Title: Involvement of long non-coding RNAs in beta cell failure at the onset of type 1 diabetes in NOD mice.
Authors: Motterle A, Gattesco S, Caille D, Meda P, Regazzi R
Journal: Diabetologia
Year: 2015 Aug
Volume: 58
Issue: 8
Pages: 1827-35
DOI: 10.1007/s00125-015-3641-5

Involvement of long non-coding RNAs in beta-cell failure at the onset of type 1 diabetes in NOD mice

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Number of words :

Abstract : 245

Main text and figure legend: 4004

Abstract

Aims/hypothesis: Exposure of pancreatic beta-cells to cytokines released by islet-infiltrating immune cells induces alterations in gene expression, leading to impaired insulin secretion and apoptosis in the initial phases of Type 1 diabetes. Long non-coding RNAs (lncRNAs) are a new class of transcripts participating in the development of many diseases. Since little is known about their role in insulin-secreting cells, this study aimed at evaluating their contribution to beta-cell dysfunction.

Methods: The expression of lncRNAs was determined by microarray in the MIN6 beta-cell line exposed to proinflammatory cytokines. The changes induced by cytokines were further assessed by real-time PCR in islets of control and NOD mice. The involvement of selected lncRNAs modified by cytokines was assessed after their overexpression in MIN6 cells and primary islet cells.

Results: MIN6 cells were found to express a large number of lncRNAs, many of which were modified by cytokine treatment. The changes in the level of selected lncRNAs were confirmed in mouse islets and an increase of these lncRNAs was also seen in pre-diabetic NOD mice. Overexpression of these lncRNAs in MIN6 and mouse islet cells, either alone or in combination with cytokines, favored beta-cell apoptosis without affecting insulin production or secretion. Furthermore, overexpression of lncRNA-1 promoted nuclear translocation of NF-κB.

Conclusions/interpretations: Our study shows that lncRNAs are modulated during the development of type 1 diabetes in NOD mice, and that their overexpression sensitizes beta cells to apoptosis, likely contributing to their failure during the initial phases of the disease.

Keywords

apoptosis, beta cell, cytokines, diabetes, islet, lncRNAs, long non coding RNAs.

Abbreviations

IFN-γ	interferon-γ
IL-1β	interleukin 1β
lncRNAs	long non coding RNAs
miRNAs	microRNAs
TNF-α	tumor necrosis factor α

Introduction

Fine-tuning of insulin release from beta-cells is essential to maintain blood glucose homeostasis. Type 1 diabetes is an autoimmune disease characterized by progressive infiltration of pancreatic islets by mononuclear cells, an inflammatory process called insulitis which leads to gradual destruction of beta-cells [1]. During the initial phases of the disease, beta-cells are chronically exposed to cytokines and other pro-apoptotic mediators, released by the immune cells infiltrating the islets of Langerhans and by the islet cells themselves [1]. Prolonged exposure to pro-inflammatory cytokines such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ has deleterious effects on specialized beta-cell functions, which leads to a decreased capacity to produce and release insulin in response to secretagogues, and ultimately to beta-cell loss by apoptosis [1-4]. A deeper understanding of the mechanisms occurring during this inflammatory process is of paramount importance to identify new strategies for preventing and treating the disease.

Chronic exposure of beta-cells to cytokines is known to modulate the expression of many genes, resulting in severe impairment of key signaling pathways [5-7]. Previous studies focused mainly on protein-coding genes, giving minor attention to a newly identified set of regulatory factors, the non-coding RNAs (ncRNAs). In the context of diabetes, the most studied ncRNAs are the microRNAs (miRNAs) [8]. These molecules, which are important controllers of gene networks, are critical regulators of specialized beta-cell functions [8] and are involved in beta-cell damage during the development of type 1 diabetes [9, 10]. In addition to miRNAs, recent transcriptome analysis have identified another class of functional molecules, the long non-coding RNAs (lncRNAs) [11-13]. Although the role of lncRNAs remains largely unknown, members of this RNA family have been involved in diverse gene-regulatory mechanisms such as transcription, imprinting, splicing, protein degradation, epigenetic marks on chromatin [14-19], and their dysregulation has been implicated in many human diseases [20, 21]. Little is known about the role of lncRNAs in the maintenance of beta-cell activities and there are no data regarding the possible contribution of these molecules to the development of type 1 diabetes.

The aim of this project was to identify the lncRNAs that are differentially expressed under pathophysiological conditions favoring the development of type 1 diabetes, and to study their possible involvement in cytokine-mediated beta-cell damage. We found that pro-inflammatory cytokines induce considerable changes in the transcriptional landscape of lncRNAs, some of which were confirmed also in islets of pre-diabetic NOD mice, a well-known model of type 1 diabetes [22]. Studies on the beta-cell line MIN6 and in dissociated islet cells showed that overexpression of a selected subset of lncRNAs, either alone or in combination with cytokines, promotes beta-cell apoptosis. In this study, we show for the first time that lncRNAs are modulated by pro-inflammatory cytokines and contribute to beta-cell demise during the initial phases of murine type 1 diabetes.

Materials and Methods

Chemicals: recombinant mouse IL-1β, hexadimethrine bromide, collagenase and Histopaque 1119 and 1077 were purchased from Sigma-Aldrich (St Louis, MO, USA). Recombinant mouse TNF-α was purchased from Enzo Life sciences (Farmingdale, NY, USA), and recombinant mouse IFN-γ from R&D systems (Minneapolis, MN, USA). Hoechst die 33342 was from Invitrogen (Basel, Switzerland).

Isolation, culture and dissociation of primary islet cells: Mouse pancreatic islets were isolated from female NOD mice (Jackson laboratory, Bar Harbor, ME), male NOD SCID mice (Janvier Labs) and C57BL/6 mice (13-14 weeks-old; Charles River Laboratories, L'arbresle, France). All animal procedures were performed in accordance with the National Institutes of Health guidelines and protocols were approved by the Swiss research council and veterinary offices. Mice islets were isolated by collagenase digestion [23] of the pancreas followed by Histopaque density gradient. Details about islet handling are provided in supplementary methods 1.

MIN6B1 cell culture: the murine insulin-secreting cell line MIN6B1 [24] was cultured in DMEM-Glutamax medium (Invitrogen), supplemented with 15% FCS, 50µg/ml streptomycin, 50IU/ml penicillin (Invitrogen) and 70µmol/l β-mercaptoethanol (Sigma). Cells were seeded at a density of $10^5/cm^2$ for insulin secretion and RNA isolation, and at $5x10^4/cm^2$ for cell death measurements.

Microarray profiling: total RNA was isolated with the RNeasy kit (Qiagen, Basel, Switzerland) from MIN6 cells treated with 0.1ng/ml IL-1β, 10 ng/ml TNF-α and 30ng/ml IFN-γ for 24h. Global mRNA and lncRNA expression profiling using the Mouse LncRNA Array v2.0 and data analysis were carried out by Arraystar (Rockville, MD, USA). Differentially expressed LncRNAs and mRNAs were identified through Volcano Plot filtering (Fold Change ≥ 1.5, P-value ≤ 0.05).

Measurement of lncRNAs expression: total RNA was isolated using the RNeasy kit followed by DNase treatment on column (Qiagen). 1µg RNA was reverse transcribed using M-MLV reverse transcriptase, RNAse H minus (Promega). Quantitative PCR was performed using iQ SYBR Green mix and samples were

amplified using the CFX Connect Real-time system. The full primer list is presented in supplementary table 1.

