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Published in:
Non-Coding RNAs in Endocrinology

DOI:
[10.1515/micrnado-2018-0002](https://doi.org/10.1515/micrnado-2018-0002)

Publication date:
2018

Document Version
Publisher's PDF, also known as Version of record

Citation for published version (APA):
Jaksch, C., Thams, P. G., Iessi, I., Poulsen, S. S., Levin, B., Nielsen, J. H., & Dalgaard, L. T. (2018). The long noncoding RNA Bsr in the genomically imprinted DLK1-DIO3 region is suppressed in newborn rat pancreas by gestational obesity. *Non-Coding RNAs in Endocrinology*, 2018(3), 5-17. <https://doi.org/10.1515/micrnado-2018-0002>

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Research Article

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The long noncoding RNA *Bsr* in the genomically imprinted *Dlk1-Dio3* region is suppressed in newborn rat pancreas by gestational obesity

<https://doi.org/10.1515/micrnado-2018-0002>

Received December 11, 2017; accepted March 22, 2018

Abstract: Background: Fetal metabolic programming imposed by maternal obesity and impaired glucose tolerance predisposes the offspring to metabolic disease and beta cell dysfunction as adults. The aim of this study was to assess the whole pancreas RNA changes in neonatal offspring exposed to fetal programming. Methods: The outcome of fetal programming on offspring was tested using the selectively bred Diet Induced Obese (DIO) and the Diet Resistant (DR) strains. DIO and DR rats were fed either chow or high fat, high sucrose (high energy, HE) diet during gestation and the differences in pancreas RNA expression at two days after birth were measured by microarray. Pancreas sections were stained and analyzed for alpha and beta cell numbers. Neonatal islets were treated with cytotoxic cytokines and RNA measured by RT-Q-PCR. Results: Morphometric analyses revealed significant differences in alpha and beta cell numbers per pancreas or per islet by HE diet. Microarray analyses revealed 11 fold downregulation of the long noncoding RNA *Bsr* in whole pancreas by HE feeding of DIO rat dams. MicroRNAs from the same locus, the *Dlk1-Dio3* imprinted region, were also decreased by HE diet.

Moreover, treatment of isolated neonatal islets with inflammatory cytokines, IL-1 β and IFN- γ , led to reduction of *Bsr* transcript in a time and dose dependent manner. Conclusions: Our data suggest that fetal programming of *Bsr* may play a role in beta cell dysfunction in obesity and type 2 diabetes.

Keywords: Pancreas, perinatal, microRNA, *Bsr*, small nucleolar RNA, long non-coding RNA, imprinting, fetal programming, obesity, gestation, islets of Langerhans, beta cell, gene expression, exocrine pancreas, endocrine pancreas, maternal overnutrition, diet-induced obesity, inflammation

1 Introduction

Female obesity during gestation has a number of harmful effects on the metabolic profile of the offspring: Children of mothers exposed to overnutrition, obesity, undernutrition, type 1 and type 2 diabetes as well as gestational diabetes during pregnancy are at higher risk of developing type 2 diabetes later in life; a phenomenon termed ‘fetal programming’ [1-5]. In order to curb the present global diabetes epidemic it is imperative to elucidate the molecular mechanisms behind fetal programming. Identifying the mechanisms behind fetal programming may lead to new prevention and treatment strategies for adult type 2 diabetes. Fetal programming is complex and may affect several organs in the offspring. Maternal obesity is in addition to impaired glucose homeostasis, also associated with increased risk of cardiovascular disease, psychiatric disorders and non-alcoholic fatty pancreas in the offspring [4, 6, 7].

In the period around birth, the pancreas undergoes marked morphological and metabolic changes as the fetus transitions from placental nutrition to a suckling

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diet high in fat and carbohydrates. In rats, the perinatal period is characterized by expansion and maturation of the endocrine cells. The beta cell number increases considerably between embryonic E20 and P2 [8, 9]. A major part of this beta cell expansion occurs by neogenesis from progenitors or stem cells, which are not located inside the islets of Langerhans [10, 11], and it is thus essential to describe the entire pancreas tissue in the perinatal period to identify the mechanisms in action.

In the current study, we investigated the offspring outcome of fetal programming using an established rat model of diet-induced obesity: Selectively bred Diet Induced Obese (DIO) rat strain compared with the Diet Resistant (DR) strain [12-16]. DIO and DR rats were fed either chow or high fat, high sucrose (high energy, HE) diet during gestation and we assessed the differences in total pancreas RNA expression of pups a two days after birth. Here, we report that maternal HE diet resulted in marked suppression of the non-coding RNA transcripts located in the imprinted *Dlk1-Dio3* genomic region as well as increased expression of genes involved in inflammatory processes in the offspring.

2 Methods

2.1 Animal study

Selectively bred DIO and DR rat dams from the in-house colonies at the Veterans Administration Medical Center, East Orange, New Jersey, USA [16] were fed chow or HE diet before and during pregnancy. 22 DIO and 22 DR rat dams were kept at 23-24°C on a 12:12-h light-dark cycle with free access to food and water. Half of the DIO and DR rat dams were fed Purina rat chow (Research diets #5001, Ralston Purine, St. Louis, MO, USA). Half of the DIO and DR rat dams were fed high energy (HE) diet (Research Diets #D12266B, NJ, USA) containing 4.41 kcal/g with 17 % of total calories as protein, 32 % as fat, and 51 % as carbohydrate, of which 50 % is sucrose. Rat dams were fed these diets from 5 weeks before gestation and throughout the study period.

Food intake was measured up to the time for mating. At 4 weeks, after an overnight fast, all dams underwent tail bleeding for measurement of plasma glucose, insulin, and leptin levels. At 5 weeks they were mated with chow-fed males of the same genotype. A second blood sample was drawn at gestation day 10 after an overnight fast. DR and DIO rat dams were fed chow or HE diet 5 weeks before and during gestation. All litters were culled to 10

pups (6 males, 4 females when possible); pups from litters larger than 10 were discarded. There were no significant differences in litter sizes among the groups. Two days after the pups were born they were weighed, killed by decapitation, and the pancreas was stored in RNA Later for coming microarray and qPCR studies. The pancreas of two male pups from each litter was used for RNA extraction. Animal usage was in compliance with and approved by the Institutional Animal Care and Use Committee of the East Orange Veterans Affairs Medical Center, NJ, USA.

Plasma insulin and leptin levels were analyzed with radioimmunoassay kits (Linco, MO, USA) using antibodies to authentic rat insulin and leptin, respectively, while glucose was measured with a glucometer (AccuChek, NY, USA).

