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Regulation of Core CLOCK genes in human islets

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Abstract

Objective: Nearly all mammalian cells express a set of genes, known as clock genes. These regulate the circadian rhythm of cellular processes by means of negative and positive auto-regulatory feedback loops of transcription and translation. Recent genome-wide association studies have demonstrated an association between a polymorphism near the circadian clock gene CRY2 and elevated fasting glucose. To determine whether clock genes could play a pathogenetic role in the disease, we examined mRNA expression of core clock genes in human islets from donors with or without Type 2 Diabetes.

Materials/Methods: Microarray and qRT-PCR analysis were used to assess expression of the core clock genes CLOCK, BMAL-1, PER1-3 and CRY1-2 in human islets. Insulin secretion and insulin content in human islets were measured by radioimmunoassay.

Results: The mRNA levels of PER2, PER3 and CRY2 were significantly lower in islets from donors with Type 2 Diabetes. To investigate the functional relevance of these clock genes, we correlated their expression to insulin content and HbA1c levels: mRNA levels of PER2 (rho=0.33; p=0.012), PER3 (rho=0.30; p=0.023) and CRY2 (rho=0.37; p=0.0047) correlated positively with insulin content. Of these genes, expression of PER3 and CRY2 correlated negatively with HbA1c levels (rho= -0.44; p=0.0012; rho= -0.28; p= 0.042). Furthermore, in an in vitro model mimicking pathogenetic conditions, the PER3 mRNA level was reduced in human islets exposed to 16.7 mM glucose/1 mM palmitate for 48h (p=0.003). Conclusions: Core clock genes are regulated in human islets. The data suggest that perturbations of circadian clock components may contribute to islet pathophysiology in human Type 2 Diabetes.

Keywords: Circadian rhythm, Type 2 Diabetes, Insulin secretion, Islets
Abbreviations  *CLOCK*: Circadian Locomotor Output Cycles Kaput; *BMAL1*: Brain and muscle Arnt-like protein 1; *PER*: Period; *CRY*: Cryptochrome.
Introduction

Virtually all living cells exhibit intrinsic rhythmic behaviour. This has an impact on the regulation of fundamental biological processes, including cellular metabolism [1, 2]. Of these rhythms, circadian rhythm has been most extensively studied. It has a frequency of ~24 h, and is endogenously generated. Importantly, circadian rhythm is subject to modification by exogenous signals. This is known as entrainment.

During the last few years, global and unbiased genetic analyses have produced a wealth of new information about the pathogenesis of Type 2 Diabetes [3]. This is very important because the incidence of the disease is soaring. Appropriate and successful treatment of Type 2 Diabetes is facilitated by thorough knowledge about the pathogenesis of the disease. To this end, it was recently observed that a common variant in the gene of the melatonin receptor 1B (MTNR1B) is associated with increased risk of Type 2 Diabetes [4-7]. The hormone melatonin has its peak secretion from the pineal gland, which is embedded within the brain, during night time. It serves as a “zeitgeber”, entraining circadian rhythm in peripheral tissues. We could demonstrate that mRNA expression of MTNR1B in pancreatic islets was increased in carriers of the risk variant [4]. Because melatonin exerts an inhibitory effect on glucose-stimulated insulin secretion (GSIS) [4, 8, 9], we hypothesized that the observed impairment of insulin secretion in the examined patient cohorts was due to a gain of function of the risk allele [10].

The molecular machinery of circadian rhythm is progressively being unraveled [11]. Fundamentally, it consists of oscillating loops of transcription factor expression. They exert alternatively positive and negative feedback on expression of core clock genes. Central to this mechanism are the transcription factors CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1). Their key targets are the Period (PER1-3) and Cryptochrome (CRY1-2) genes. Very recently, members of this group of core clock genes
were implicated in the pathogenesis of Type 2 Diabetes. A variant near \textit{CRY2} was found to associate with elevated fasting glucose in humans [12], and disruption of circadian rhythm specifically in pancreatic β-cells leads to diabetes owing to impaired insulin secretion in rodents [13, 14].