Transfection and lentiviral infection: MIN6B1 cells were transfected with a control pcDNA3 plasmid or with the same plasmid containing the sequences to translate the lncRNAs (pcDNA3_lncRNA-1; pcDNA3_lncRNA-2, pcDNA3_lncRNA-3, pcDNA3_lncRNA-4), using Lipofectamine 2000 (Invitrogen). Plasmids containing the sequence of the lncRNA of interest or a GFP mRNA were used to produce lentiviral vectors, as described [25]. More information is provided in supplementary method 2.

Insulin secretion: Insulin secretion of MIN6B1 cells transfected with the relevant plasmids for 48h was carried out as previously described ([10]).

Assessment of cell death: the percentage of apoptotic cells was determined by scoring the fraction of cells displaying pycnotic nuclei under fluorescence microscopy (AxioCam MRc5, Zeiss, Feldbach, Switzerland) after incubation with 1µg/ml Hoechst. At least 500 cells were inspected for each condition.

Nf-κB nuclear translocation: MIN6B1 cells were transfected with a plasmid expressing a GFP-tagged form of the human Nf-κB subunit p65 (Rela) and/or the plasmid expressing the lncRNA of choice. 24h after transfection, the cells were treated with the indicated cytokines for 3h, fixed and mounted on a coverslip for microscopic examination. The fraction of cells displaying nuclear localization of p65 was calculated after analyzing around 1500 cells for each condition.

Statistical analysis: Data are presented as mean ±sem. Statistical differences were assessed by two-tailed paired Student's *t*-test when only two sets of data were present (Fig.2, Fig.6 a-d) or by one-way ANOVA followed by Tukey's post hoc test, with a discriminating *P* value of 0.05 (GraphPad Prims).

Results

To investigate the possible contribution of lncRNAs to beta-cell dysfunction, MIN6 cells were incubated for 24h with a mix of cytokines. The expression profile of protein-coding genes and of previously annotated lncRNAs was determined by microarray analysis. In agreement with previous reports in INS1E cells [5, 26], rat beta-cells [6], mice islets [27] and human islets [7], cytokines modified the expression of numerous protein-coding genes involved in inflammatory responses (i.e. *Ccl2*, *Cxcl1*, *Cxcl2*, *Icam1*, *IL15*), IFN-γ signaling (i.e. *Irf1*, *Irf7*, *Igtp*, *Stat1*, *Stat2*, *Stat3* and *Stat4*, *Jak3*), NF-kB regulation (i.e. *Nfkbia*, *Nfkb2*, *Nfkbiz*), endoplasmic reticulum stress and apoptosis (*Atf3*, *Atf6*, *Atf2*, *Bid*, *Bik1*, *Casp1*, *Casp4*, *Chop*) and others (Supplementary Table 2). Hierarchical clustering showed a distinguishable lncRNAs expression profile among the two group of samples obtained from MIN6 cells (Fig.1a). Control MIN6 cells expressed 18.066 of the 31.000 transcripts included in the array. Upon treatment with cytokines, 467 transcripts were up-regulated and 219 were down-regulated (Fig.1b and Supplementary Table 3). Under control conditions, the average raw expression level of lncRNAs was about three times lower than that of protein-coding genes, and six times lower than that observed after exposure to cytokines (data not shown). Four up-regulated lncRNAs, which for simplicity will be hereafter referred as lncRNA-1, -2, -3 and -4, were selected for further analysis. LncRNA-1, -2 and -3 were chosen because of they displayed the most significant expression changes. LncRNA-4 was selected because it is intergenic and the full sequence was available. The fold changes of these lncRNAs in response to cytokine treatment, and their genomic location are displayed in Fig.1c.

The increase in the four lncRNAs up-regulated by cytokines was confirmed by qRT-PCR (Fig.2 a-d). These lncRNAs were almost undetectable in control MIN6 cells, and after 24h incubation with cytokines their expression raised by 1072, 148, 209 and 3.8 folds, for lncRNA-1, -2, -3 and -4, respectively (Fig.2 a-d). Similar results were observed in islets isolated from C57BL/6N mice incubated with the same cytokines for 24h, with lncRNA-1, -2, -3 and -4 being up-regulated 520, 26, 36 and 3.6 times, respectively (Fig.2 e-h). Similar data were obtained when the data were normalized using 18s instead of Gapdh (Supplementary fig.

1). These changes appeared to be specifically induced by cytokines, since they were not observed when MIN6 cells were incubated with palmitate or with the GLP1 analog Exendin-4 (Supplementary Fig.2). The expression of these lncRNAs raised rapidly after treatment with cytokines, the induction of lncRNA-1 and -2 was significant after 6h treatment, whereas that of lncRNA-3 and -4 was already significant after 2h treatment (Fig.3). Incubation of MIN6 cells with different combinations of pro-inflammatory cytokines showed that IFN-γ alone was sufficient to induce the expression of lncRNA-1, lncRNA-3 and lncRNA-4 (Fig.4). The changes seen for lncRNA-2 reached statistical significance only in the presence of a mix of cytokines (Fig.4).

Subsequently, we studied whether changes in the level of islet lncRNAs precede the development of type 1 diabetes in NOD mice. Although a minor infiltration of lymphocytes has been reported as early as at 4 weeks of age, in these mice peri-insulitis (a leukocytic lesion on the pole of the islet) becomes evident between 6-8 weeks and by week 12 is present in the majority of the islets [22, 28]. In this study, we used pre-diabetic female NOD mice displaying normal blood glucose levels and we isolated the islets at 4, 8 and 13 weeks of age. We found that the levels of lncRNA-1 and lncRNA-2 were significantly higher in the islets of 8 and 13 weeks-old mice than in those of 4 weeks-old mice. LncRNA-3 expression was also higher in the islets of 8 than 4 week mice while lncRNA-4 did not reach statistical significance (Fig.5). The expression of these lncRNAs is not modified in NOD-SCID mice that do not develop type 1 diabetes (Supplementary Fig.3), demonstrating that their up-regulation is linked to the autoimmune reaction.

We then tested whether the changes in the expression of these lncRNAs affect specific beta-cell functions. Overexpression of the four lncRNAs in MIN6 cells (Supplementary Fig.4) did neither modify the insulin mRNA level (Supplementary Fig.5), the insulin content (Fig.6 a-d) nor insulin release (Fig.6 e-h). Prolonged exposure of beta-cells to cytokines sensitizes them to apoptosis [1, 3]. The expression of the four lncRNAs is controlled by cytokines and raises concomitantly with the increase of insulitis (Fig.5), suggesting that they may be involved in the events causing beta-cell failure. Therefore, we investigated whether the

modulation of these lncRNAs affects beta-cell survival. We found that overexpression of lncRNA-3 and lncRNA-4 in MIN6 cells is sufficient to increase the number of apoptotic cells, and that this effect is not increased in the presence of cytokines (Fig.7 c-d). In contrast, overexpression of either lncRNA-1 or lncRNA-2 alone did not modify the proportion of apoptotic cells. However, the overexpression of these lncRNAs increased apoptosis when the cells were concomitantly incubated with low doses of IL-1β or TNF-α, which alone did not affect cell survival (Fig.7 a-b), suggesting that the combination of both signals is necessary to trigger apoptosis. Similar results were obtained in mice islets (Fig.7 e-f), transduced with lentiviral vectors (Supplementary Fig.6). We then investigated whether the overexpression of these lncRNAs, either alone or in combination with IL-1β, modifies the level of some members of the Bcl-2 family (*Bik1* and *Bid*) or of the downstream endoplasmic reticulum stress related genes *Chop* and *Atf3*. No significant effect was observed on any of these genes (supplementary fig.7).