2.2 RNA extraction and cDNA synthesis

RNA from whole pancreas and isolated islets was extracted using TRIreagent (Sigma-Aldrich, MO, USA) followed by miRNeasy Mini kit (Qiagen, København, Denmark) according to the manufacturer's instructions. The miRNeasy Mini kit purifies total RNA including microRNA. RNA content and quality was evaluated using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies, DE, USA). Samples with a 260/280 ratio smaller than 1.9 were discarded. cDNA from 0.5-µg RNA was prepared with qScript cDNA SuperMix (Quanta, MD, USA) using manufacturers' instructions. For qPCR analysis on microRNA levels, cDNA synthesis was performed using microRNA specific reverse-transcription primers [17].

2.3 Morphometry of pancreatic islets

Offspring pancreata (one pancreas per litter and four pancreases per group) were stained for insulin and glucagon and counter-stained with Mayers haematoxylin. Sections were imaged with an Olympus BX43 microscope (10 x objective)(Olympus, Tokyo, Japan). Five images/slice (covering 3.8 ± 0.016 mm²) were sampled. Insulin stained sections were used to indicate presence of an islet and whole islets were measured using ImageJ software and the islet area of total pancreatic area was calculated. The percentage of insulin and glucagon positive cells per pancreas was calculated as: (% of insulin or glucagon positive cells per islet x % of islet area per pancreas)/100 = % of insulin or glucagon positive cells per pancreas. The insulin/glucagon cell ratio was calculated as: (% of insulin positive cells per pancreas)/(% of glucagon positive cells per pancreas).

2.4 Reverse transcription quantitative polymerase chain reaction (Q-PCR)

For validation of the microarray results, qPCR was performed on RNA extracted from whole pancreas from male littermates to the offspring that were used for the microarray analysis and were considered biological replicates. Genes for further studies were chosen for their possible or known impact on islet or beta-cell development, function or viability. The following genes were tested for validation by qPCR: *Bsr*, *DIO3*, *Pdx1*, *Insulin*, *MafA*, *MafB*, *Ngn3*, *DLK1*, *miR-431* and *miR-495*. qPCR was performed on 4-16 ng of cDNA/well in a 10 μ l reaction on the ViiA7 real time PCR system (Applied Biosystems) in three technical replicates. Taqman Fast Advanced Mastermix (ThermoFisher Scientific) was used with the following Taqman gene expression assays (Probe/primer sets): *Bsr* (Rn03456339), *DIO3* (Rn00568002_s1), *Pdx1* (Rn00755591), *Ppia* (Rn00690933_m1), all from ThermoFisher Scientific. Fast SyBR Green Master Mix (Applied Biosystems) was used with primers indicated in Suppl. Table S1. Samples were loaded in triplicates. A no template control (NTC) and a no reverse transcriptase control (NRT) was used. Gene expression was normalized to *PPIA* (cyclophilin A) that proved more reliable compared to the other tested housekeeping genes: *TFIIB* (General transcription factor IIB) and *HPRT* (Hypoxanthine-guanine phosphoribosyltransferase).

2.5 Microarray analysis

Microarray analysis was performed on whole pancreas from rat offspring. Pancreas RNA from three male offspring from three different litters (except for the DIO HE offspring group where siblings were used due to lack of litters) was pooled into one sample. Three of such samples from each of the four different rat offspring groups were used for microarray analysis. All RNA sample pools underwent RNA integrity analysis and were accepted for microarray analysis at the Center for Genomic Medicine, Rigshospitalet, Copenhagen. Biotin-labeled cDNA was synthesized from 1 μ g pancreas total RNA according to the Affymetrix protocol (Affymetrix, Santa Clara, CA, USA) and hybridized to Rat Gene 2.0 ST array and scanned. Gene expression data were normalized with the robust multi-array average (RMA) method using Bioconductor [18]. One-way ANOVA was performed on logarithmically transformed gene expression values. Genes that were differentially expressed between the offspring groups, with a False Discovery Rate (FDR) of less than 10% and with a minimum fold change of 1.3 were selected for further investigation.

2.5.1 Pathway analysis of microRNA targets

Predicted mRNA targets of the five miRNAs significantly down-regulated by HE diet were retrieved from TargetScan 7.1. Only mRNAs with Total Context score $++>0.15$ were included to create a data set of predicted mRNA targets with high quality binding sites. A total list of all predicted mRNA targets of these five miRNAs (2554 genes) underwent DAVID functional annotation analysis using Gene Ontology Biological Processes (GO:BP) using default settings (BP TERM ALL, high stringency, and adjusted for multiple testing by Benjamini) [19]. Venn diagrams were created with Venny [20]. Microarray data of fold changes between DIO HE and DIO Chow (DIO HE/DIO Chow) were split into two groups based on whether the gene was a target of any of the five miRNAs down-regulated by HE diet. The average fold change by HE diet was compared for each group (targets vs non-targets) and statistical significance calculated by a Mann-Whitney Rank test.

2.6 Neonatal islet isolation

Islets were isolated from 3-5 day old Wistar rat pups (both male and female). Litter sizes were normal (9-12 pups) and for each experiment, islets were isolated from 2 different litters and pooled. Pups were killed by decapitation, their pancreas were dissected and placed in ice-cold serum-free Hank's balanced salt solution (HBSS; Lonza, Verviers, Belgium). The pancreata were distributed into tubes, and partially dissociated shaking in collagenase (Roche, Penzberg, Germany) at a final concentration of 1.5 mg/ml. Islets were isolated using Histopaque (1.077 g/ml density) (Sigma, St Louis, MO, USA) for 20 min at 1,400 g without brake. Islets were collected from the interface, washed with ice-cold HBSS containing 10 % FBS (Biosera, Ringmer, UK) and handpicked under a microscope. Islets were resuspended in RPMI 1640 with L-glutamine and 11 mmol/l glucose (Lonza) with 10 % FBS, 100 U/ml penicillin and 100 U/ml streptomycin (Lonza), plated in bacteriological petri dishes (around 1000 islets/dish) (Nunc, Roskilde, Denmark) and cultured at 37°C and 5 % CO₂.

2.7 Islet cytokine exposure experiment

For cytokine treatment, neonatal rat islets were cultured in 6 cm dishes (300-500 islets/dish) and the fetal calf serum concentration was lowered to 0.5 % for 24 hrs before the addition of IL-1 β (75-150 pg/ml) or IFN- γ (5 ng/ml, R&D Systems, Abingdon, Oxon, UK). Islets were incubated for 3-24 h as specified in results text. After treatment, islets

were lysed in TRI reagent (Sigma-Aldrich) and frozen until RNA extraction and Q-PCR as described above.

