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Quantitative real-time PCR detection of insulin signalling-related genes in pancreatic islets isolated from healthy cats

Eric Zini a*, Marco Franchini b, Melania Osto c, Andrea Vögtlin b, Franco Guscetti d, Philippe Linscheid a, Karin Kaufmann a, Brigitte Sigrist a, Mathias Ackermann b, Thomas A. Lutz c, Claudia E. Reusch a

* Corresponding author. Tel.: +41 44 6358746; fax: +41 44 6358930.
E-mail address: ezini@vetclinics.uzh.ch (Eric Zini).
Abstract

The cat has been recently proposed as a valuable model for type 2 diabetes mellitus (T2DM), because feline diabetes shares several similarities to the disease in humans. Impaired β-cell function, decreased β-cell mass, insulin resistance that is often related to obesity, and pancreatic amyloid deposition, are among the most important common features. In this study and to further develop the cat as a model of T2DM, we aimed to isolate feline pancreatic islets and establish real-time PCR quantification of mRNA transcripts of genes central to β-cell function and survival. In particular, mRNA quantification systems were determined for insulin, the insulin enhancer pancreatic duodenal homeobox-1 (PDX-1), the insulin suppressor CCAAT/enhancer binding protein-beta (C/EBPβ), glucose transporter isoform 2 (GLUT2), Fas receptor, the caspase-8 inhibitor FLIP (FLICE [caspase-8]-inhibitory protein) and two chemokines, interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1). Pancreatic islets were isolated by collagenase digestion from healthy cat donors. Partial feline mRNA sequences were determined for PDX-1, C/EBPβ, GLUT2 and FLIP using primers identified from conserved regions of human, dog and rat mRNA on feline pancreatic islet cDNA. These novel and the previously available sequences (insulin, Fas receptor, IL-8 and MCP-1) were used to design feline-specific primers suitable for real-time PCR in isolated pancreatic islets. The adopted protocol of collagenase digestion yielded pancreatic islets that were frequently surrounded by acinar cells. Quantification of mRNA transcripts was simple and reproducible in healthy cats. Characterisation of genes related to insulin signalling in cats will prove useful to better understand the pathogenesis of feline diabetes and possibly of human T2DM.

Keywords: Feline; Diabetes; Beta-cell; Transcript; mRNA.
Introduction

The domestic cat has been proposed as an attractive model of T2DM (Lutz and Rand, 1995; Henson and O’Brien, 2006). In contrast to commonly used rodent models, cats spontaneously develop a form of diabetes that is clinically and pathologically very similar to human T2DM. Among shared features, diabetic cats are often middle aged, genetics play a role in diabetes risk, obesity leads to insulin resistance, and impaired β-cell function can be recognized in healthy and diabetic cats. The most striking histological similarity between human T2DM and diabetes in cats occurs at the pathological level in the pancreatic islets. As in diabetic humans, islet amyloidosis is found in more than 80% of diabetic cats and most of them have around 50% β-cell loss (Lutz and Rand, 1997; Appleton et al., 2001, Butler et al., 2003; Rand et al., 2004; Henson and O’Brien, 2006). Late complications similar to those occurring in humans, such as diabetic retinopathy and polyneuropathy are also described in the feline species (Linsenmeier et al., 1998; Mizisin et al., 2002). The study of feline diabetes is often hampered by the lack of tools to characterize the molecular mechanisms through which glucose metabolism is disturbed in diabetic cats, particularly in pancreatic islets and β-cells.

