-starved STZ-treated transgenic and non-treated wild-type mice were intraperitoneally injected with 1mg/kg body weight glucose, and serum glucose was determined at the indicated time.

Fig. 3. Progressive depletion of germ cells in testes of *Insl3-hIns* transgenic mice with advancing age. H&E stained testis sections of 4-month-old wild-type (A), 4- (B), 8- (C) and 12-month-old transgenic mice (D) show a progressive degeneration of germ cells in the seminiferous tubules. In testis of 4-month-old transgenic mice (B), there was no gross morphological difference between wild-type (A) and transgenic seminiferous tubules (B). Panels show testicular sections of 8- (C) and 12-month-old transgenic mice (D). A significant proportion of tubules in 8- and a large majority of tubules in 12-month-old transgenic mice showed impairment of spermatogenesis with formation of vacuoles, release of premature germ cells and focal as well as full degeneration of germ cells (*). Magnification, X10.

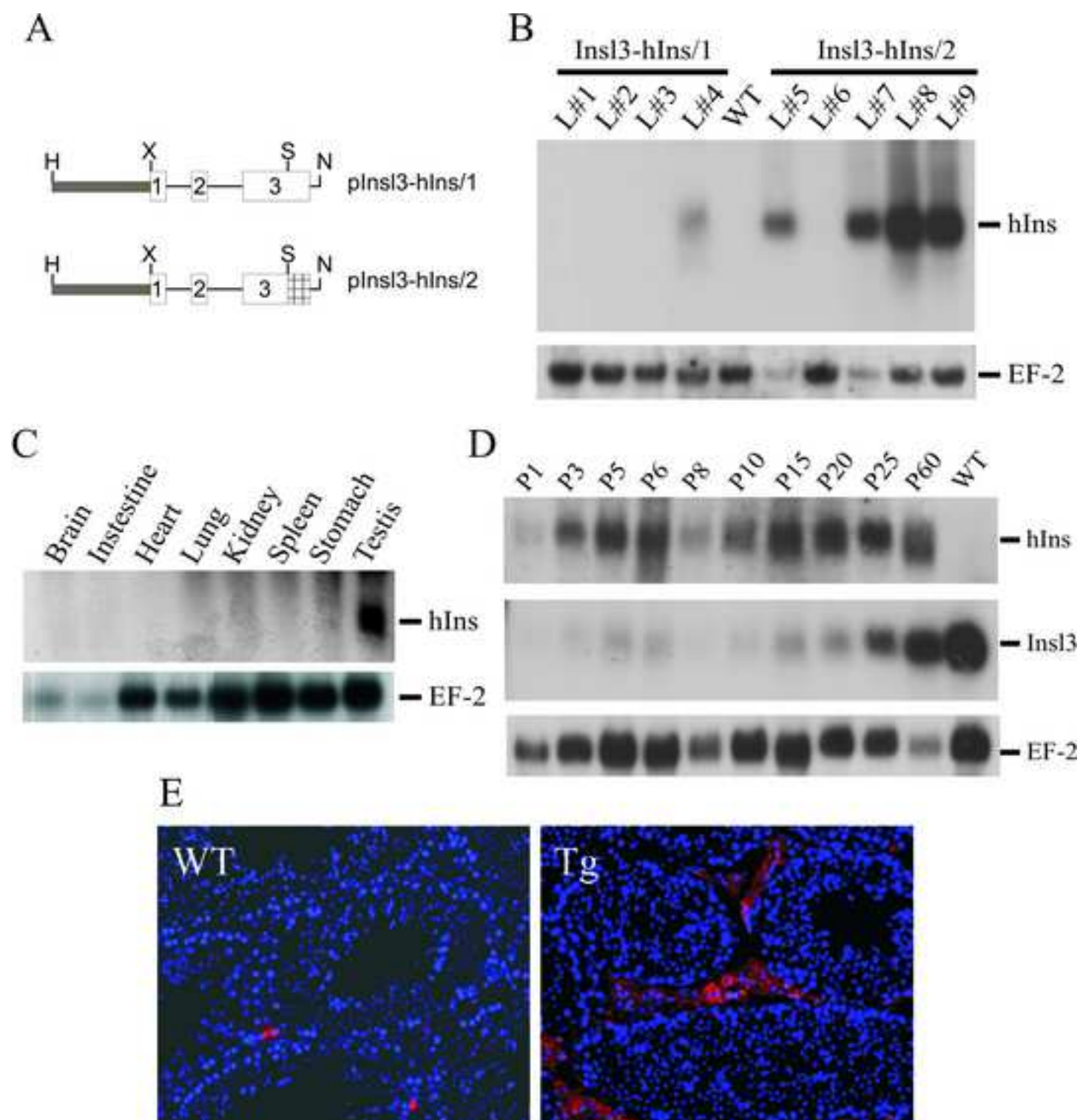
Table 1

Fertility and sperm parameters of transgenic and wild-type mice during postnatal development