Although basal nuclear factor-κB (NF-κB) activity is required for normal insulin release [29], induction of the NF-κB pathway plays a central role in cytokine-induced apoptosis [30]. Translocation of NF-κB to the nucleus is one of the initial events occurring shortly after exposure to IL-1β [31]. In view of their potential crosstalk with the NF-κB pathway, we transfected MIN6 cells with a GFP-tagged form of p65, one of the NF-κB subunits in the predominant dimers (p65/p65 and p65/p50) found in IL-1β–exposed beta-cells [32], and we monitored its translocation to the nucleus upon overexpression of each of the four lncRNAs. We found that incubation of the cells in the presence of low doses of IL-1β (0.1ng/ml) or IFN-γ (30ng/ml) does not affect the intracellular distribution of NF-κB (Fig.8, supplementary fig.8). In contrast, incubation with higher doses of IL-1β (10ng/ml) or IFN-γ (300ng/ml) led to an increase in the fraction of cells in which NF-κB is localized in the nucleus. Of the four lncRNAs studied, only overexpression of lncRNA-1 mimicked the effect caused by high doses of IL-1β or IFN-γ, and increased the fraction of cells in which p65 is localized to the nucleus (Fig.8). This effect was not further potentiated by exposing the cells to IL-1β (Fig.8).

Discussion

Type 1 diabetes is characterized by progressive islet infiltration by immune cells releasing cytokines and other pro-apoptotic mediators that contribute to beta-cell death during the initial phases of the disease [1]. In diabetes-prone NOD mice beta-cell apoptosis precedes substantial lymphocytic infiltration [33, 34], supporting the notion that inflammatory mediators released by infiltrating cells play a major role in beta-cell death. Accordingly, chronic exposure to pro-inflammatory cytokines has detrimental effects on specialized beta-cell functions [1-4] and affects the expression of protein-coding genes and of small non-coding RNAs [5-7]. We previously showed that changes in the level of miRNAs can affect the secretory capacities of beta-cells, and their sensitivity to apoptosis [9, 10]. Here, we not only demonstrate that incubation of MIN6 cells with a mix of Th1 cytokines results in considerable changes in the expression of protein-coding genes involved in inflammatory responses, IFN-γ signaling, NF-kB regulation, endoplasmic reticulum stress and apoptosis similar to those shown in previous reports [5-7, 26, 27] but we also reveal for the first time that it causes major modifications in lncRNA expression. Despite extensive research, the role of the majority of these transcripts is still unknown and no information is available regarding their contribution to the maintenance of beta-cell function and the development of type 1 diabetes. We found that MIN6 cells express many previously annotated lncRNAs, and that a considerable fraction of these transcripts is modulated in the presence of pro-inflammatory cytokines. In agreement with previous studies [35, 36], we found that the average levels of lncRNAs expression in MIN6 cells was lower than that of protein-coding genes.

We focused on four lncRNAs and demonstrated that the pro-inflammatory cytokines modulate their expression also in mouse islets. IFN-γ was sufficient to induce the expression of these lncRNAs. Interestingly, in NOD mice during the initial phases of the disease, the islet genes displaying the higher expression changes are all induced by interferons [28]. The expression of the four tested lncRNAs is rapidly increased by cytokines, suggesting that these lncRNAs act early in the cascade of events activated by the inflammatory mediators, and culminating in cell death. This is not surprising since the expression of many

protein-coding genes involved in beta-cell apoptosis is already modified after 2h exposure to cytokine [5, 26].

Subsequently, we investigated whether changes in the level of these lncRNAs precede the development of diabetes and we measured their expression in islets of normoglycemic NOD mice of increasing age. We found that the levels of lncRNA-1, lncRNA-2 and lncRNA-3 raise with age, paralleling the development of insulitis in the early phases of the disease [22, 28]. This was not observed in NOD-SCID mice, which are immunodeficient and do not develop diabetes, suggesting that the induction of these lncRNAs is a consequence of the autoimmune reaction. In NOD mice autoimmune destruction of beta-cells is preceded by defective insulin secretion [37]. However, none of the lncRNAs investigated had an impact on insulin biosynthesis and release. As in NOD mice beta-cell apoptosis precedes substantial lymphocytic infiltration [33, 34], and prolonged exposure to cytokines sensitizes beta-cells to apoptosis [1-4], we assessed whether the modulation of lncRNAs affects the survival of insulin-secreting cells. Indeed, overexpression of lncRNA-3 and lncRNA-4 was sufficient to increase apoptosis of MIN6 and mouse islet cells. In contrast, lncRNA-1 and lncRNA-2 induced apoptosis only in cells concomitantly exposed to low doses of IL-1β or TNF-α. This suggests that lncRNA-1 and -2 trigger apoptosis only when the beta-cells receive a combination of signals generated by the activation of different inflammatory pathways. This is reminiscent of the events occurring in the initial phases of type 1 diabetes when beta-cell death is believed to be elicited by prolonged exposure to a combination of inflammatory mediators, rather than to low doses of a single cytokine [38]. Cytokines modify the level of many genes belonging to the Bcl2 family [6, 39] and activate the ER stress response [40]. Overexpression of the four lncRNAs, alone or in combination with IL1-β, did neither alter the expression of the pro-apoptotic Bcl2-family members *Bik1* and *Bid*, nor they affected the levels of the downstream ER stress-related genes *Chop* and *Atf3*, suggesting that they may not operate through the activation of these genes.

Stimulation of beta-cells with either IL-1β or TNF-α results in the activation of NF-κB [41], a key regulator of gene networks controlling cytokine-induced beta-cell dysfunction and death [30]. Indeed, inhibition of

NF-κB activation *in vitro* by overexpression of inhibitory κB (IκB) protects beta-cells against cytokine-induced apoptosis [42, 43]. Furthermore, beta-cell specific NF-kB blockade *in vivo* prevents streptozotocin-induced diabetes development [44]. The translocation of NF-κB into the nucleus is one of the initial events occurring shortly after exposure to IL-1β or TNF-α [31]. In INS1E cells, IL-1β triggers a stronger induction of NF-κB target genes compared to TNF-α, partly because it elicits an earlier translocation of NF-kB into the nucleus [31] and a more stable activation of this signaling pathway [32]. We found that lncRNAs-1 promotes the translocation of p65 into the nucleus, mimicking the effects observed when the cells are exposed to high doses of IL-1β and IFNγ. Upon overexpression of lncRNA-1, low doses of IL-1β are sufficient to induce apoptosis. Thus, sensitization of beta-cells overexpressing lncRNA-1 to apoptosis may at least in part be caused by NF-κB nuclear translocation. Our findings indicate a possible crosstalk of IFN-γ with the NF-κB pathway in beta-cells. IFN-γ exerts its functions mainly through the JAK-STAT cascade [45], but evidence are accumulating indicating that in some cell types IFN-γ can also activate the NF-kB pathway [46, 47]. The mechanisms through which the NF-kB pathway may mediate the actions of IFN-γ remain largely unknown. Our data suggest that lncRNAs could provide a link between the two signaling cascades. Further studies will be needed to elucidate the precise mechanism through which lncRNA-1 induces the nuclear translocation of NF-κB and, in general, to understand the role of lncRNA-2, lncRNA-3 and lncRNA-4 in beta-cell apoptosis.