2.8 Statistics and data analysis

The differences between the rat dams' body weight, food intake and calorie intake were measured using repeated measures ANOVA test. To detect differences in effects of diet, rat strain or their interaction two-way ANOVA was used with Sidaks post-hoc tests for individual comparisons. If standard deviations were significantly different, non-parametric Kruskal-Wallis test was used followed by Bonferroni's post hoc test. The significance level used was $p < 0.05$. Graph pad prism 5.0 was used for statistics and results are given as Mean \pm SEM.

3 Results

3.1 Phenotype characterization of the dams of the DR-DIO model during gestation

The diet sensitive DIO rat dams gained more body weight than DR dams when fed either chow or a HE diet before and during gestation (Fig. 1A and Fig. 1B) (DR vs DIO,

$P < 0.001$). There was no effect of HE diet to increase body weight of DR dams, as previously reported (Fig. 1A and Fig. 1B), while HE diet fed DIO dams gained significantly more weight than DIO chow fed dams from the first week of treatment ($P < 0.05$ at Week 1 and $P < 0.001$ for all time points). Moreover, DIO rats had a significantly larger caloric intake compared to DR rats at all time points regardless of diet ($P < 0.05$) (Fig. 1C). Furthermore, HE fed dams had significantly higher intake of calories compared to the chow fed dams; for DIO rats this was significant from the first week of HE diet and for DR rats this increase was significant after 3 weeks (Fig. 1C).

HE diet significantly increased fasting blood glucose values of dams prior to gestation independent of rat strain ($P < 0.01$ by two-way ANOVA), while during gestation fasting blood glucose values were not significantly changed by diet (Fig. 1D). Fasting insulin levels more than doubled in the DIO dams when fed a HE diet compared to a chow diet, both before and during pregnancy (Fig. 1E, $P < 0.001$, both comparisons). Insulin levels were not different between chow fed and HE fed DR rats. Fasting leptin levels were increased 3 fold in the HE fed DIO group compared to the DIO chow and DR HE groups before and during pregnancy (Fig. 1F, $P < 0.001$).

We observed that the pregnancy rates were significantly different in the groups: 11 out of 11 DR chow

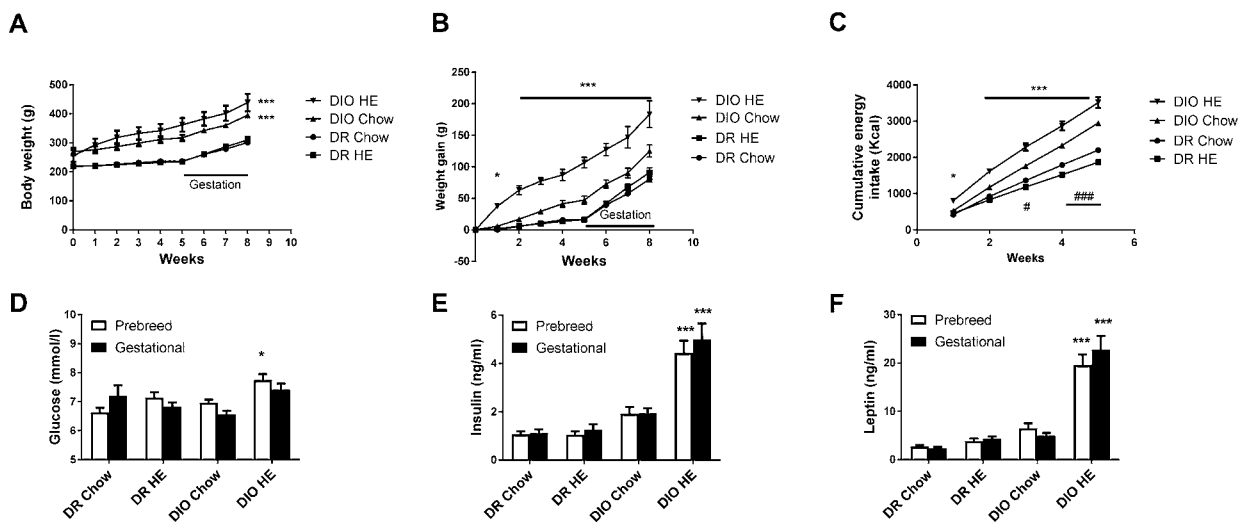


Figure 1: Phenotype characterization of rat dams. A) Body weight of dams prior to and during gestation. HE diet was started at week 0 and continued throughout gestation. ***: $P < 0.001$ for the comparisons: DIO HE vs DR HE rats, DIO HE vs DIO chow, DIO chow vs DR chow. B) Cumulative weight gain during the study. ***: $P < 0.001$ for the comparisons DIO HE or DIO Chow vs DR HE at the indicated time points. C) Cumulative energy intake calculated based on measured food intake. *: $P < 0.05$ for DIO HE vs DIO chow, ***: $P < 0.001$ for DIO HE vs DIO chow, #: $P < 0.05$ for DR HE vs DR chow, ###: $P < 0.001$ for DR HE vs DR chow at the indicated time points. D-F) Fasting hormone values in dams prior to gestation (prebreed, white bars) and during gestation (Gestational, black bars), D) Fasting glucose, *: $P < 0.05$ vs DIO chow, prebreed, E) Fasting serum insulin, ***: $P < 0.001$ for the comparisons DIO HE vs DR chow, DR HE or DIO chow, prebreed and gestational. F) Fasting serum leptin, ***: $P < 0.001$ for the comparisons DIO HE vs DR chow, DR HE or DIO chow, prebreed and gestational. DIO: Diet induced obese strain. DR: Diet resistant strain. HE: High energy diet. Data are given as Mean \pm SEM, $n = 11$ dams per group.

dams, 9 out of 11 DR HE dams and 9 out of 11 DIO chow dams were successfully impregnated, whereas only 4 out of 11 DIO HE dams were impregnated ($P < 0.01$ by Chi-square analysis).

3.2 Body weight of offspring of the DR-DIO model during gestation

At two days after birth, the body weight and percentage body fat was measured. The body weight of DIO strain offspring was significantly increased compared with DR strain offspring ($P < 0.001$ by two-way ANOVA). Both male and female offspring from HE fed DIO dams had a significantly increased body weight compared with offspring of HE fed DR dams (males $P < 0.05$, females $P < 0.01$), but not significantly higher compared to DIO chow offspring (Fig. 2).

3.3 Islet morphometry analysis in offspring

The percentage of insulin-expressing cells per islet was in general increased by HE diet ($P < 0.05$ by two-way ANOVA), while single comparison indicated more insulin positive cells in DR HE offspring compared to DR chow offspring (Fig. 3A, $P < 0.01$ (% insulin per islet)). The number of glucagon positive cells was also significantly increased by HE diet (Fig. 3B, $P < 0.05$ by two-way ANOVA), but for glucagon single individual comparisons were not significantly different. There was no significant difference in insulin-to-glucagon positive cell ratio (Fig. 3C). The percentage of insulin and glucagon positive cells per pancreas tended to be higher in HE fed DR offspring compared to chow fed DR offspring (Fig. 3D, $P = 0.05$ DR HE vs DR chow by two-way ANOVA, Fig. 3E, $P = 0.05$ DR HE vs

DR chow by two-way ANOVA). Furthermore, the islet mass was not changed by rat strain or diet (Fig. 3F).