Given the growing interest in the role of circadian rhythm in metabolic regulation and potentially the development of Type 2 Diabetes, we decided to examine expression of core clock genes in human islets. We can confirm that most core clock genes are expressed in human islets. Expression of \textit{PER2}, 3 and \textit{CRY2} mRNA was altered in islets from donors with Type 2 Diabetes and correlated with some functional parameters. Expression of said genes was also changed in human islets under experimental conditions mimicking pathogenetic processes in β-cells [15]. Our findings support the notion that altered expression of genes involved in control of circadian rhythm in islets may play a pathogenetic role in Type 2 Diabetes.
Materials and Methods

Human Islets

Islets from deceased human donors were received from the Nordic Center of Islet Transplantation and processed at the Human Tissue Laboratory at Lund University Diabetes Centre. For analysis of islet gene expression in Type 2 Diabetes, we used islets from 9 donors; islets from 5 donors without Type 2 Diabetes were used for comparisons. A summary of the characteristics of the donors is given in Table 1. The islets were cultured in CMRL 1066 medium (ICN Biomedicals, Costa Mesa, CA) containing 10 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone (Invitrogen), 20 μg/ml ciprofloxacin (Bayer Healthcare, Leverkusen, Germany), and 10 mM nicotinamide at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. They were kept for 1–9 days prior to RNA preparation. All donors had given consent to donate organs for medical research.

To examine the impact of gluco-lipotoxic conditions on gene expression in human islets, they were first cultured overnight in 3.3 mM glucose. For gluco-lipotoxic conditions, the medium concentration was changed from 3.3 mM glucose to 16.7 mM glucose together with 1 mM palmitate complexed to 1% bovine serum albumin for 48 h. All procedures with human islets were approved by the ethical committees in Uppsala and Lund.

Expression analysis

Total RNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen GmbH, Hilden, Germany). RNA quality and concentration was measured using an Agilent 2100 bioanalyzer and Nanodrop ND-1000 equipment, respectively. cDNA was synthesized using SuperScript II RNase H-Reverse Transcriptase and random hexamer primers (Invitrogen Corporation, Carlsbad, CA) and QuantiTect Reverse Transcription kit (Qiagen). mRNA levels were quantified by TaqMan Real-Time PCR with an ABI Prism
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7900 HT system (Applied Biosystems), using gene specific probes and primer pairs (Assays-on-demands, Hs00256144_m1, Hs00997925_m1, Hs00323654_m1 Applied Biosystems). The transcript quantity was normalized to the mRNA level of HPRT.

The microarrays were performed following the Affymetrix standard protocol. Briefly, total RNA 100-200 ng was processed following the GeneChip® Expression 3’-Amplification Reagents One-cycle cDNA synthesis kit instructions to produce double-stranded cDNA. This was used as a template to generate biotin-targeted cRNA following manufacturer’s specifications. 15 μg of the biotin labeled cRNA was fragmented to strands between 35 and 200 bases in length, 10 μg of which was hybridized onto the GeneChip® Human Gene 1.0 ST whole transcript based assay overnight in the GeneChip® Hybridization oven 6400 using standard procedures. The arrays were washed and stained in a GeneChip® Fluidics Station 450. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was performed using GeneChip® Operating Software. The array data was summarized and normalized with Robust Multi-array Analysis (RMA) method using the software “Expression Console” (Affymetrix).

Insulin mRNA expression was analyzed as previously described [16].

The aim of the study was to examine whether the seven core clock genes CLOCK, BMAL1, PER1-3, and CRY1-2 [11, 17, 18] were expressed in human pancreatic islets. These genes are a selection from an ongoing comprehensive analysis of gene expression in human islets (Jalal Taneera and Leif Groop; manuscript in preparation).

**Insulin secretion and content**

Insulin secretion from islets was measured in Krebs-Ringer bicarbonate buffer (KRBB) containing (in mM): 115 NaCl, 4.7 KCl, 2.6 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 20 NaHCO3, 16 HEPES and 2 mg/ml BSA; pH 7.4. Islets were preincubated in KRBB with 3.3 mM glucose at 37°C for 60 min. For static measurements of insulin release, groups of
10 islets were incubated at 37°C for 1 h in 300 μl of the same buffer containing 3.3 or 16.7 mM glucose. Released insulin was measured in the buffer by radio immunoassay (RIA) as described [16]. Insulin content was extracted by acid ethanol and analyzed by RIA as previously described [16].