Overall our findings show that pro-inflammatory cytokines induce extensive changes in the expression of previously annotated lncRNAs. The study of four selected lncRNAs shows that the expression of these non-coding transcripts raises with the development of insulitis in NOD mice and that their modulation increases beta-cell apoptosis, suggesting that they may contribute to cytokine-mediated beta-cell dysfunction occurring during the initial phases of type 1 diabetes. The four lncRNAs investigated in this study are most probably not the only long non-coding transcripts contributing to beta-cell demise. In fact, our data have highlighted dramatic changes in the level of many other lncRNAs elicited by cytokines and these inflammatory mediators may potentially also affect the expression of some of the recently

described beta-cell specific mouse lncRNAs [48] that were not included in the array. Future studies will have not only to elucidate the contribution of all these lncRNAs in the development of type 1 diabetes in mice but will also need to assess whether the same mechanism operates in human. This promises to be a difficult task because although the function of lncRNAs is likely to be maintained, the conservation of the sequence throughout species can be as little as 21%, with exons being under less evolutionary constraint than in protein-coding genes [35, 49]. The genomic loci from which the four lncRNAs are generated produce a large number of partially overlapping transcripts and it was not possible to identify with certainty their human orthologues. New computational approaches based on the analysis of the secondary structure rather than on sequence conservation will be necessary to precisely identify the human transcripts corresponding to the lncRNAs investigated in this study.

Acknowledgments

We would like to thank the group of Christian Widmann for kindly providing the vector expressing Rela and Arraystar for the microarray service provided.

Funding

This work was supported by the European Foundation for the Study of Diabetes and Lilly research fellowship (AM), by grants of the Swiss National Science Foundation (310030-146138 to RR and 310000-141162 to PM) and by the "Fondation Francophone pour la Recherche sur le Diabète" (RR).

Duality of interest

The authors declare that there is no duality of interest associated to this manuscript.

Contribution statement

AM conceived the experiments, generated the research, analyzed the data, wrote the manuscript and approved its final version. SG DC and PM contributed to the acquisition of data, reviewed the manuscript and approved its final version. RR conceived the experiments, analyzed the research data, wrote the manuscript and approved its final version. AM is responsible for the integrity of the work as a whole.

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Figure legends

Fig.1. **a**. Hierarchical clustering of the samples analyzed by microarray. The dendogram shows the relationship between the expression of control samples (Ctrl) and samples exposed for 24h to 0.1ng/ml IL-1β, 10ng/ml TNF-α and 30ng/ml IFN-γ (Cyt Mix) (Red=high expression; blue= low expression). **b**. Volcano plot summarizing the results obtained by microarray, in which control and cytokine-exposed MIN6 cells are compared. Significantly up- or down-regulated transcripts are in red. **c**. List of studied lncRNAs with fold changes, p-values and genomic locations.

Fig.2. The four lncRNAs are induced by pro-inflammatory cytokines. MIN6 cells (a-d) and mouse islets (e-h) were incubated in the presence or absence of 0.1ng/ml IL-1β, 10ng/ml TNF-α and 30ng/ml IFN-γ. The expression levels were measured by qRT-PCR and normalized to those of Gapdh. The results are means ±SEM of 3-6 independent experiments. **P*< 0.05, ***P*< 0.01, ****P*<0.001 versus the control.

Fig.3. Time-course of the induction of the lncRNAs. MIN6 cells were incubated for the indicated periods with 0.1ng/ml IL-1β, 10ng/ml TNF-α and 30ng/ml IFN-γ (a-d). The expression was measured by qRT-PCR and normalized to that of Gapdh. The results are means ±SEM of 3 independent experiments. **P*< 0.05, ****P*<0.001 versus the control at 0h.

Fig.4. IFN-γ is sufficient to induce the expression of the lncRNAs. MIN6 cells were incubated in the presence or absence of different cytokines at the concentrations given in the previous figures (a-d). The expression were measured by qRT-PCR and normalized to that of Gapdh. The results

shown are means ±SEM of 3 independent experiments. **P< 0.01, ***P<0.001 versus ctrl or versus the indicated condition.

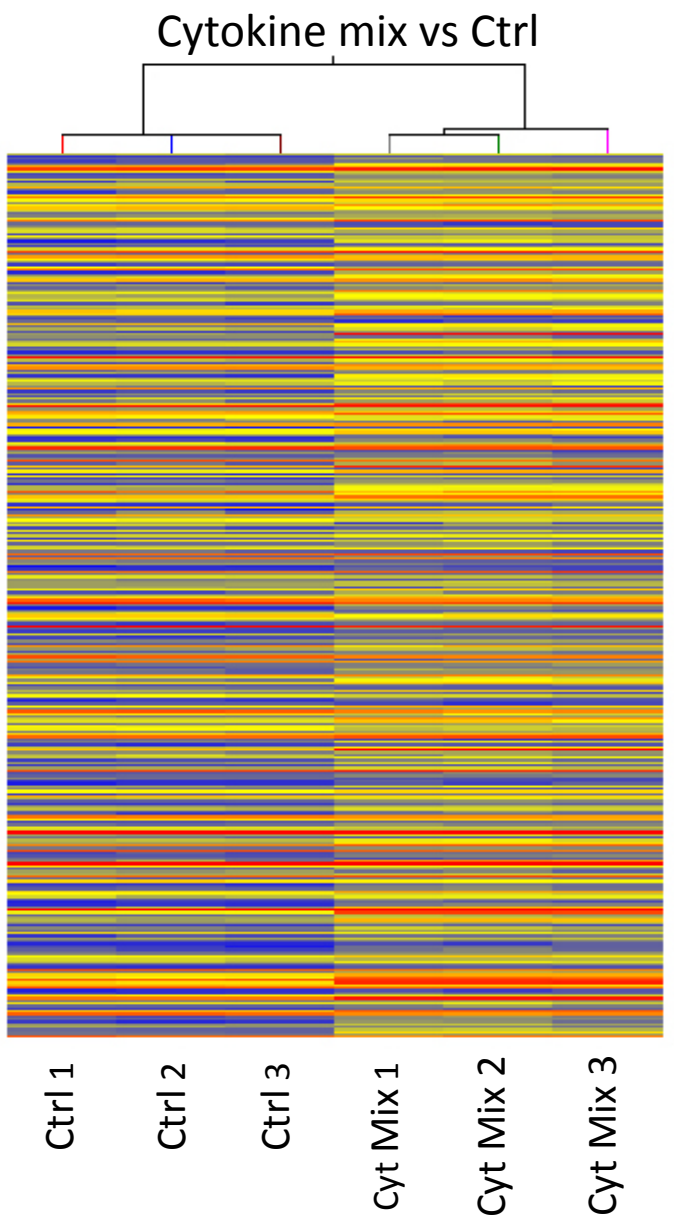
Fig.5. The expression of lncRNA-1, -2 and -3 increases with insulitis in islets of NOD mice. Islets were isolated from female mice aged 4, 8 and 13 weeks. The level of the four lncRNAs was measured by qRT-PCR and normalized to that of Gapdh (a-d). **P< 0.01 versus the levels measured in 4 week-old animals.

Fig.6. Overexpression of the lncRNAs does not affect insulin content or secretion. MIN6 cells were transfected with an empty plasmid (vector) or a plasmid permitting to transcribe each individual lncRNA for 48h. The cellular insulin content was measured by ELISA (a-d). To test their secretory properties, the cells were incubated with 2 or 20mmol/l glucose for 45 min. Basal and glucose-induced insulin release were assessed by ELISA (e-h). The results represent means ±SEM of 3-6 independent experiments.