Based on studies in affected humans and in rodent models of the disease, a defect in the β-cell insulin secretory machinery is considered one of the most important processes leading to T2DM (Maedler and Donath, 2004). It has been demonstrated that chronic high glucose levels per se can impair the insulin stimulus-secretion coupling. This occurs, at least in part, due to the direct effect of glucose excess on the regulatory elements of the insulin gene, in particular the insulin enhancer pancreatic duodenal homeobox-1 (PDX-1) and the insulin suppressor CCAAT/enhancer binding protein-beta (C/EBPβ) (Seufert et al., 1998; Marshak et al., 1999). PDX-1 is a homeodomain protein that binds to the promoter of the
insulin gene and exerts a potent stimulation of insulin transcription (Marshak et al., 1999). C/EBPβ belongs to a family of basic leucine zipper transcription factors and serves as a transcriptional repressor of insulin in pancreatic β-cells via interactions with the insulin promoter (Seufert et al., 1998).

In isolated pancreatic islets of humans and in vivo in diabetic rats, it has been shown that chronically elevated glucose levels induce β-cell exhaustion followed by decreased insulin gene expression. This change is associated with decreased expression and protein binding of the transcription enhancer PDX-1 and transcriptional upregulation of the suppressor element C/EBPβ (Seufert et al., 1998; Marshak et al., 1999). In addition to the regulatory activity of PDX-1 on the insulin promoter, the enhancer element also participates in the transcriptional control of the glucose transporter isoform 2 (GLUT2) (Waeber et al., 1996). GLUT2 is a membrane protein that facilitates glucose diffusion through cell membranes in pancreatic β-cells, liver, small intestine and kidney. In β-cells, PDX-1 binds to the GLUT2 promoter and transactivates transcription of the GLUT2 gene (Waeber et al., 1996). Similar to PDX-1, GLUT2 expression is decreased in rat and mouse models of T2DM (Johnson et al., 1990; Chankiewitz et al., 2006).

Several authors have recently highlighted the role of a reduced β-cell mass in T2DM (Butler et al., 2003; Yoon et al., 2003). In cultured islets from humans and rodents, sustained elevation of glucose concentration directly initiated β-cell apoptosis, through Fas receptor upregulation and activation (Donath et al., 1999; Meadler et al., 2001). The apoptosis-inducing receptor Fas is a sub-member of the tumor necrosis factor receptor family. Engagement of Fas receptors by Fas ligand results in β-cell apoptosis through activation of caspase-8 (Maedler et al., 2001; Maedler et al., 2002a). However, Fas receptor signalling may
also be implicated in proliferative signals. An endogenous inhibitor of caspase-8, FLIP (FLICE [caspase-8]-inhibitory protein) seems to switch Fas signalling from apoptosis to survival/proliferation (Maedler et al., 2002b). In human β-cells, FLIP is constitutively expressed but downregulated by high glucose (Maedler and Donath, 2004). Finally, increased numbers of islet-associated macrophages have been observed in rodent models and in humans with T2DM (Ehses et al., 2007). In the same study, an increased amount of chemokines, especially interleukin (IL)-8, was secreted by cultured human and mouse islets exposed to high glucose levels. Inflammatory cells migrated into pancreatic islets because of a combination of upregulated chemotactic factors are suspected to contribute to β-cell death in T2DM (Ehses et al., 2007).

The aim of the present study was to establish in cats real-time polymerase chain reaction (PCR) detection methods to quantify the expression of mRNAs of crucial genes involved in the above mentioned processes of diabetic islet pathology. The methods were established using pancreatic islets isolated from healthy cats. In particular, real-time PCR systems were established to quantify transcripts of insulin, PDX-1, C/EBPβ, GLUT2, and the chemokines IL-8 and monocyte chemoattractant protein-1 (MCP-1). Detection tools for mRNA transcripts of two genes related to apoptosis, specifically the Fas receptor and FLIP were also set-up in the cat.