3.4 Expression of genes involved in β -cell function

Messenger RNA levels of genes important for β -cell function and development were measured in RNA extracted from whole pancreas and compared between the rat offspring groups. There were no significant differences in the mRNA levels of insulin, MafA, MafB, Pdx1 or Ngn3 between the offspring groups (Fig. 4A-F). However, the mRNA levels of Regenerating islet derived 3 alpha (Reg 3 α) was significantly increased in the DIO rat strain compared with the DR strain (Fig. 3F, $P < 0.01$ by two-way ANOVA). The increase in Reg 3 α and related Reg transcripts (Reg 3 β and Reg 3 γ) was most pronounced in the DIO HE group (Fig. 3F, $P < 0.05$ for the DIO HE vs DR HE comparison). Reg proteins have been demonstrated to be upregulated in the pancreas under various forms of stress and inflammation and have been suggested to be involved in compensatory β -cell regeneration after injury [21-23].

3.5 Noncoding RNAs altered by fetal over-nutrition in the DR and DIO rat strains

Microarray analysis of whole pancreas RNA from offspring indicated that, with a fold change of ± 1.3 or more, 502 genes were significantly ($P < 0.05$), up- or down- regulated between the DR Chow and the DIO chow. For the DIO Chow versus the DIO HE group 367 genes were regulated. Between the DR Chow and DR HE group, 215 genes were regulated and between the DR HE and DIO HE group, 323 genes were regulated. Only 2 transcripts; Mast cell protease 10 (Mcpt10) and Rgs22 (RGD1565493) were differentially regulated between all groups. Mcpt10 being increased by HE diet and in the DR strain, while Rgs22 was increased by HE diet in the DIO strain, while not in the DR strain (Suppl Table S2). A complete list of all significantly regulated genes may be found in Suppl. Table S2.

Five miRNAs were significantly down-regulated by HE diet in the DIO rats (miR-410-3p, -377-5p, 431-5p, 495-3p and -543-3p) (Table 1). Predicted mRNA targets were retrieved and gene sets containing high fidelity binding sites underwent analysis for GO term enrichment (Suppl. Table S3). 25 GO terms were enriched among predicted targets of the down-regulated miRNAs, which included protein ubiquitination, cellular metabolic processes, DNA-regulation, RNA-regulation, cellular macromolecule biosynthetic process and vasculature development (Suppl. Table S3). We compared the fold regulation between DIO

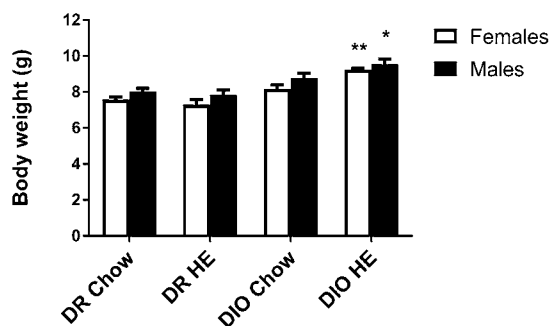


Figure 2: Body weight of neonatal rat pups. DIO: Diet induced obese strain. DR: Diet resistant strain. HE: High energy. White bars: Females. Black bars: Males. Data are given as Mean + SEM. *: $P < 0.05$, **: $P < 0.01$ for the comparison between HE DIO and HE DR groups for males and females, respectively.

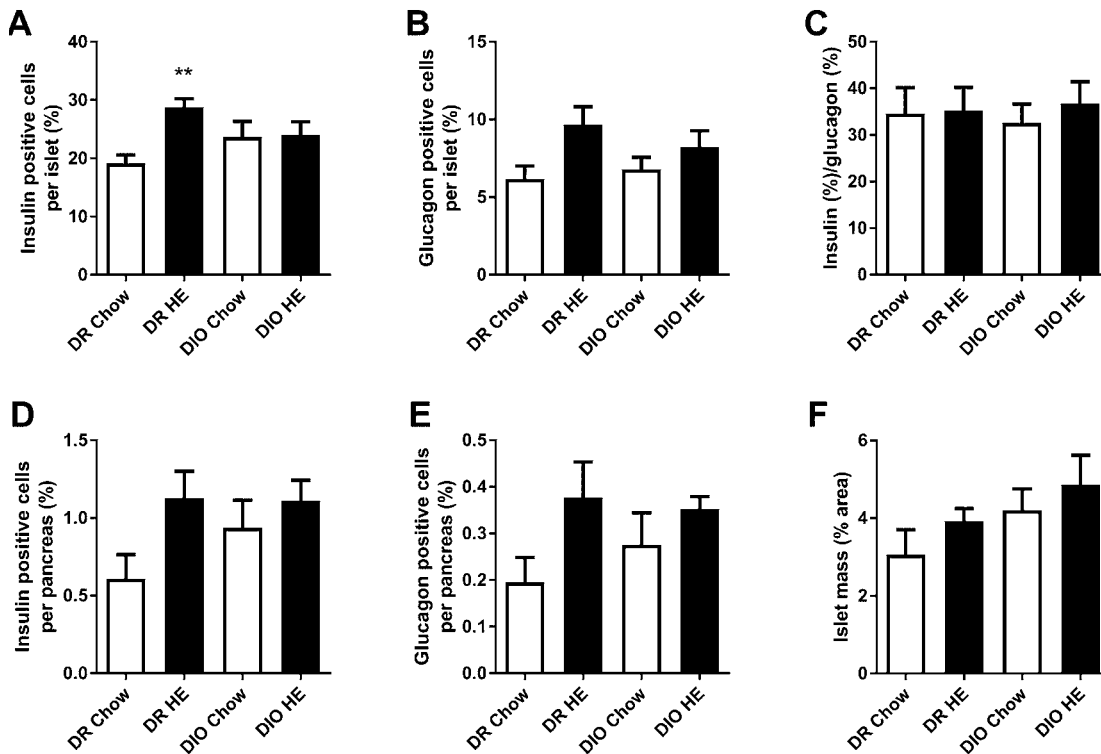


Figure 3: A-F) Pancreas and islet morphometry in neonatal offspring of DR and DIO rats. A) % insulin positive cells per islet, **: $P < 0.01$ for DR HE vs DR chow, B) % glucagon positive cells per islet, C) Ratio of insulin to glucagon positive cells (Insulin (%) / glucagon (%)), D) % insulin positive cells per pancreas, E) % glucagon positive cells per pancreas, and F) Islet mass (% area: The area of the islets compared with the whole pancreatic area of each section). DIO: Diet induced obese strain. DR: Diet resistant strain. HE: High energy. Data are given as Mean + SEM, $n=4$.