Fig.7. Overexpression of the lncRNAs triggers apoptosis. a-d, MIN6 cells were transfected with a control plasmid (vector), in grey or with plasmids overexpressing lncRNA-1 (a); lncRNA-2 (b); lncRNA-3 (c) or lncRNAs-4 (d), in black. After 24h, the cells were incubated for another 24h with or without 0.1ng/ml IL-1β, 10ng/ml TNF-α and 30ng/ml IFN-γ, alone or in combination. Dispersed mouse islet cells were transduced with a control lentiviral vector expressing GFP or a vector expressing the lncRNA of interest (e: GFP in white, lncRNA-1 in grey and lncRNA-2 in black; f: as indicated in the figure). Incubation with cytokines was performed as described above. The proportion of cells showing pycnotic nuclei was scored. The results are the means ±SEM of 4-6 independent experiments. *P< 0.05, **P< 0.01 versus ctrl.

Fig.8. Overexpression of lncRNA-1 induces the nuclear translocation of NF-κB. MIN6 cells were transfected for 24h with a plasmid expressing GFP-tagged p65 (Rela). They were then exposed for 3h to the indicated cytokines, before fixation and fluorescence microscopy analysis. The number of cells displaying nuclear NF-κB localization were scored. Data were normalized to the values of control p65 with no cytokine exposure. The results shown represent means ±SEM of 6 independent experiments. **P*< 0.05 versus the Ctrl value (p65, no cytokine exposure).

a



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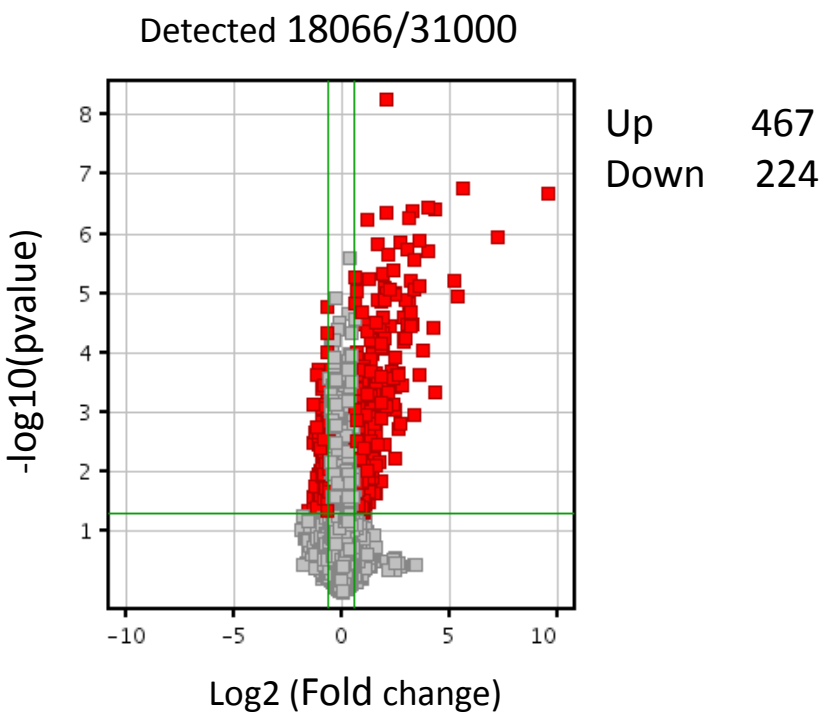

c

LncRNA	Transcript	Fold change	p-value	Genomic location
lncRNA-1	gm5970	766.9	1.98E-07	chr18:60380496-60381712
lncRNA-2	AI451557	146.4	1.1E-06	chr8:97048966-97051168
lncRNA-3	BC002288	42.1	1.07E-05	chr2:121973452-121975096
lncRNA-4	gm16675	5.4	0.00089	chr:47816325-47824869

Figure 1. **a**. Hierarchical clustering analysis of the 6 samples utilized to perform the microarray. The dendogram shows the relationship among the expression levels of the samples, 3 control samples (Ctrl) and 3 samples treated with a mix of pro-inflammatory cytokines (Cyt Mix) for 24h (IL-1β 0.1ng/ml; TNF-α 10ng/ml and IFN-γ 30ng/ml); (red=high expression; blue= low expression).
B. Volcano plot summarizing the results obtained by the microarray. MIN6 cells treated with a mix of pro-inflammatory cytokines were compared to untreated samples. In red are the transcript that were significantly up or down-regulated.
C. List of lncRNAs that were further studied in this project, with fold change, p-value and genomic location.

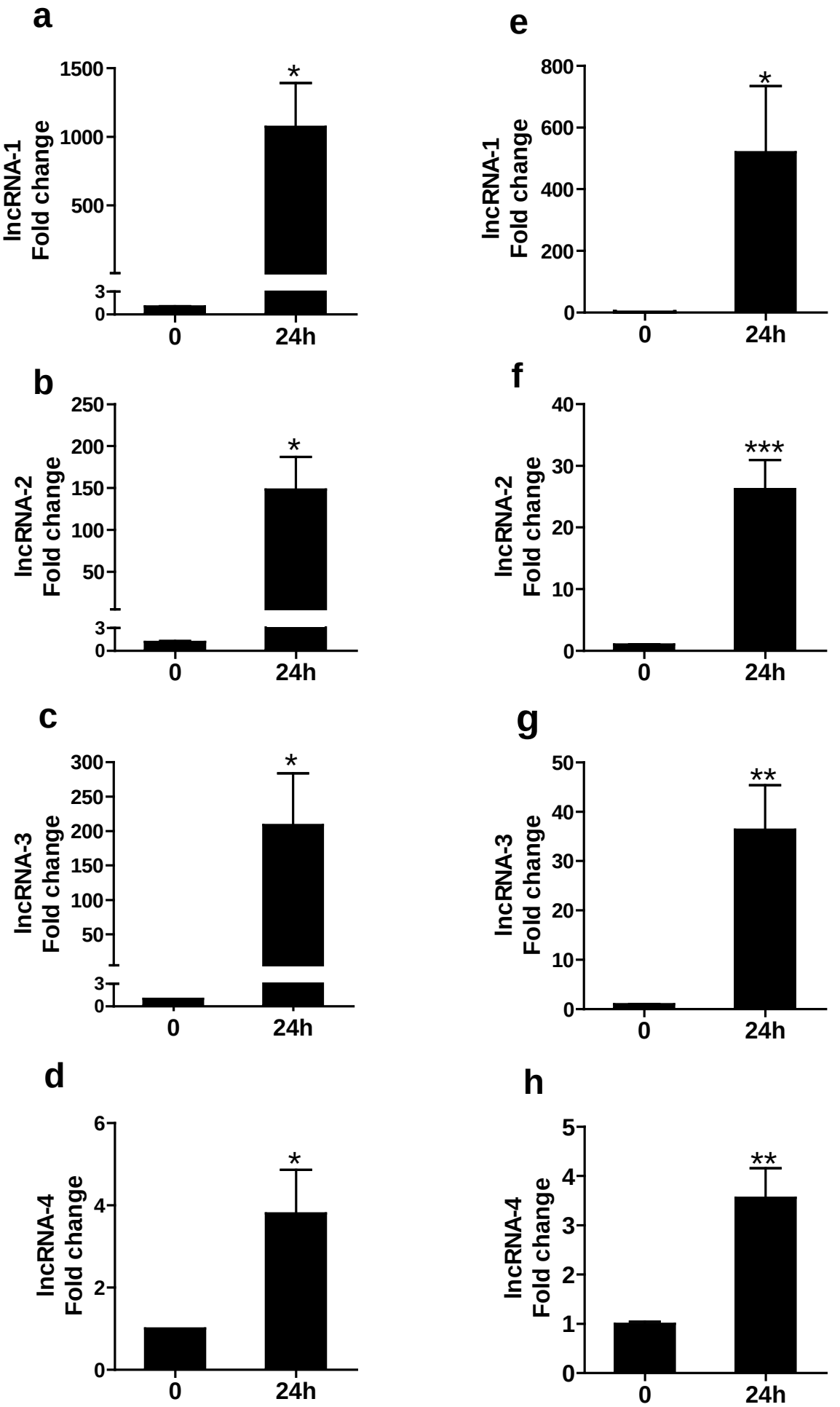

Figure 2. Proinflammatory cytokines induce the expression of lncRNA-1, -2, -3 and -4 in MIN6 cells and primary islets.
A. MIN6 cells were incubated for 6 or 24h in control medium or with a mix of cytokines (IL-1b 0.1ng/ml; TNF-a 10ng/ml and IFN-g 30ng/ml). **B**. Primary mouse islets were incubated for 24h in the presence or absence of the cytokine mix described above. The expression levels were measured by qRT-PCR and normalised to the expression of Gapdh. The results shown are the means ±SEM of 3 to 6 independent experiments. **P*< 0.05, ***P*< 0.01, ****P*<0.001 versus ctrl (indicated here are the comparisons versus the 0h).