Materials and methods

Animals and isolation of pancreatic islets

A group of five healthy neutered male domestic short-hair cats, 18 month-old and weighing 3.3-4.1 kg (median 3.7 kg), was maintained at the animal care facility of the Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, University of Zürich, Switzerland.
Animal studies were approved by the Cantonal Veterinary Office of Zürich. Cats were determined to be healthy on the basis of physical examination and clinical laboratory data. After fasting for 12 h, cats were sedated with tiletamine/zolazepam (Zoletil 100, Virbac) and anaesthesia was induced with propofol (Propofol 1%, Fresenius-Kabi). An intravenous (IV) overdose of sodium pentobarbital (Esconarkon, Streuli Pharma) was injected to euthanase the cats. Immediately thereafter, the whole pancreas was surgically excised under sterile conditions from each donor.

The isolated tissue was immersed in Hank’s balanced salt solution (HBSS) and care was taken to remove fat tissue surrounding the pancreas with sterile surgical scissors. The pancreas was weighed and injected with a chilled HBSS solution containing 1 unit/mL of collagenase NB8 (Serva Electrophoresis), using a 27G needle connected to a 10 mL syringe. The injected solution volume (mL) to pancreas weight (g) ratio was approximately 5 to 1 and the solution was injected in equal aliquots through 5-10 different punctures. The inflated pancreas was placed in a Falcon tube containing the same amount of injected solution and was incubated at 37 °C for 35-40 min. The digested tissue was washed with an ice-cold solution of HBSS and filtered through a stainless steel screen with a 1 mm mesh. The residual non-filtered tissue remaining on the steel screen was placed in an additional tube containing 30 mL solution of HBSS and collagenase for a second digestion, incubated for 5-10 min and then filtrated again. Immediately after filtration, the two filtrates were placed in separate Falcon tubes and filled up with HBSS containing 10% fetal calf serum (FCS) in a 1 to 1 volume ratio. The tubes were left on ice for 5 min allowing sedimentation of the digest. Thereafter the supernatants were aspirated and the pellets washed 2-3 times at 5 min intervals with HBSS. Rinsed pellets were then transferred into a single tube.
After collagenase digestion, the isolated tissue was rapidly frozen in liquid nitrogen and stored at −80 °C until further use. In addition, from each isolate an aliquot was placed in vials containing 4% buffered formaldehyde for 24 h and embedded into paraffin using standard methods.

To verify whether collagenase treatment yielded free pancreatic islets, sections of formalin-fixed, paraffin-embedded digests were prepared and subsequently immunohistochemically stained for insulin and cytokeratin, using a polyclonal guinea pig anti-swine insulin antibody (Dako Cytomation) and a monoclonal mouse anti-human cytokeratin clone MNF116 antibody (Dako). Paraffin sections (3 µm) were deparaffinized in xylene and rehydrated through graded ethanol to water. Antigen retrieval consisted of a 5 min (for insulin) and a 10 min (for cytokeratin) incubation with protease (REAL Proteinase K, Dako) diluted in Tris buffer (pH. 7.5) as indicated by the manufacturer. The immunohistochemical reactions were performed using a commercially available detection kit (Dako REAL™ Detection system, peroxidase/AEC rabbit/mouse) according to the manufacturer's instructions. All steps were performed at room temperature in an automated device (Dako Autostainer). Primary antibody incubation conditions were 1:200 for 1 h for insulin and 1:50 for 30 min for cytokeratin. The sections were counterstained with Mayer's hematoxylin.

RNA isolation and reverse transcription

Total RNA from pancreatic islets was extracted using RNeasy Mini Kit (Qiagen). Pancreatic pellets (30 mg) were homogenised using the Mixer Mill MM 300 (Qiagen) for 1 min at 30 Hz. Possible genomic DNA contamination in islet-derived RNA samples was eliminated by including DNase-treatments (DNase-Free DNase Set, Qiagen). RNA was
quantified spectrophotometrically (ND-1000 Spectrophotometer, NanoDrop) and the quality was assessed by identifying 18S and 28S rRNA bands on gel electrophoresis. cDNA was obtained from 1 μg samples of islet-derived RNA (Omniscript RT Kit, Qiagen) in the presence of 13 U of RNasin (Promega). cDNA was subjected to PCR using PCR Taq core kit (Qiagen) on a conventional thermal cycler (T-personal, Biometra).