Statistical analyses

Data are shown as mean ± SD. Differences in core clock gene expression in pancreatic islets from donors with and without Type 2 Diabetes were analyzed using Mann-Whitney U-tests. A χ²-test was used to analyze if core clock genes showed significantly reduced gene expression in pancreatic islets from patients with Type 2 Diabetes. The test examined if the observed number of core clock genes with differential gene expression in human pancreatic islets due to Type 2 Diabetes (n=3) is significantly higher than the expected number of core clock genes (n=0.42). The expected number of genes is calculated based on the total number of core clock genes analyzed in the microarray (n=7), the total number of genes analyzed in the microarray (n=21764) and the total number of genes that show reduced gene expression in the islets from donors with Type 2 Diabetes with p≤0.05 (n=1289). Differences in gene expression in islets from donors (no diagnosis of diabetes) kept under gluco-lipotoxic conditions compared to islets kept under normal conditions were calculated by Related-Samples Wilcoxon Signed Ranks Test. Correlations were calculated using Spearman correlation coefficient. All p-values were two-tailed and p-values less than 0.05 were considered significant. The presented p-values have not been corrected for multiple testing. Statistical calculations were performed using PASW Statistics 18 for Windows (SPSS, Chicago, IL, USA). For HbA1c, insulin mRNA and insulin content correlations, we have used data from the microarray and for the fold-change of insulin secretion we have used qRT-PCR data.
Results

Islet expression of core clock genes

First, we examined whether core clock genes were expressed in human islets, using a microarray analysis. We found that CLOCK, BMAL1, PER1-3, CRY1-2 were expressed in the human islets (Figure 1).

Core clock gene expression in islets in Type 2 Diabetes

We have at our disposal islet mRNA from 9 donors that suffered from Type 2 Diabetes (Table 1). The first question we asked was whether expression of core clock genes in these islets was changed compared with that in islets from 55 control donors. We found that expression of PER2 and 3, as well as CRY2 was significantly reduced in islets from the donors with Type 2 Diabetes, using data from our microarray (Figure 1).

Since the reduced expression of these three genes was only nominally significant, we further used a χ² test to examine if the identified number of core clock genes with reduced expression exceeded that to be expected. The expected number of core clock genes with reduced gene expression is 0.42, and the χ² of observed over expected core clock genes is 16.12 with p=5.94·10⁻⁵.

Next, we performed a technical validation of the microarray data for PER2 and 3, as well as CRY2, using quantitative real time-PCR (qRT-PCR). We found that expression levels for PER2-3 and CRY2 correlated significantly when determined by microarray and qRT-PCR (Table 2).

Core clock gene expression correlation with functional parameters

An inherent problem with expression data derived from islets from patients with Type 2 Diabetes is that a causal relationship between the changes observed and the disease state can not be assumed. Clearly, the hyperglycemic environment could in itself affect expression of key genes. To further elucidate these possibilities, we investigated whether
expression of the core clock genes that was changed in islets from donors with Type 2 Diabetes correlated with HbA1c, an indicator of long term glucose homeostasis in the donors. For this purpose, we included data from all donors of islets available to us. Interestingly, we found that expression of PER3 and CRY2 but not PER2, was negatively correlated with HbA1c (rho=-0.44; p=0.0012 and rho=-0.28; p=0.042, regardless of whether data from patients with Type 2 Diabetes were included or not. Thus, the poorer the metabolic control (elevated HbA1c), the lower the expression of the respective core clock gene (Figure 2).

In the insulin-resistant state, β-cells will try to overcome the increased demand for insulin by increasing release of the hormone. To accommodate this, increased production of insulin is required. This may be reflected by increased levels of insulin mRNA and protein. To examine whether core clock genes could play a role in such processes, we asked whether islet expression of the core clock genes measured in the microarray correlated with that of insulin mRNA and protein. Indeed, we found a positive correlation of expression of CLOCK, CRY2, PER2 and 3 with the level of insulin mRNA (Figure 3). Interestingly, a similar correlation was found with insulin protein for PER2 and 3 and CRY2, but not for CLOCK (Figure 4).