Fig 3

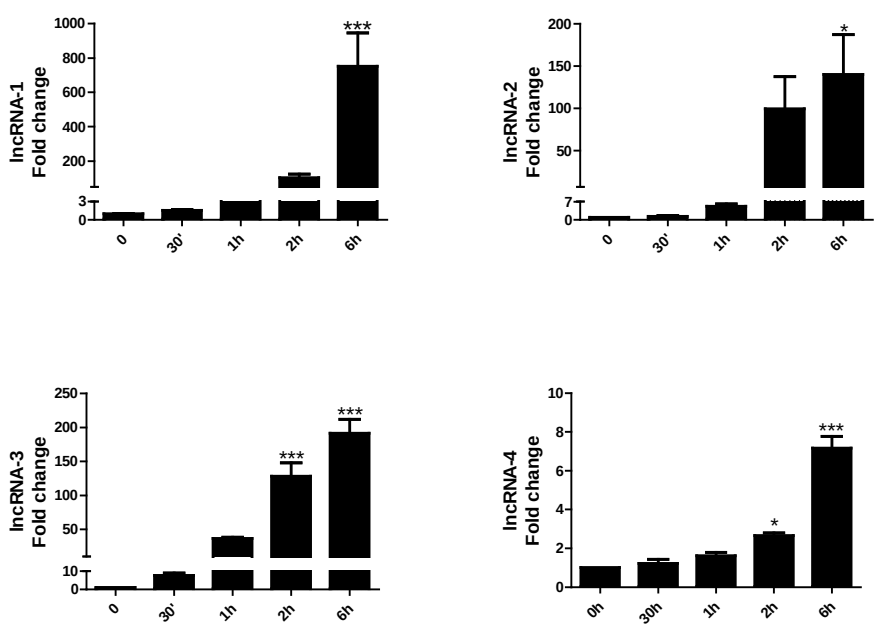

Figure 3. Proinflammatory cytokines induce the expression of lncRNA-1, -2, -3 and -4 within 1 to 6 hours from the beginning of the incubation. MIN6 cells were incubated for 0, 0.5, 1, 2 and 6 h with a mix of cytokines (IL-1β 0.1ng/ml; TNF-α 10ng/ml and IFN-γ 30ng/ml). The levels of expression were measured by qRT-PCR and normalised to those of Gapdh. The results shown are the means $\pm$SEM of 3 independent experiments.
**P*< 0.05, ***P*< 0.01, ****P*<0.001 versus ctrl (indicated here are the comparisons versus the 0h).

Fig.4

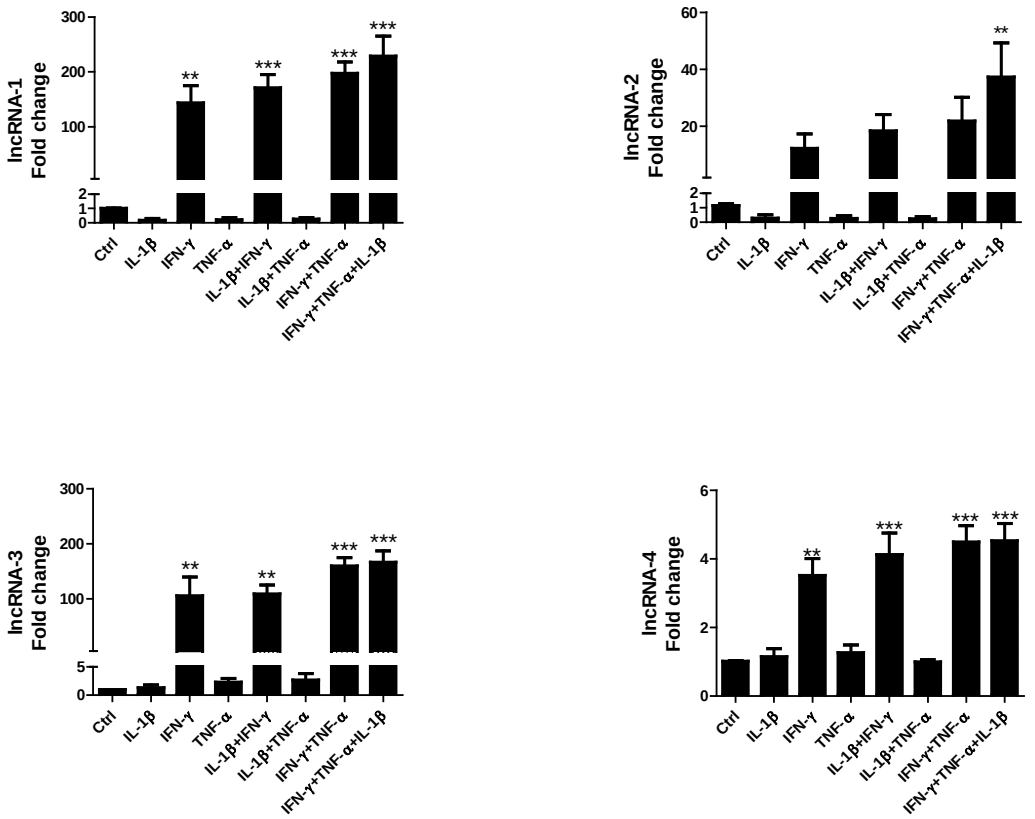

Figure 4. IFN-γ is sufficient to induce the expression of lncRNA-1, -2, -3, -4. MIN6 cells were incubated either in absence or presence of different combinations of pro-inflammatory cytokines with the following concentrations: IL-1β 0.1ng/ml; TNF-α 10ng/ml and IFN-γ 30ng/ml. The levels of expression were measured by qRT-PCR and normalised to the expression of Gapdh. The results shown are the means ±SEM of 3 independent experiments.
*P< 0.05, **P< 0.01, ***P<0.001 versus ctrl or versus the indicated condition.