Partial sequencing of feline-specific mRNAs

Partial or complete mRNA sequences of feline insulin (NM_001009272), Fas receptor (NM_001009314), IL-8 (AF158598) and MCP-1 (DQ835566) have been previously deposited in GenBank. For PDX-1, C/EBPβ, GLUT2 and FLIP whose mRNA sequences are not available in cats, conserved regions were identified from human, canine and rat sequence alignments. PCR primers were designed with a web-based tool (http://bibiserv.techfak.uni-bielefeld.de/genefisher/) using canine or human sequences. Feline pancreatic islet-derived cDNA was subjected to PCR amplification with canine or human primer pairs located in conserved sections (Table 1). A total volume of 25 μL contained 1 μL cDNA template, 0.2 mM dNTPs, 0.025 units/μL Taq DNA polymerase and 2.5 μL reaction buffer (PCR Taq Core Kit, Qiagen), with specific sense and antisense primers, each at a final concentration of 300 nM. To perform the PCR, for each target, an initial denaturation step of 3 min at 94 °C was followed by 40 cycles of 30 s at 94 °C, an annealing of 45 s at 60 °C and extension of 60 s at 72 °C.

When present, multiple amplicons were eliminated by optimising PCR conditions or by replacing the primers until single products of the expected size were obtained. Amplicons were purified with QIAquick PCR purification kit (Qiagen) and both strands were sequenced using the same primers (Microsynth).
For PDX-1 mRNA, single amplicons were not achieved (Electronic supplementary material_1). Therefore, the expected PDX-1 band was extracted from a 2% agarose gel using the QIAex gel extraction kit II (Qiagen), followed by cloning. To clone the PCR fragments the TOPO TA cloning kit (Invitrogen) was used. Briefly, 4 mL of cleaned PCR product, 1 mL of salt solution and 1 mL of vector were mixed and processed further according to the manufacturer’s instructions. Two clones were sent for sequencing using the M13 forward and reverse primers (M13 forward: 5’-TGTTAAACGACGGCCAG-3’; M13 reverse: 5’- CAGGAAAACACGCTATGACC-3’) (Microsynth).

Quantitative analysis of mRNA

cDNA obtained from feline tissues was subjected to quantitative real-time PCR analysis using feline-specific oligonucleotides (Table 2). Primers for insulin, PDX-1, C/EBPβ, GLUT2, Fas, FLIP, IL-8 and MCP-1 sequences were designed within the newly determined or previously published feline-specific mRNA sequences, using Primer Express 3.0 software (Applied Biosystems) or Primer3 software (http://frodo.wi.mit.edu/). Primer pair selection criteria were set to generate short amplicons of 67-217 base pairs (bp) with annealing temperature of 60 °C and without predicted dimer formation. Published feline-specific oligonucleotides were used for detection of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Kipar et al., 2001). As an additional housekeeping gene, primers were designed to quantify cyclophilin A using the published mRNA sequence (AY029366). PCR reactions were prepared with 10 μL Power SYBR-Green Master Mix (Applied Biosystems), a final concentration of 500 nM of each primer and 5 μL of template diluted 1:50 in a total reaction volume of 20 μL.
Using the iCycler iQ sequence detection system (BioRad, Hercules), an initial
denaturing step was performed for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s,
60 °C for 30 s and 80 °C for 10 s, then 95 °C and 60 °C for 1 min each and 80 cycles of 10 s,
starting from 60 °C with a 0.5 °C increase after each cycle. At the end of the programme,
PCR product identity was confirmed by melting curve analysis and DNA sequencing
(Microsynth).

