Methods in Molecular Biology (2016) 1433: 21–54
DOI 10.1007/7651_2015_291

Published online: 03 March 2016

# Pancreatic Beta Cell Survival and Signaling Pathways: Effects of Type 1 Diabetes-Associated Genetic Variants

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## Abstract

Type 1 diabetes (T1D) is a complex autoimmune disease in which pancreatic beta cells are specifically destroyed by the immune system. The disease has an important genetic component and more than 50 *loci* across the genome have been associated with risk of developing T1D. The molecular mechanisms by which these putative T1D candidate genes modulate disease risk, however, remain poorly characterized and little is known about their effects in pancreatic beta cells. Functional studies in in vitro models of pancreatic beta cells, based on techniques to inhibit or overexpress T1D candidate genes, allow the functional characterization of several T1D candidate genes. This requires a multistage procedure comprising two major steps, namely accurate selection of genes of potential interest and then in vitro and/or in vivo mechanistic approaches to characterize their role in pancreatic beta cell dysfunction and death in T1D. This chapter details the methods and settings used by our groups to characterize the role of T1D candidate genes on pancreatic beta cell survival and signaling pathways, with particular focus on potentially relevant pathways in the pathogenesis of T1D, i.e., inflammation and innate immune responses, apoptosis, beta cell metabolism and function.

**Keywords:** Type 1 diabetes, Diabetes candidate genes, Pancreatic beta cells, Pancreatic islets, Inflammation, Apoptosis, Cytokines, Small interfering RNA (siRNA), Overexpression vector

## 1 Introduction

Genome wide association studies (GWAS) have identified more than 50 genomic regions associated with type 1 diabetes (T1D) risk [1]. Many candidate genes have been proposed within these regions but few have been confirmed as true etiological genes, and the molecular mechanisms by which these candidate genes modulate disease risk remain poorly characterized [1]. The big challenge in the field is to define how candidate genes for T1D interact with the environment and modulate the development of diabetes.

Chronic and excessive inflammation contributes to tissue damage in diseases such as lupus erythematosus, rheumatoid arthritis and T1D. There is thus a major unmet need for therapies that modulate excessive inflammation without affecting immune defenses against invading pathogens. Achieving this goal will require a comprehensive understanding of molecular pathways and mechanisms that regulate the expression of pro-inflammatory

genes [2], and the role of the individual's genetic background in these processes. An increased innate inflammatory state, due to inheritance of potentiating genetic variants in immune pathways that is independent of auto-antibodies, HLA status and disease progression, is likely to exist in T1D families [3]. Until recently nearly all studies dealing with genetic risk for T1D focused on the putative role of candidate genes on the immune system [4]. We and others have shown, however, that T1D genes regulate pathways that may be crucial for the pathogenesis of diabetes at the pancreatic beta cell level [5–12]. These observations suggest that four major genetically regulated pathways play a role in pancreatic beta cell dysfunction and death in T1D, e.g., innate immunity and antiviral activity [6, 10], and pathways related to beta cell phenotype and susceptibility to pro-apoptotic stimuli [7, 8, 10–12].

Assessment of the real contribution of a given T1D susceptibility gene to the pathogenesis of the disease requires a multistage procedure, combining the use of genetic association data, gene expression profiles and other transcriptomic data, advanced bioinformatics techniques, and functional assays in in vitro and in vivo models [8, 10, 13].

### 1.1 Selection of Genes for Functional Studies

Genetic risk to develop T1D has been associated with several regions along the genome [1, 4]. Causal candidate genes have been proposed based on the localization of the association signal (positional candidates) or on their function (functional candidates), but most of these regions contain more than one protein-coding gene and several noncoding features, such as long noncoding RNAs (lncRNAs) or microRNAs (miRNAs). Thus, selecting candidate genes for functional studies is not trivial and requires an accurate multistage selection procedure.

In our laboratory we are following a "minimalist" selection procedure composed of three steps that will be discussed in detail here: (a) Identification of genes falling into GWAS association signals; (b) Determination on whether these genes are expressed in human and rodent beta cells; (c) Identification of genes with a potential relevant function in three key signaling pathways for T1D pathogenesis: inflammation/innate immunity, apoptosis and beta cell metabolism/function (Fig. 1). The reader is referred to additional and elegant approaches for candidate gene selection based on in silico "phenome-interactome analysis" and integration of GWAS data with protein–protein interactions to construct biological networks of putative relevance for T1D [8–10, 13]. Moreover, the relevance of understanding candidate genes inside functional pathways has led to the development of novel tools that include pipelines to test for enrichment of T1D association signals among the targets of a given set of transcription factors [14]. To select potential causal candidate genes, other approaches take advantage of the great amount of available GWAS data to identify functionally