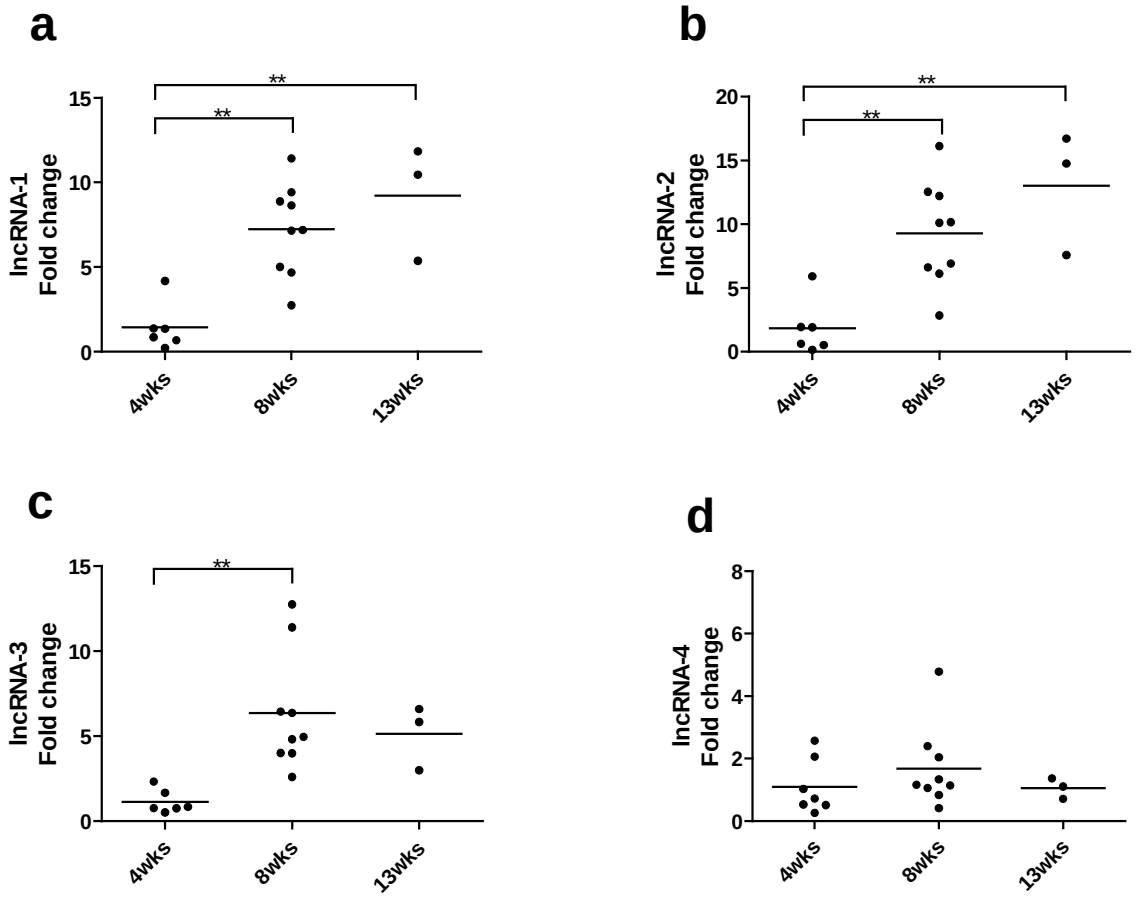

Figure 5. The expression of lncRNA-1, -2 and -3 increases with insulitis in pancreatic islets of NOD mice. Pancreatic islets were isolated from female mice of 4, 8 and 13 weeks. Their level of expression was measured by qRT-PCR and normalised to the expression of Gapdh. **P*< 0.05, ***P*< 0.01, ****P*<0.001 versus 4wks.

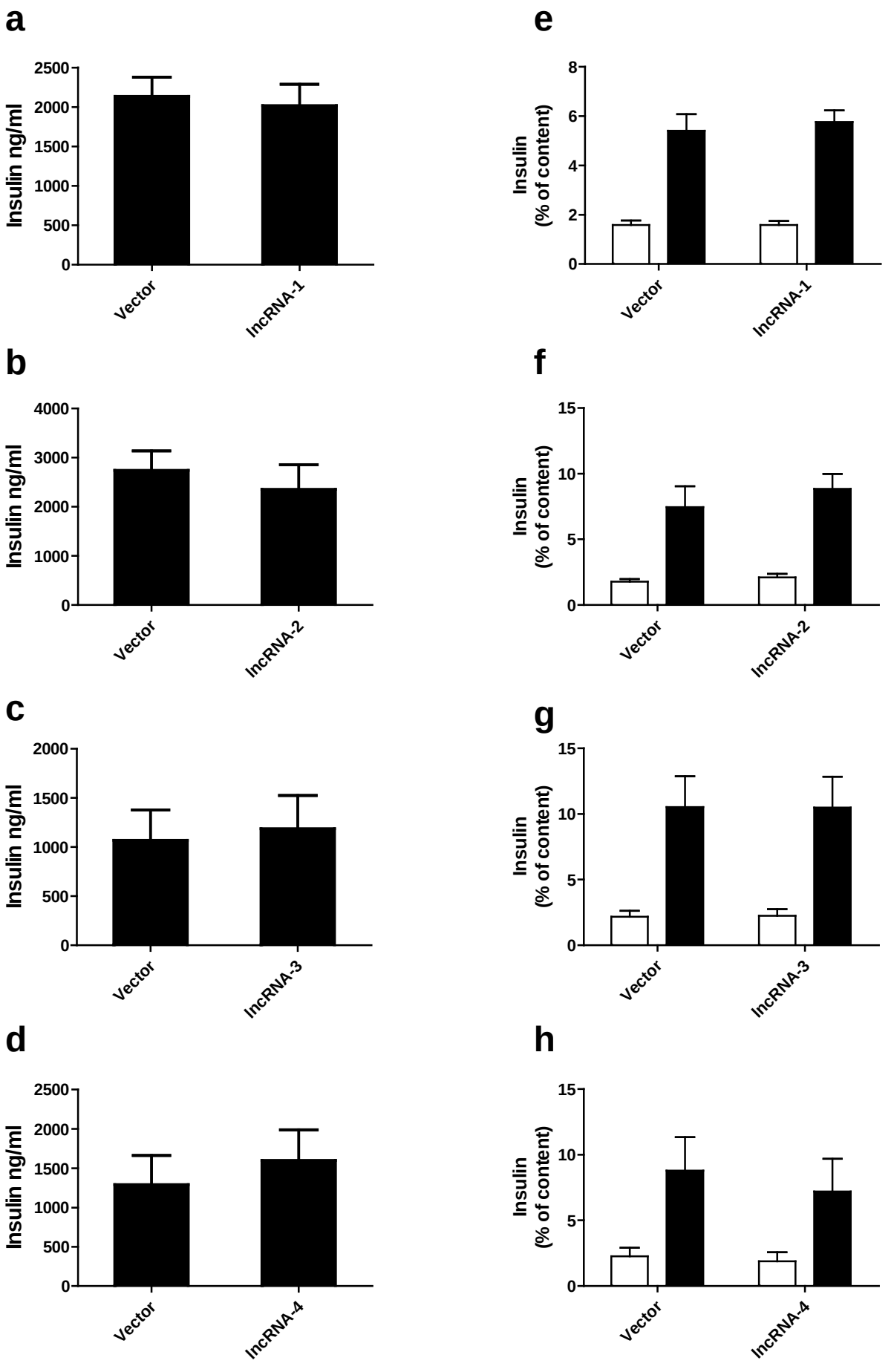

Figure 6. The overexpression of the four lncRNAs does not change insulin content or glucose-induced insulin secretion. **A**. MIN6 cells were transfected either with an empty pcDNA3 plasmid (vector) or a plasmid containing the sequence to transcribe each individual lncRNA for 48h. The insulin content of the cells, measured by ELISA, was not significantly altered. **B**. Under the same conditions, the cells were incubated with either 2 or 20 mmol/L glucose for 45 min. No significant differences were detected in either basal or glucose-induced insulin release. The results shown are the means ±SEM of 3-6 independent experiments.