A crucial component of the pathogenesis of Type 2 Diabetes is failing insulin secretion. Hence, we examined whether expression of core clock genes correlated with insulin secretion in the human islets. We found that the expression levels of the core clock genes derived from the microarray were not significantly correlated with the fold-response of insulin secretion. However, when we correlated expression of each of the three genes that we found were altered in islets from patients with Type 2 Diabetes (PER2-3 and CRY2), using results from qRT-PCR analyses, with the fold-response of insulin secretion, we found that expression of PER2 (rho=0.46; p=0.001), PER3 (rho=0.34; p=0.017) and
CRY2 (rho=0.88; p=0.004) correlated positively with insulin secretion upon a rise in glucose from 3.3 to 16.7 mM.

Core clock gene expression under experimental diabetes-like conditions
Since we found that the expression of core clock genes was altered in islets from patients with Type 2 Diabetes, we asked whether pathogenetic conditions induced experimentally in normal human islets would affect core clock genes in a similar fashion. In addition, this could also indicate whether a regulation of core clock genes is a primary or secondary phenomenon in a diabetic situation. To this end, we examined the impact of an elevated level of glucose and palmitate in the culture medium, a condition known as gluco-lipotoxicity. Under these experimental settings, GSIS was altered: basal insulin release was attenuated (0.25±0.11 ng/islet/h versus 0.45±0.10 ng/islet/h) in islets challenged by gluco-lipotoxicity versus control islets (-80%; p<0.05), and the fold response to a rise in glucose from 3.3 mM to 16.7 mM was reduced from 1.32 to 1.17 in control islets versus glucose- and palmitate-exposed islets. We found that expression of PER3 was reduced in human islets exposed to an elevated level of glucose (16.7 mM) and palmitate (1 mM) compared to islets kept under normal conditions (Figure 5). In contrast, expression of PER1 was increased while that of PER2 was unaffected.
Discussion

It has become increasingly clear that defective insulin secretion due to deficient function and/or reduced mass of β-cells is the primary perturbation leading to Type 2 Diabetes. This notion is largely based on the findings from the recent genome-wide association studies of Type 2 Diabetes and its associated traits. Insulin secretion from pancreatic β-cells is triggered and amplified by the intracellular metabolism of glucose and, likely, other fuels [20]. The fuels are transported into the β-cells in proportion to their extracellular concentrations. The triggering of exocytosis is a result of the increase in the ATP/ADP ratio that fuel metabolism brings about. It is less clear how other metabolic stimuli amplify exocytosis. Nevertheless, given the pivotal role of metabolism in β-cells, an interest in a potential regulatory role of circadian rhythm in the pancreatic β-cell is growing.

The recent study by Marcheva et al. [13] provides robust evidence for the existence of a self-sustained clock within pancreatic islets. This implies a not yet clearly elucidated role for the core clock components CLOCK and BMAL1 in control of insulin secretion. Using different knock out and transgenic mouse models, they showed at both the mRNA and protein level that loss of these central components of the circadian machinery has a huge impact on the function of pancreatic β-cells and insulin secretion. Moreover, these results are in accordance with recent findings that BMAL1 is required in pancreatic β-cells for normal insulin secretion and consequently glucose homeostasis [14].

The core circadian clock has been described at the molecular and genetic level in several independent studies [11,17,18]. It is a highly regulated mechanism, consisting of transcription/translation-based negative and positive feedback loops. These loops involve a well-concerted regulation of the so-called core clock genes, CLOCK, BMAL1, PER1-3, CRY1-2, and their gene products, which were chosen for the present investigation. To confirm published findings and to shed some more light on the role of this group of genes
in human β-cell function and Type 2 Diabetes, we performed expression profiling of these seven core clock genes in human islets from donors with and without Type 2 Diabetes. Our data confirmed that the core clock genes are expressed in human islets. Moreover, mRNA expression of three of the circadian machinery components downstream of CLOCK and BMAL1, i.e., PER2, PER3 and CRY2, were down-regulated in islets from Type 2 Diabetes donors. We found that mRNA expression of PER3 and CRY2, a downstream component of the circadian machinery, correlated negatively with HbA1c levels. Glycated hemoglobin (HbA1c) is considered the gold standard for monitoring metabolic control in diabetes. This parameter provides a measure of the individual’s mean glucose concentrations over a period of three months, i.e., the life span of the erythrocyte. Future studies will dissect the mechanism by which elevated plasma glucose as well as other metabolic abnormalities is regulating expression of these clock genes. Like PER2 and CRY2, PER3 also correlated positively with the secretory capacity of the human islets, assessed as fold increase of GSIS.