Because SYBR-Green PCR detection for PDX-1 and GLUT2 mRNAs was not
satisfactory (melting curves with 2-3 peaks), primers and probe sets were designed (Table 2).
Criteria for primer selection were as above. Probes were designed such as G was not present
at the 5’-end of the sequence, and melting temperature was 70 °C. Probes were labelled at the
5’-end with FAM (6-carboxyfluorescein) and at the 3’-end with TAMRA (5,6-
tetramethylrhodamine). For probe-based quantification of GAPDH mRNA, published feline
sequences were used (Kipar et al., 2001). PCR reactions consisted of 12.5 μL qPCR
Mastermix (Eurogentec, Seraing, Belgium), primers and probe to final concentrations of 900
nM and 250 nM, respectively, and 5 μL of cDNA diluted 1:50 in a 25 μL total reaction
volume. Cycling parameters were an initial denaturation of 10 min at 95 °C, followed by 45
cycles of 95 °C for 15 s and 60 °C for 1 min. PCR product identity was confirmed by DNA
sequencing as above.

Parallel reactions were performed in triplicate for every feline cDNA sample. A
template-free control and a sample without reverse transcriptase were included in each
amplification run. For each reaction, triplicate standard curves were generated with five 10-
fold dilutions of the purified specific PCR product previously obtained by conventional PCR.
Standard curves were also used to assess amplification efficiency of each PCR assay. Target
gene mRNA was quantified using the relative standard curve method (Perkin-Elmer Cetus User Bulletin no. 2, 1997). Gene expression was normalised to the respective quantities of GAPDH and cyclophilin A.

To assess the reproducibility of the listed quantitative real-time PCR assays, within- and between-run precision were assessed by multiple measurements of several dilutions of the cDNA. Three cDNA samples were evaluated in seven replicates (within-run precision) and in four separate experiments (between-run precision). For the within-run precision, coefficients of variation (\(CV\)) were calculated using the threshold cycle (CT) value (\(CV_{CT}\)). Because cDNA degradation due to repeated sample melting/freezing procedures may modify the CT values, between-run precision was assessed with the CV calculated for the relative copy number (\(CV_{rel}\)).

Results

Isolation of pancreatic islets in cats

With the adopted protocol of isolation, the amount of retrieved islet tissue appeared to be considerably enhanced by exposing the pancreas to two consecutive collagenase digestions. Indeed, on average, with the second digestion the volume of digest contained in Falcon tubes seems to double. With immunohistochemical staining for insulin all clumps were shown to contain a pancreatic islet. However, as demonstrated by cytokeratin immunostaining, residual acinar cells surrounded most islets with an approximate exocrine to endocrine pancreatic area ratio of 3-4 to 1 (Fig. 1).

Partial mRNA sequences of feline genes
By applying a PCR-based approach, we partially determined the feline-specific mRNA sequences, and deduced the corresponding protein sequences, of the insulin gene suppressor C/EBPβ, GLUT2 and the caspase-8 inhibitor FLIP. For the insulin gene enhancer PDX-1, sequencing was achieved after more than 20 unsuccessful different primer sets and cloning into a vector was performed. The GenBank accession numbers and the degree of homology of the feline mRNA and protein sequences to the human, canine and rat counterparts are given in Table 3.

mRNA expression in isolated pancreatic islets in cats

Expression of insulin, PDX-1, C/EBPβ, GLUT2, IL-8, MCP-1, Fas receptor and FLIP transcripts were quantified in isolated islets of healthy cats (Fig. 2). For targets quantified with SYBR-Green detection, similar expression levels were achieved when GAPDH or cyclophilin A was used as the housekeeping gene (Fig. 3).

To verify real-time PCR assay specificity, PCR products were analysed by gel electrophoresis. All targets produced a single band at the expected bp length. In addition, for SYBR-Green detection, primer specificity was confirmed after each run by melting curve analysis. A single melting peak and at the expected melting temperature was observed in all cases (Fig. 4). Finally, sequencing of the PCR products revealed identical nucleotide sequences to those newly determined or formerly available for each target (Electronic supplementary material_3).