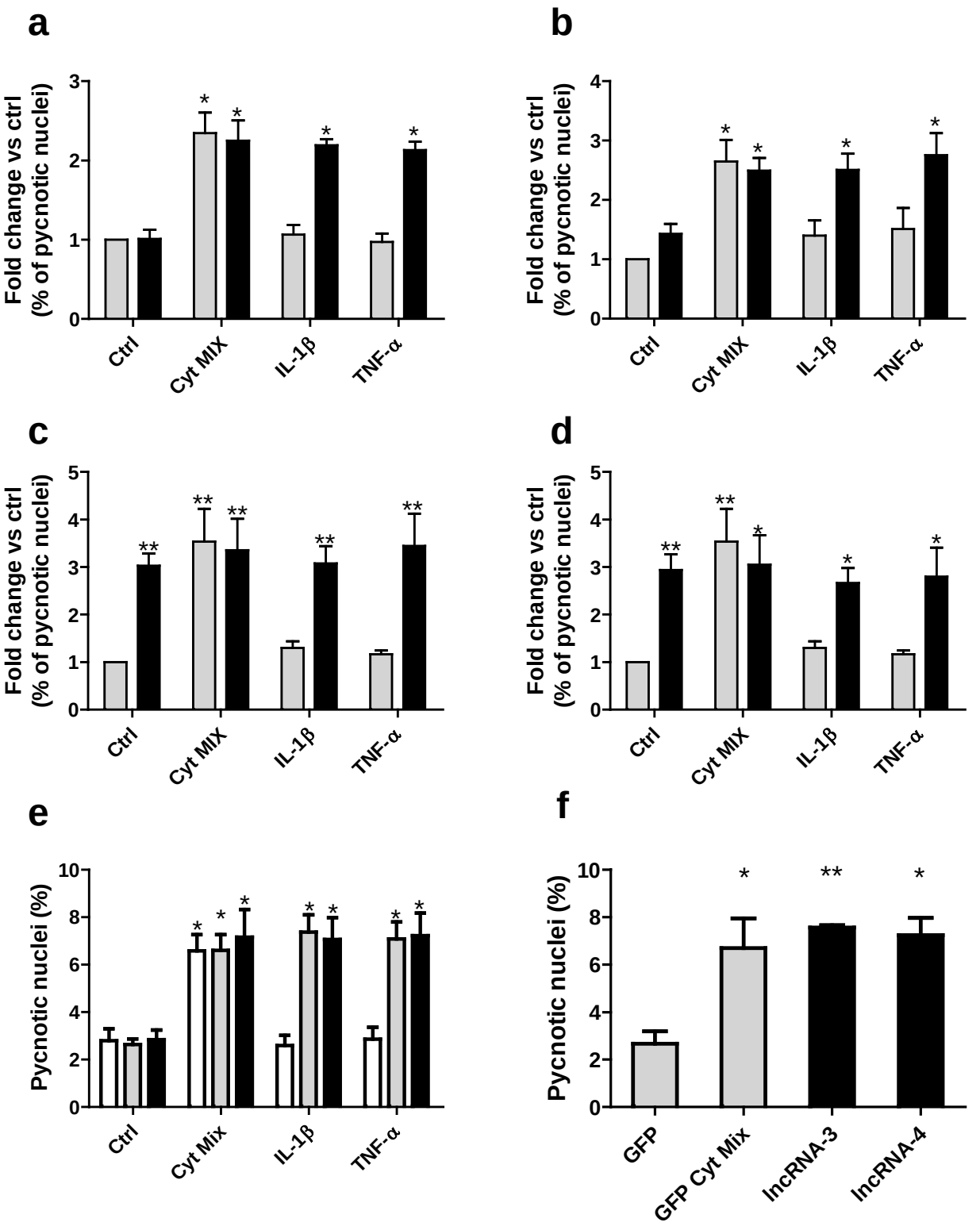

Figure 7. Overexpression of lncRNA-1, -2, -3 and -4 in MIN6 cells and mouse islets modulates apoptosis. **A**. MIN6 cells were transfected with a control plasmid (vector) or a plasmid overexpressing either lncRNA-1, -2, -3 or -4. After 24h, the cells were treated or not for another 24h with IL-1β 0.1ng/ml; TNF-α 10ng/ml and/or IFN-γ 30ng/ml alone or in combination, as indicated. Pycnotic nuclei were scored upon Hoechst staining. **P*< 0.05, ***P*< 0.01, ****P*<0.001 versus Ctrl or versus the conditions indicated in the figure. **B**. Dispersed mouse islet cells were infected with a control lentivirus (expressing GFP) or a lentivirus expressing the lncRNA of interest. Incubation with cytokines and assessment of apoptotic cells was performed as described above. The results shown are the means ±SEM of 4 to 6 independent experiments. **P*< 0.05, ***P*< 0.01, ****P*< versus ctrl or versus the indicated condition.

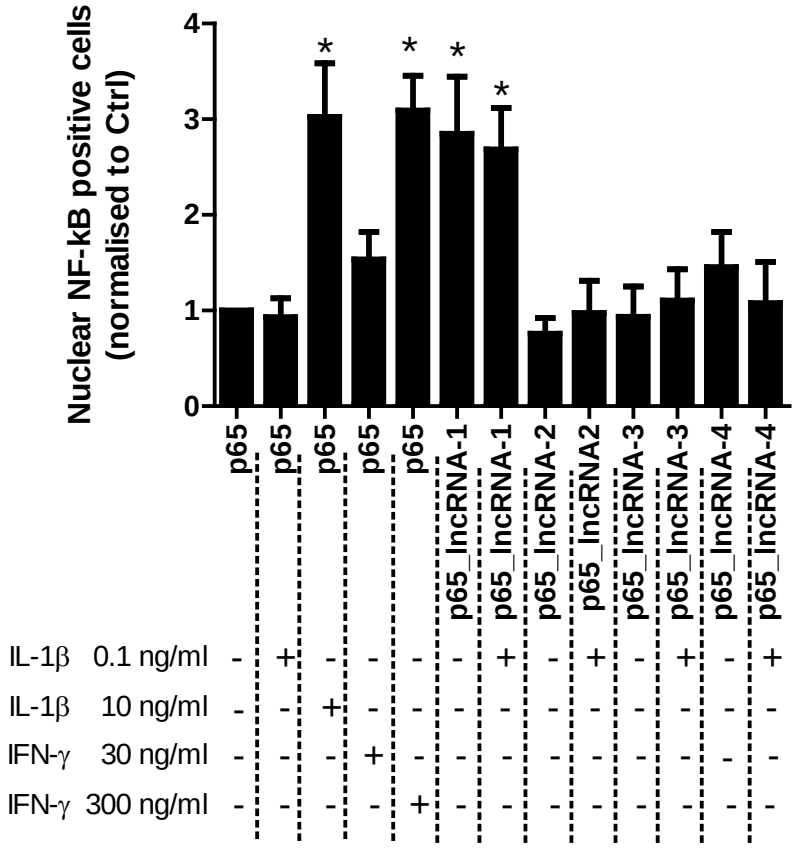

Figure 8. Overexpression of lncRNA-1 induces nuclear localization of NF-kB. Cells were transfected for 24h with a plasmid containing the sequence to express p65 (Rela) conjugated with GFP. They were then treated with different cytokines at the concentrations indicated in the figure for 3h before they were fixed and analyzed by fluorescence microscopy. The number of cells displaying a nuclear NF-kB localization were scored. Data were normalized to the values of the control p65, no treatment. The results shown are the means ±SEM of 6 independent experiments. *P< 0.05 versus the Ctrl value (p65, no cytokine exposure).