Admittedly, the present data are descriptive and correlative and they can not distinguish between cause and effect. It can not be ruled out that observed changes in expression are secondary rather than primary mechanisms in the events leading to Type 2 Diabetes. Nevertheless, it has been previously shown in cultured rat fibroblasts that PER1 and PER2 mRNA levels are downregulated when cells are cultured under high glucose conditions [19]. This finding suggests that these core clock genes are indeed sensitive to ambient glucose. Moreover, in an attempt to further elucidate causality, in human islets, we experimentally induced in vitro pathogenic conditions, i.e. gluco-lipotoxicity. This mimics to some extent the stressful metabolic microenvironment of Type 2 Diabetes in vivo. Here, we found that PER3, but not PER2 and CRY2, showed reduced expression in islets exposed to high glucose and fatty acids compared to healthy matched ones. Clearly, there is a discrepancy with the in vivo data from the human islets, where PER2, PER3 and
CRY2 were downregulated at the mRNA level. This discrepancy may be viewed in the perspective of the different time frames of the conditions, where the islets from the patients with Type 2 Diabetes represent the situation *in vivo* for a prolonged period of time versus the, by comparison, short time period of 48 h in the *in vitro* study. However, other possibilities cannot be excluded.

Since islets are derived from donors deceased for different reasons, and kept in culture for variable times prior to transport to our facility for experiments, no conclusions can be drawn from our data regarding circadian rhythm in human islets. Furthermore, this would have required synchronization, a procedure we thought was too harsh for the material at our disposal. Instead, our goal has been to highlight a possible correlation between the expression levels of core clock genes and functional parameters relevant for Type 2 Diabetes. All together, our data suggest an involvement of the control of the circadian machinery by the core components *PER2, PER3* and *CRY2* in Type 2 Diabetes. Since no differences at the mRNA level of the two main transcription factors and core clock genes *CLOCK* and *BMAL1* were observed, further investigations will indicate whether instead post-transcriptional mechanisms are involved in the regulation of these two core clock genes. Indeed, post-transcriptional and -translational mechanisms could also play a role in the described changes of *PER2, PER3* and *CRY2*. In sum, our data underscore the possible role of core clock genes in the pathogenesis of human Type 2 Diabetes.

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Author Contributions: J.S. performed and analyzed most of the experiments in this study, with assistance from C.N. and S.M. A.H.O. performed statistical analysis and analysis of the microarray data. M.D.N. conducted the gluco-lipotoxicity experiments. H.M. and C.L. provided critical intellectual input in the preparation of the manuscript.

J.S., A.H.O., C.L. and H.M. wrote the paper.

Duality of interest: The authors declare that there is no duality of interest associated with the manuscript.
References


Figure Legends

**Figure. 1.** mRNA expression of seven core clock genes in human pancreatic islets determined by microarray. Three genes showed reduced mRNA expression with $p<0.05$ in islets from patients with Type 2 Diabetes (Type 2 Diabetes; $n=9$) compared with donors not suffering from Type 2 Diabetes ($n=55$). Since the expected number of CLOCK genes with reduced expression is 0.416, the $\chi^2$ of observed over expected CLOCK genes is 16.12 with $p = 5.94 \times 10^{-5}$. Bars represent mean values with SD of relative mRNA expression. $p$-values are based on Mann-Whitney U-test and not corrected for multiple testing; *$p \leq 0.05$; **$p \leq 0.001$.

**Figure. 2.** Correlation of mRNA expression (arbitrary units) of *CLOCK*, *BMAL1*, *PER1-3*, and *CRY1-2* with the level of HbA1c (%); data are from 52 islet donors; 8 Type 2 Diabetes donors and 44 donors without Type 2 Diabetes.