Amplification efficiency of each PCR assays was calculated by serial dilutions of a same template and ranged from 83-99%. To assess within- and between-run precision, dilutions of cDNA were assayed in replicates on the same plate or in separate experiments.
The CV\textsubscript{CT} of the within-run precision and the CV\textsubscript{rel} of the between-run experiments are reported in Table 4.

**Discussion**

In order to provide additional tools to explore the role of cats as a model for T2DM, we determined partial gene sequences, and we designed primers for quantification of mRNA levels of crucial genes that regulate insulin secretion or that are implicated in \(\beta\)-cell survival. With this study, we made accessible four new feline-specific partial mRNA sequences of insulin signalling-related genes, including PDX-1, C/EBP\(\beta\), GLUT2 and FLIP.

We then tested feline-specific real-time PCR primers nested within the mRNA sequences and determined transcript quantities using cDNA derived from pancreatic islets of healthy cats. Some of these sequences had previously been available (insulin, Fas, IL-8, MCP-1). In order for the results to be meaningful, validating the set of SYBR-Green primer pairs and of TaqMan primer and probe combinations was necessary. Amplification efficiency was evaluated using standard curves generated from 10-fold dilutions of cDNA products achieved by conventional PCR. Quantitative PCR assays were efficient and linear over the range tested. Furthermore, within- and between run variation was shown to be low and reproducible results were obtained in each case. Finally we examined product specificity by melting curve analysis for SYBR-Green targets, and gel electrophoresis and DNA sequencing for all PCR assays. Using the parameters tested here, the assays appear to be specific for the desired genes. Therefore, this study provides for the first time a set of primers and probes that are appropriate to quantify cDNA transcripts of insulin, PDX-1, C/EBP\(\beta\), GLUT2, Fas, FLIP, IL-8 and MCP-1 in feline pancreatic tissue.
It has been demonstrated that β-cell mRNA expression of commonly used internal control genes such as GAPDH and β-actin can be upregulated by high glucose levels in INS-1 β-cell line and in vivo in rats (Roche et al., 1997; Rodriguez-Mulero and Montanya, 2005). In contrast, internal controls such as the minor ribosomal particle 18S (18S rRNA) and, to a lesser extent, cyclophilin A seem less susceptible to the effects of hyperglycemia (Rodriguez-Mulero and Montanya, 2005). Because it is yet unknown whether the reference gene GAPDH is constitutively transcribed at a constant level in the feline pancreas, in particular during diabetes, we sought to establish real-time PCR assay for a second internal control gene. Based on the above studies and on GenBank available housekeeping gene sequences in cats we designed primers for cyclophilin A (AY029366). Normalization of mRNA transcripts with Cyclophilin A yielded similar results to GAPDH in healthy cats. The two reference genes have not been studied in diabetes in cats. We believe that the use of a second internal control gene will be useful for comparison of gene expression differences in feline pancreatic islets, possibly under different experimental conditions.

To establish real-time PCR quantification of the above mRNA transcripts in cats, isolation of pancreatic islets was mandatory. To our knowledge, isolation of islets has been attempted in the feline species by a single group (Maeno et al., 2006). Different from the reported technique, a second exposure to collagenase was performed. We expected to increase the amount of retrieved islet tissue. Even though the modified procedure substantially augmented the volume of digest, the average quality of isolated islets was only moderately satisfactory. Indeed, a majority of isolated islets was surrounded by residual acinar cells. As suggested by Maeno et al. (2006), feline islets are difficult to separate completely from acinar tissue by collagenase digestion because they are surrounded only minimally by extracellular matrix. This feline anatomic feature likely accounted for the incomplete isolation. In order to
improve the purity of isolates and perform more reliable molecular studies in the field of feline diabetes, it will be necessary to compare different protocols of islet isolation in cats in future work. In particular, the use of additional collagen digestion solutions, pure or mixed with collagenase and followed or not by Ficoll centrifugation of the filtrate may represent an alternative.