Chow and DIO HE (DIO HE/DIO Chow) in the two groups of transcripts: Those without target sites ($n=18832$) for the down-regulated miRNAs and those with predicted targets sites for one of the down-regulated miRNAs ($n=1925$). Transcripts harboring binding sites for down-regulated miRNAs increased expression by, on average, 0.054 fold ± 0.024 fold, while transcripts that were not targets decreased their expression in the DIO HE group by 0.103 fold ± 0.008 fold ($P < 0.0001$), which is concordant with miRNA target genes undergoing target gene derepression when their cognate miRNAs decrease in expression.

We observed that a large number of noncoding RNAs were differentially expressed following fetal over-nutrition in the DR and DIO rat strains. The most regulated gene found in the microarray analysis was *Bsr*, a long noncoding RNA, expressed from the maternal allele in the imprinted *Dlk1-Dio3* genomic region. *Bsr*, also known as *Rian*, was 11 fold downregulated in the DIO HE offspring group compared to the DR HE and DIO chow group ($P < 0.005$, Table 1). Moreover, biological replication by Q-PCR also showed a significant downregulation of *Bsr* in the DIO HE group ($p < 0.01$) compared to the DIO Chow offspring group (Fig. 5A).

Several miRNAs from the *Dlk1-Dio3* region tended to be or were significantly downregulated in the DIO HE group compared to the DIO chow group and in the DR HE group compared to the DR Chow group (Table 1), although results were not consistent between all miRNAs from the region. Biological validation by Q-PCR indicated down-regulation by HE diet of miR-431 (Fig. 5B, $P < 0.05$ by two-way ANOVA) and miR-495 (Fig. 5C $P < 0.05$ by two-way ANOVA) although no individual comparisons were significantly different. Furthermore, *Dlk1* or *Dio3* RNA levels between the groups were unchanged although there was a tendency to lower expression of *Dlk1* in the HE groups (Fig. 5 D-E).

3.6 Regulation of *Bsr* by cytokines in neonatal rat islets

The microarray data showed changes in expression of both pro- and anti-inflammatory genes including phospholipase A2 (*Pla2g2a*), IL-1 receptor antagonist (*IL1ra*), caspase 3, glutathione S-transferase theta 2 (*Gstt2*), glutathione peroxidase 3 (*Gpx3*), Mast cell protease 1 (*Mcpt1*), Mast cell protease 2 (*Mcpt2*), secretory leukocyte peptidase inhibitor (*Slpi*) and neutrophilic granule protein

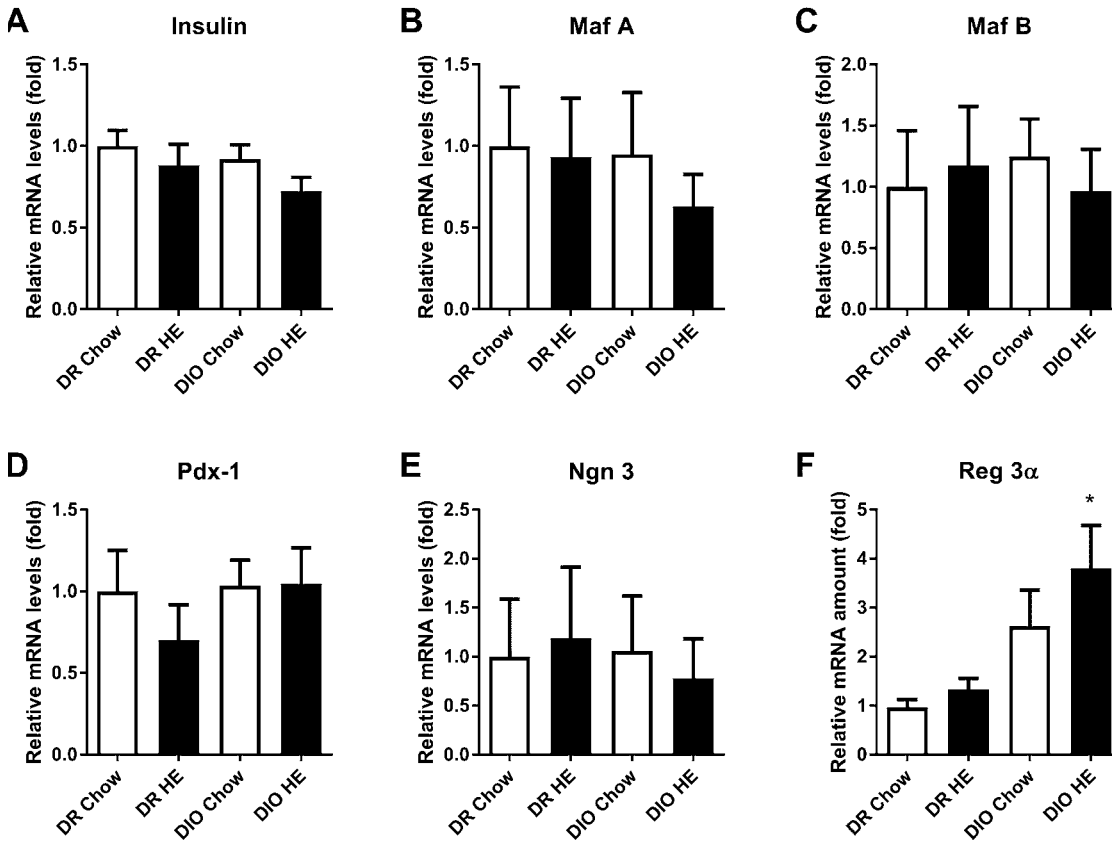


Figure 4: A-F) mRNA steady state levels of transcripts encoding markers of beta cells, progenitor cells or beta cell regeneration. Messenger RNA levels were normalized to the levels of *Ppia* mRNA and the DR Chow group was set to 1.0. A) Insulin, B) MafA, C) MafB, D) Pdx1, E) Ngn3 and F) Reg α 3, * $P < 0.05$ for DIO HE vs DR He. DIO: Diet induced obese strain. DR: Diet resistant strain. HE: High energy. Data are given as Mean + SEM, $N = 6$ /group.

(Ngn), which indicates increased inflammatory responses or increased infiltration of immune cells in the pancreas [24, 25] (Suppl. Table S2). The expression of *Pla2g2a* was increased in DIO rats independent of diet compared with DR rats as confirmed by qPCR (Fig. 6A). The expression was also upregulated in a time-dependent manner in neonatal rat islets exposed to IL-1 β (Fig. 6B).