**Figure. 3.** Correlation of mRNA expression (arbitrary units) of *CLOCK*, *BMAL1*, *PER1-3*, and *CRY1-2* with the level of insulin mRNA (arbitrary units; qRT-PCR); data are from 56 islet donors; 9 Type 2 Diabetes donors and 47 without Type 2 Diabetes.

**Figure 4.** Correlation of mRNA expression (arbitrary units) of *CLOCK*, *BMAL1*, *PER1-3*, and *CRY1-2* with the insulin content (normalized to DNA); data are from 58 islet donors; 8 Type 2 Diabetes donors and 50 without Type 2 Diabetes.

**Figure 5.** mRNA expression of core clock genes in human pancreatic islets from donors without Type 2 Diabetes ($n=14$) kept under gluco-lipotoxic conditions (high glucose + palmitate; HGP) compared to islets kept under normal conditions (low glucose; LG). $p$-values are based on a Related-Samples Wilcoxon Signed Ranks Test.
Tables

<table>
<thead>
<tr>
<th></th>
<th>Type 2 Diabetes</th>
<th>Control</th>
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<tbody>
<tr>
<td>Gender (male/female)</td>
<td>9 (5/4)</td>
<td>55 (29/26)</td>
</tr>
<tr>
<td>Age</td>
<td>57 ± 13.0</td>
<td>56 ± 10.0</td>
</tr>
<tr>
<td>BMI</td>
<td>28 ± 5.0</td>
<td>26 ± 3.5</td>
</tr>
<tr>
<td>HbA1c</td>
<td>7.3 ± 1.2</td>
<td>5.7 ± 0.8</td>
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<tr>
<td>Basal insulin secretion (ng/islet/h)</td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.3</td>
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<tr>
<td>GSIS (ng/islet/h)</td>
<td>1.0 ± 1.5</td>
<td>1.1 ± 1.0</td>
</tr>
</tbody>
</table>

**Table 1.** Clinical characteristics of donors with and without Type 2 Diabetes

**Table. 1.** Values represent means ± standard deviation
Table 2. Correlation of mRNA levels

<table>
<thead>
<tr>
<th>Gene</th>
<th>R</th>
<th>p</th>
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<tbody>
<tr>
<td>Cry2</td>
<td>0.33</td>
<td>0.013</td>
</tr>
<tr>
<td>Per2</td>
<td>0.59</td>
<td>1.51x10^-6</td>
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<tr>
<td>Per3</td>
<td>0.55</td>
<td>9.98x10^-6</td>
</tr>
</tbody>
</table>

**Table. 2.** Correlations between mRNA levels derived from the Affymetrix GeneChip® Human Gene 1.0 ST assay and qT-PCR data analyzed by Spearman correlation coefficient (rho; for details, see Materials and Methods).
Figure 1.
Figure 2

- BMAL1 expression: 
  - rho = -0.16
  - p = 0.27

- CLOCK expression: 
  - rho = -0.13
  - p = 0.35

- CRY1 expression: 
  - rho = 0.056
  - p = 0.69

- CRY2 expression: 
  - rho = -0.28
  - p = 0.042

- PER1 expression: 
  - rho = -0.20
  - p = 0.16

- PER2 expression: 
  - rho = -0.20
  - p = 0.16

- PER3 expression: 
  - rho = -0.44
  - p = 1.2 \times 10^{-3}
Figure 3

- **BMAL1 expression** vs. INS expression
  - rho = 0.090
  - p = 0.51

- **CLOCK expression** vs. INS expression
  - rho = 0.31
  - p = 0.019

- **CRY1 expression** vs. INS expression
  - rho = 0.25
  - p = 0.063

- **CRY2 expression** vs. INS expression
  - rho = 0.58
  - p = 3.4 \times 10^{-6}

- **PER1 expression** vs. INS expression
  - rho = 0.21
  - p = 0.11

- **PER2 expression** vs. INS expression
  - rho = 0.39
  - p = 2.7 \times 10^{-3}

- **PER3 expression** vs. INS expression
  - rho = 0.47
  - p = 2.5 \times 10^{-4}
Figure 4

![Graphs showing correlation between insulin content and DNA with expression of BMAL1, CLOCK, CRY1, CRY2, PER1, PER2, and PER3 genes.](image)
Figure 5