Conclusions

In conclusion, we determined feline-specific mRNA sequences of important insulin signalling-related genes and provided a simple and reproducible method to quantify gene transcripts in isolated pancreatic islets by real-time PCR. Isolation of pancreatic islets by collagenase digestion is feasible in cats even though surrounding acinar cells accompany most islets. The present tools developed for cats should prove useful to better understand the pathogenesis of feline diabetes and, possibly, some of the mechanisms underlying β-cell dysfunction and decreased β-cell mass in T2DM.

Acknowledgements

We are grateful to Dr. Valentino Cattori, Clinical Laboratory, University of Zurich, Switzerland, for kindly providing primer sequences for feline cyclophilin A.

Conflict of interest Statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.
References


2003. Selective beta-cell loss and alpha-cell expansion in patients with type 2 diabetes
**Table 1**

Primers used for amplification of feline cDNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (5′-3′)</th>
<th>Antisense (5′-3′)</th>
<th>Approximate product size (bp)</th>
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<tbody>
<tr>
<td><em>PDX-1</em></td>
<td>CAGCTCAGCTCGCGCTCCCCC</td>
<td>GRCGGTTYTGRAACCAGATYT</td>
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<tr>
<td><em>C/EBPβ</em></td>
<td>CTACCAGGCAGGTGCCGAGCG</td>
<td>GTTCCGCAGGGTGCTGAGCT</td>
<td>300</td>
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<tr>
<td><em>GLUT2</em></td>
<td>TCCAGTTTGGATATGACATT</td>
<td>CTGCCCTTCTCCACAAGGAATA</td>
<td>950</td>
</tr>
<tr>
<td><em>FLIP</em></td>
<td>CAGGTCGAAGAGGCACTTGA</td>
<td>CATCCACCTCCAGGAAGCTGC</td>
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### Table 2

**Feline-specific primers**

<table>
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<th>Gene</th>
<th>Sense (5′-3′)</th>
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<th>Probe (5′-3′)</th>
<th>Product size (bp)</th>
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<td>Insulin</td>
<td>TTCGTCAACCAGCACCTGTG</td>
<td>CACAGCATGCTCCACGATG</td>
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<tr>
<td>PDX-1</td>
<td>TCCCGTGATGAACTTACC</td>
<td>CGGCTCGAGATGTATTTTG</td>
<td>TACACTCGCGCGAGCTGCT</td>
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<tr>
<td>C/EBPβ</td>
<td>GCGACAAGGCCAACAGATGC</td>
<td>GGCAGCTGCTGAAACAGTIC</td>
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<tr>
<td>GLUT2</td>
<td>GCGCCCTTGGGACACTT</td>
<td>AGGCGAATCTGACATGATG</td>
<td>ACCAGCTGGCATTTCACAGGC</td>
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<tr>
<td>Fas</td>
<td>AGACCTGCGTTAGAAGTGGAA</td>
<td>CTGGAGAGCGTTACAAAAAAGTGTGA</td>
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<tr>
<td>FLIP</td>
<td>GCAAGCCTGGGAATCT</td>
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<tr>
<td>IL-8</td>
<td>TCGATGCCAGTGCAAAAAACT</td>
<td>GTGGGCACTGCTAATCA</td>
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<tr>
<td>MCP-1</td>
<td>TGGCTAGCGCCAGAGCTAA</td>
<td>TGGGTCAGCGCAGATCTCA</td>
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<tr>
<td>Cyclophilin A</td>
<td>CAAAGTTCCAAGACAGCAGAGA</td>
<td>AGTGCCATTATGGCGTGGTA</td>
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</table>
Table 3

GenBank accession numbers and degrees of mRNA and protein homology of genes partially sequenced in the cat