Thus, we investigated the regulation of *Bsr* in isolated neonatal rat islets by cytotoxic cytokines (IL-1 β and IFN- γ), which are increased in states of inflammation. Islet *Bsr* was downregulated significantly by both IL-1 β (150pg/mL), IFN- γ (5ng/mL) and their combination after 24hrs of treatment (Fig. 6C). Moreover, in a time series IL-1 β (75pg/mL) alone down-regulated *Bsr* after 24hrs (Fig. 6D) and IFN- γ (5ng/mL) in combination with IL-1 β also decreased *Bsr* after 24hrs of treatment (Fig. 6E). Thus, cytotoxic cytokines, most notably IL-1 β , suppress *Bsr* RNA levels.

To summarize, a maternal HE diet downregulated the expression of *Bsr* and several miRNAs from the *Dlk1-Dio3*

locus, in the pancreas of DIO offspring and inflammatory cytokines suppressed *Bsr* in neonatal rat islets *in vitro*.

4 Discussion

In the present study, the effect of maternal high energy (HE) feeding and obesity on the pancreas of offspring was investigated with focus on gene expression and changes in islet mass. As a model for the metabolic syndrome and fetal programming, we used selectively bred DR and DIO rat strains. This rat model does not develop frank diabetes, but shares many clinical features with the human metabolic syndrome [26]. The development of the β -cell dysfunction seen in HE fed DIO rats agrees well with descriptions of the developing β -cell dysfunction observed in human subjects during progression to diabetes [27]. Over time, the HE fed DIO rats lose their ability to compensate for insulin resistance leading to increased plasma glucose levels [12, 26]. The DIO/DR rat

Table 1: Microarray results of non-coding RNAs from the DLK1-DIO3 region.

RNA species	DR Chow	DR HE	DIO Chow	DIO HE	Significance
Bsr (Rian)	290	474	458	42**	** P<0.0 vs DIO chow and DR HE
miR-544a	153	154	222	154	
miR-493-5p	204	168	204	179	
miR-431-5p	86	82	117*	91	*P<0.05 vs DR chow
miR-665	171	139	164	140	
miR-134	151	144	154	129	
miR-337-5p	114	100	114	102	
miR-127-5p	99	89	109	85	#
miR-154-5p	77	92	104	64*	* P<0.05 vs DIO chow and DR HE
miR-485-5p	102	92	104	81	
miR-770-5p	79	78	100	84	
miR-410	100	72*	99	69	*P<0.05 vs DR chow
miR-758	87	82	94	89	
miR-668	90	91	87	79	
miR-433	82	66	81	64	
miR-136-5p	85	77	79	80	#
miR-300	74	51	74	52	
miR-377-5p	57	57	65	48*	*P<0.05 vs DIO chow
miR-329	57	56	62	60	
miR-494	52	53	55	56	
miR-495	52	32	55	37*	*P<0.05 vs DIO chow, #
miR-370	45	44	50	48	
miR-487b	52	50	50	47	#
miR-299-5p	31	27	41	30	
miR-380-5p	37	33	37	26	
miR-379-5p	34	32	34	33	
miR-412	28	33	32	26	
miR-323a-5p	26	24	28	22	
miR-543	24	30	27	21*	*P<0.05 vs DR HE, #
miR-411-5p	22	19	18	17	#

miRNAs annotated with # were also down-regulated in islets from type 2 diabetics (Kameswaran et al., 2014) [40].

model has a polygenic mode of inheritance [28], similar to human obesity and type 2 diabetes. The HE diet used is a high-fat high-sucrose diet similar to human, westernized diet.

Offspring of HE fed DIO rats became more obese and had higher plasma levels of glucose and leptin later in

life compared to offspring of chow fed DIO or HE fed DR offspring [14]. Since the DIO rat dams, but not the DR rat dams develop obesity when they fed a HE diet, it is possible to study the effects of a maternal HE diet with and without obesity on fetal programming of the pancreas when using this rat model. Thus, the selectively bred DR and DIO rat

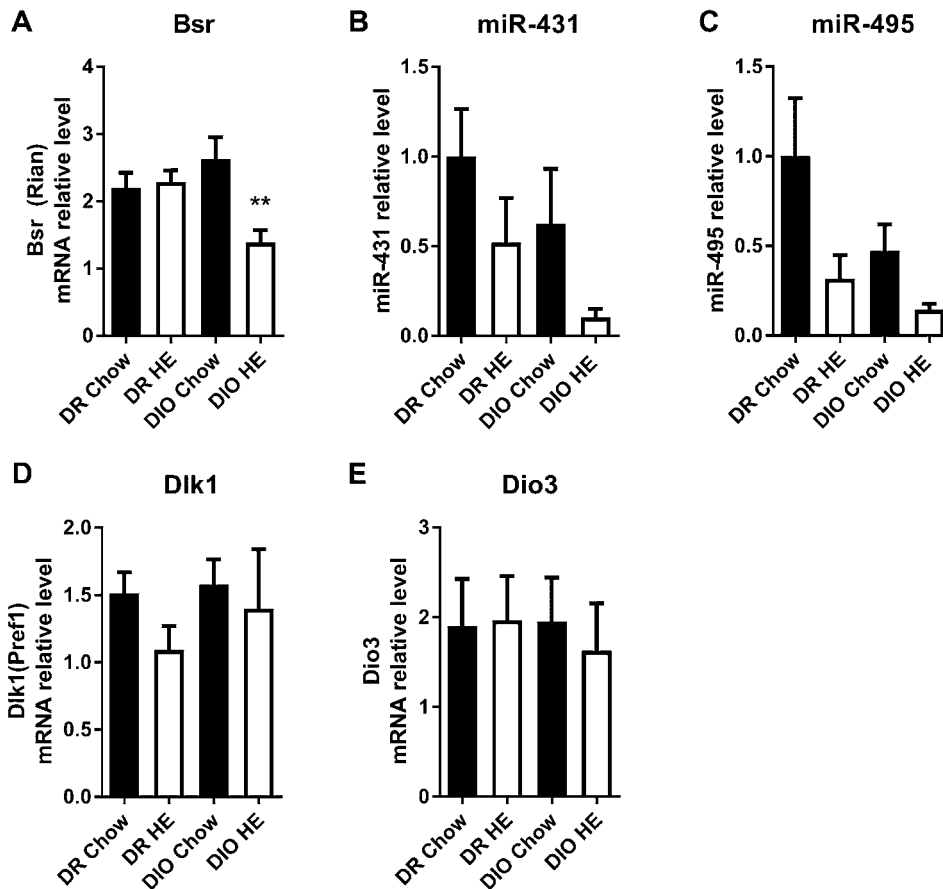


Figure 5: Biological validation by RT-Q-PCR of transcripts identified in the microarray analysis. A) RNA levels of long-noncoding RNA *Bsr*, **: $P < 0.05$ for DIO HE vs DIO chow, B) Levels of miR-431, C) Levels of miR-495, D) mRNA levels of *Dlk1*, and E) mRNA levels of *Dio3*. Messenger RNA levels were normalized to the levels of *Ppia* mRNA. DIO: Diet induced obese strain. DR: Diet resistant strain. HE: High energy. Data are given as Mean+SEM. N=7-9 in each group.

model may be useful to study fetal programming of the pancreas.