		4-month	8-month	12-month
Litter size	Wild-type mice	6.6 ± 1.2 (6)	5.8 ± 0.8 (6)	5.2 ± 1.3 (6)
	Transgenic mice	4.8 ± 0.8(8)	2.8 ± 1.0* (8)	1.5 ± 0.7* (6)
No. of sperm (x = 10 ⁶)	Wild-type mice	13.2 ± 3.1 (3)	8.6 ± 3.4 (3)	5.7 ± 3.4 (3)
	Transgenic mice	5.2 ± 0.6(3)	4.5 ± 0.3* (4)	1.2 ± 0.8* (5)
Sperm motility (%)	Wild-type mice	61 ± 4.6 (3)	45 ± 6.3 (3)	33.6 ± 6.4 (3)
	Transgenic mice	48 ± 8.2 (3)	30.3 ± 7.0 (4)	13.6 ± 7.4* (5)
Progressive motility	Wild-type mice	42.9 ± 4.6 (3)	27.3 ± 8.3 (3)	21 ± 4.6 (3)
	Transgenic mice	29.7 ± 3.6 (3)	19.2 ± 5.8 (4)	6.2 ± 6.1* (5)

Data are the mean ± SD of the number of individual measurements indicated in parentheses.

* The value for this parameter for transgenic mice is significant different compared with nontransgenic at same ages ($p < 0.001$, Student's t test).



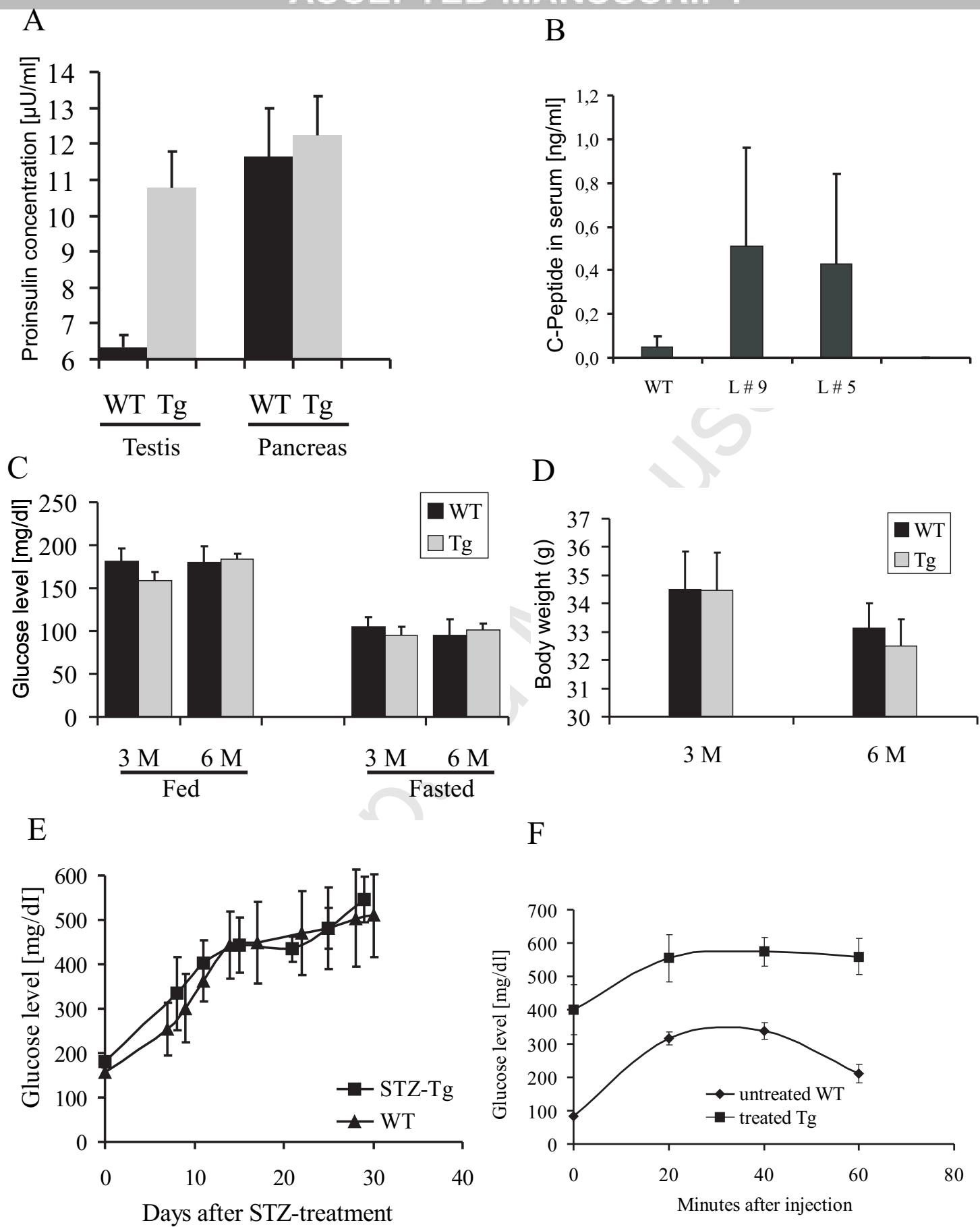


Figure 3