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Human mRNA</th>
<th>Human Protein</th>
<th>Canine mRNA</th>
<th>Canine Protein</th>
<th>Rat mRNA</th>
<th>Rat Protein</th>
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<tbody>
<tr>
<td>PDX-1*</td>
<td>EU286552</td>
<td>91%</td>
<td>95%</td>
<td>95%**</td>
<td>96%</td>
<td>83%</td>
<td>89%</td>
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<tr>
<td>C/EBPβ</td>
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<td>94%</td>
<td>100%</td>
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<tr>
<td>GLUT2</td>
<td>EF451817</td>
<td>87%</td>
<td>85%</td>
<td>91%**</td>
<td>87%</td>
<td>82%</td>
<td>84%</td>
</tr>
<tr>
<td>FLIP</td>
<td>EF451818</td>
<td>84%</td>
<td>76%</td>
<td>90%**</td>
<td>87%</td>
<td>79%</td>
<td>70%</td>
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</table>

n.a., not available

(*), alignment between feline PDX-1 partial sequence and corresponding sequence in dog, human and rat is provided in Electronic supplementary material_2.

(**), predicted canine sequence
## Table 4

Within- and between-run precision of real-time PCR assays. Coefficients of variation are expressed as mean ± SD.

<table>
<thead>
<tr>
<th>Real-time PCR assay</th>
<th>Within-run precision</th>
<th>Between-run precision</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CV&lt;sub&gt;CT&lt;/sub&gt; (%)</td>
<td>CV&lt;sub&gt;rel&lt;/sub&gt; (%)</td>
</tr>
<tr>
<td>Insulin</td>
<td>0.9 ± 0.2</td>
<td>15.1 ± 7.1</td>
</tr>
<tr>
<td>PDX-1</td>
<td>1.1 ± 0.4</td>
<td>24.0 ± 7.6</td>
</tr>
<tr>
<td>C/EBPβ</td>
<td>1.1 ± 0.5</td>
<td>16.8 ± 7.3</td>
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<tr>
<td>GLUT2</td>
<td>1.3 ± 0.5</td>
<td>26.1 ± 7.0</td>
</tr>
<tr>
<td>Fas</td>
<td>0.6 ± 0.1</td>
<td>20.3 ± 7.6</td>
</tr>
<tr>
<td>FLIP</td>
<td>0.8 ± 0.3</td>
<td>12.7 ± 5.7</td>
</tr>
<tr>
<td>IL-8</td>
<td>0.8 ± 0.4</td>
<td>25.4 ± 5.0</td>
</tr>
<tr>
<td>MCP-1</td>
<td>0.8 ± 0.2</td>
<td>24.2 ± 7.4</td>
</tr>
<tr>
<td>Cyclophilin A</td>
<td>0.9 ± 0.2</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

CV<sub>CT</sub>, coefficient of variation for the threshold cycle

CV<sub>rel</sub>, coefficient of variation for the relative copy number

n.d., not determined
Figure legends

**Fig. 1.** Immunohistochemistry of paraffin-embedded pancreatic digest. (A) insulin immunostaining (pink) and (B) cytokeratin “Lu5” immunostaining (pink-red) indicate that the isolation procedure produced tissue clumps consisting of pancreatic islets surrounded by acinar cells.

**Fig. 2.** mRNA transcript levels of genes in isolated pancreatic islets of five healthy cats. Values are normalized to GAPDH and expressed relative to an internal calibrator (cat 1).

**Fig. 3.** mRNA transcript levels of one sample target (MCP-1) normalized to GAPDH and to cyclophilin A in five healthy cats. Expression profiles calculated with the two reference genes are comparable. Values are expressed relative to an internal calibrator (cat 1).

**Fig. 4.** Melting curve of SYBR-Green based real-time PCR assays in isolated pancreatic islets of five healthy cats. Mean threshold cycles (mCT) are given for each target. For the TaqMan based PDX-1 and GLUT2 assays, mCT are 25.2 and 29.3, respectively.