In the current study, we studied whole pancreas, because the development of the islets occurs, at least partly, from non-endocrine cells regulated by genes that may not be expressed in the islets but in e.g. duct cells. Thus, it has been shown that in the rat perinatal period a large part of the β -cell expansion occur by neogenesis rather than proliferation [11]. In our study, DIO rat dams became more obese than DR rat dams when fed either chow or a HE diet for 5 weeks before and during pregnancy (Fig. 1A). The DR rats were, as previously shown [16], resistant to diet-induced obesity and did not increase body weight when fed a HE diet (Fig. 1A, Fig. 1B), despite significantly increased caloric intake (Fig. 1C). These results confirm earlier studies of this rat model [14, 16]. The increased food intake of DIO rats is associated with leptin resistance previously reported in these rats [13]. The HE fed DIO dams had a large increase in fasting insulin

and leptin levels (Fig. 1 E-F) together with slightly higher glucose levels (Fig. 1 D). Thus, this group of rats were obese and pre-diabetic, confirming earlier results from this rat model [14].

In the current study, we observed that only 4 out of 11 HE fed DIO rats became pregnant after mating, while fertility in the other groups was higher (9-11 pregnant out of 11). It is tempting to speculate that the pre-diabetic state of the HE fed DIO rats dams led to decreased fertility. In humans, insulin resistance and hyperinsulinemia is a central feature of polycystic ovarian syndrome (PCOS) that leads to abnormal ovulation and infertility [29].

Offspring of HE fed DIO dams had a higher body weight at birth than offspring of HE fed DR dams (Fig. 2). In humans, maternal glucose levels correlate with fetal size and adiposity [30]. Although the fasting glucose levels were not significantly different between the rat dam groups during pregnancy, a possible impaired glucose tolerance in the DIO HE rat dams may have led to a higher

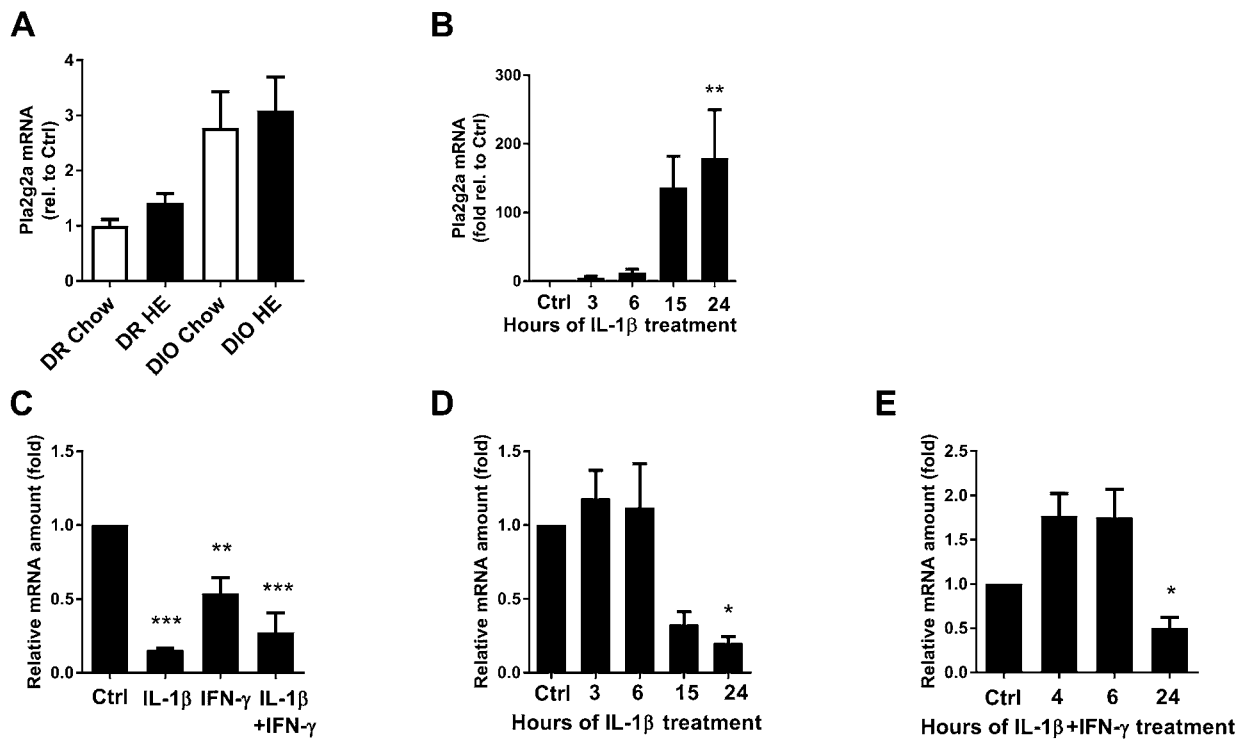


Figure 6: Regulation of the Phospholipase A2 Group IIA (Pla2g2a) mRNA and the long noncoding RNA *Bsr A* qPCR results on mRNA levels of Pla2g2a in A) pancreas from DIO HE vs DR HE, DIO chow vs DR chow. $p < 0.05^*$ and B) normal rat islets after 3-24 hours of IL-1 β treatment (75pg/ml). $P < 0.01^{**}$ vs ctrl. Data are shown as means \pm SEM ($n = 3-9$). C) Culture of neonatal islets for 24 hrs with IL-1 β (150 pg/mL) and/or IFN γ (5 ng/mL). Statistical comparisons are between treatments and Ctrl. D) Neonatal islets exposed to 3-24 hrs of IL-1 β treatment (75pg/mL). Statistical comparisons are between treatment at 24hrs and 3 or 6hrs. E) Neonatal islets treated for 4- 24 hrs with IL-1 β (150 pg/mL) and IFN γ (5 ng/mL). Statistical comparisons are between treatments and Ctrl. Data are shown as Means \pm SEM ($n = 3-4$ unless otherwise noted). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

exposure of glucose to the offspring after a meal, leading to a higher body weight and possible increase in body fat in offspring.

We studied islet cell morphometry by immunohistochemical staining for insulin and glucagon in pancreata from two day old rat pups. The percentage of insulin and glucagon per islet (Fig. 3A, Fig. 3B) and per pancreas (Fig. 3D, Fig. 3E) increased by HE diet, but there were no significant differences in islet mass between the groups.

Others have shown an increase in the neonatal β -cell mass after a maternal high fat diet, which was associated with impaired β -cell function and glucose intolerance in adult rodents [31-33]. Type 2 diabetic patients typically have an increased secretion of glucagon during hyperglycemia, contributing to excessive hepatic glucose production [34]. A low insulin/glucagon ratio increases glycogenolysis and gluconeogenesis, leading to higher blood glucose levels and promotes adipose tissue lipolysis [35]. In our study, however, there was no difference in the insulin/glucagon cell ratio between the rat offspring groups (Fig. 3C) at

postnatal day 2. Thus, at postnatal day 2 morphometric analyses did not show impaired islet mass, but pointed to increased α and β -cell prevalence by HE diet.

The lncRNA *Bsr* (Brain specific repetitive), which is expressed from the maternal allele in the *Dlk1-Dio3* genomic region was the most regulated transcript in the microarray analysis. The microarray analysis showed an 11-fold downregulation, while qPCR studies showed a close to 50% downregulation in the DIO HE offspring group compared to the other offspring groups. The differences observed in *Bsr* in microarray (11 fold) vs 50% in qPCR could be due to the use of different techniques (fluorescent hybridization vs TaqMan assays). Moreover, microarray results showed that several miRNAs from the *Dlk1-Dio3* region were significantly downregulated in the DIO HE offspring compared to DIO chow offspring (Table 1). All the maternally expressed ncRNAs in the *Dlk1-Dio3* locus are most likely processed from the same primary transcript [36-38]. Hypermethylation of either of the two differentially methylated regions (DMRs) in this locus leads to a decreased expression of the maternal transcript

[39]. The differences in expression levels found between the different noncoding RNAs in our study, may be due to differences in processing of the primary transcript. *Bsr* is the rat homolog of the human long noncoding RNA Rian, harboring >80 repeats of the RBII-36 snoRNA and a *Bsr* lncRNA exon [40, 41]. The specific molecular role of *Bsr* has not been investigated in detail, but this noncoding RNA is generally localized to the nucleus [40] and the mouse homolog Rian has been shown to interact with polycomb proteins [42].

Dlk1/Pref-1 has previously been implicated in β cell growth and function. It is highly expressed in islets from 2-day-old rats and pregnant rats [43]. It is also highly expressed in adult human β cells [44]. It is not a β cell growth factor in itself [45, 46], but may have a permissive effect on other growth factors as it is upregulated by growth hormone and prolactin [43]. Recently it was shown that overexpression of Dlk1 increases the expression of Pdx-1 leading to differentiation of human ductal cell to β cells [47]. Thus the tendency to reduced expression in the HE offspring may suggest impaired capacity of the β cells to compensate for the insulin resistance. The soluble form of Dlk1/Pref-1 does not affect β -cell function directly, but is associated with impaired insulin sensitivity [53]. As Dlk1/Pref-1 is known to inhibit adipocyte differentiation the reduced expression may thus promote adipocyte formation and thus contribute to the obesity. On the other hand Dlk1 depletion in pregnancy has been shown to be associated with impaired fetal growth [48]. However, this apparent discrepancy does not exclude that the ratio between fat and lean body mass is increased as has been reported in offspring of mothers suffering from malnutrition during pregnancy [49].

The DLK1-DIO3 imprinted region has previously been associated with fetal metabolic programming: Individuals exposed to wartime famine prenatally had increased methylation of MEG3 in whole blood taken at the age of 58 years [50]. Knockdown of MEG3 in mice resulted in impaired glucose tolerance and decreased insulin secretion as well as a reduction of insulin positive cells. In addition, islets from MEG3 knockout mice showed a decrease in mRNA and protein levels of Pdx1 and MafA that are important for β -cell function [51]. In rats, MEG3 is not annotated and thus we do not have microarray or qPCR results on this neighboring lncRNA from our study. However, a general downregulation of the DLK1-DIO3 locus during development of the fetus may be speculated to predispose to pancreatic islet dysfunction later in life.

Previous studies have shown that several of miRNAs encoded by this locus target proinflammatory and pro-apoptotic genes like IAPP and TP53INP1 in human islets

[38] suggesting that their downregulation may promote inflammation and apoptosis. However, in our data IAPP and TP53INP1 are not regulated by HE diet (Suppl Table S2). We investigated predicted target mRNAs of the miRNAs that were down-regulated significantly by HE diet in the DIO group (miR-410-3p, -377-5p, 431-5p, 495-3p and -543-3p) (Table 1). Gene Ontology analysis showed enrichment of predicted target genes in categories such as protein ubiquitination, metabolic processes, nucleic acid metabolic processes and vasculature development, suggesting that these five miRNAs preferentially regulates these processes. Moreover, when we looked at mRNAs that were predicted targets of the five HE diet down-regulated miRNAs, we observed that these mRNAs had indeed increased their expression by on average 0.05 fold compared with mRNAs, which were not predicted targets of the down-regulated miRNAs and which decreased their average expression by 0.1 fold (10%). Due to large number of observations ($n=1925$ target genes and 18832 non-target genes), this comparison is highly significant ($P<0.0001$ by Mann-Whitney). While this is not a proof that the predicted target genes are de-repressed by the HE diet down-regulated miRNAs, the observation is concordant with this hypothesis.

We found an enrichment in the expression of pro- and anti-inflammatory genes Mcpt1, Mcpt2, Slpi, Ngp, Pla2g2a and IL1ra (Suppl. Table S2) that were upregulated in HE fed rats which is consistent with the low grade inflammation observed in obesity [52]. Our study shows that the proinflammatory cytokines IL-1 β and IFN γ can induce a pronounced downregulation of *Bsr* in rat islets (Fig. 6). Thus, it is tempting to speculate that low-grade inflammation present in type 2 diabetes may result in down-regulation of the DLK1-DIO3 locus in the pancreatic β -cells. Since maternal inflammation may be transferred to the offspring, an inflammatory environment may have induced the down-regulation of the locus found in HE fed DIO rat offspring in this study. Thus, our findings are compatible with the hypothesis that downregulation of the DLK1-DIO3 locus during fetal pancreatic development may promote inflammatory responses leading to type 2 diabetes later in life.

Acknowledgements: We are very grateful for the skilled technical assistance of Christa Persson, Jelena Miskovic Krivokapic, Lene Grønne Pedersen, Sunny Lee, Jacqueline Tybjerg and Susanne Sørensen. The Danish Medical Research Council supported these studies.

Conflict of interest statement: Authors state no conflict of interest

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