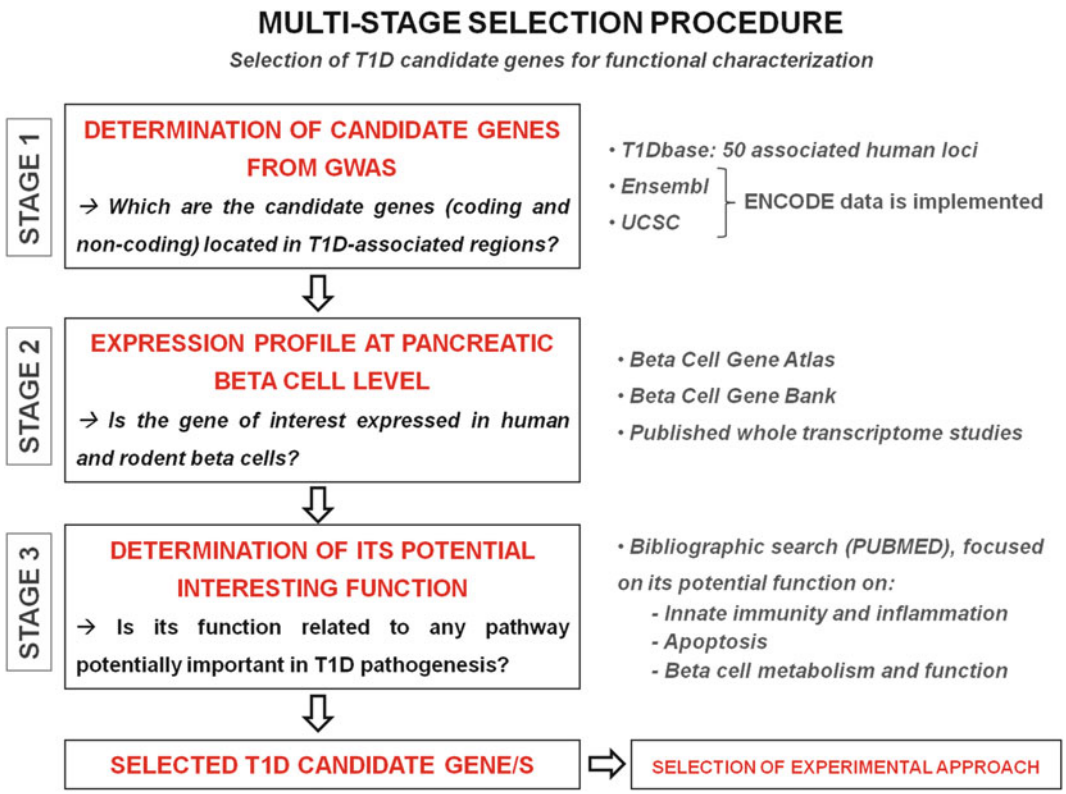


**Fig. 1** Multistage selection procedure to identify T1D candidate genes for functional characterization in pancreatic beta cells. The selection of T1D candidate genes for functional studies in pancreatic beta comprises three main steps: (1) Identification of candidate genes located in T1D-associated genomic regions; (2) Evaluation of their expression profiles in rodent and human pancreatic beta cells; (3) Mechanistic determination of their potential role in T1D pathogenesis

related genes spanning multiple GWAS *loci* by using genome-scale shared-function networks [15]. By using a similar strategy, in which a gene pathway-based analysis method is applied to summary statistics of GWAS, novel genetic associations and potential novel causal genes have been identified [16].

#### *1.1.1 Identification of Genes Falling into GWAS Association Signals*

The T1Dbase (http://www.t1dbase.org/page/Welcome/display) is a web-based resource focused on the genetics and genomics of type 1 diabetes and containing a table of human, mouse, and rat *loci* associated with T1D [17]. Associated regions are defined by starting at the associated variant of a given GWAS publication and extending out the region by ± 0.1 cM. The newly extended region is further investigated for variants of genome-wide significance; these steps are repeated until no more variants reach a significance of 1E−06 within the cited study. Thus, this table shows all genes falling into association peaks based on the data published by several GWAS [18–21]. The T1Dbase is a valuable tool to search for

potential functional candidate genes in T1D-associated genomic regions, but it is important to keep in mind that the protein-coding and noncoding genes/regions were extracted from Ensembl release 73 (we are currently at release 75) and that the latest update of the database was done in 2011 [22]. Thus, and in order to have an updated information, it is important to corroborate the data obtained from the T1Dbase using other databases such as Ensembl (http://www.ensembl.org/index.html) or UCSC Genome Browser (http://genome.ucsc.edu/) in which recent genomic information obtained by the ENCODE project has been implemented.

### 1.1.2 Expression Profile in Pancreatic Beta Cells

There are several online resources currently available to check whether a candidate gene is expressed in pancreatic beta cells. Among them, the EuroDia database (http://eurodia.vital-it.ch) contains a collection of global gene expression determinations performed on beta-cells of three organisms (human, mouse, and rat) [23]. The Gene Expression Data Analysis Interface (GEDAI) has been developed to support this database in which, in addition to the expression data repository, several tools for mining the data are available. At the time of publication (2010), the EuroDia database contained 38 curated experiments (441 hybridizations), thirteen of which were produced by members of the EC-supported EuroDia project. The database has been periodically updated and presently expression data of 50 experiments comprising 684 hybridizations are available.

The Beta Cell Gene Atlas (http://www.t1dbase.org/page/AtlasHome) is a web-accessible database in which basal expression of genes from different beta cell-related sources, including human, mouse, and rat pancreatic beta cells, islets and whole pancreas, as well as clonal beta cell lines, can be consulted [24]. The Beta Cell Gene Atlas is a collection of public microarray data generated from 131 array analyses derived from 28 experiments published between 2001 and 2006. This has not been updated in recent years, and other resources should be checked to make it sure that the gene is indeed expressed in beta cells. Another open access source is the Beta Cell Gene Bank (http://betacellgenebank.ulb.ac.be/cgi-bin/dispatcher.cgi/BCGB_Enter/display) in which array expression profiles from rodent and human pancreatic beta cells are found, both at basal condition and after exposure to a given insult (e.g., exposure to pro-inflammatory cytokines, transfection with synthetic viral double stranded RNA (Polyinosinic–polycytidylic acid; PIC) or infection with potentially diabetogenic viruses); information (based on manual curation) is provided for >500 genes. The expression data available in this database is based on microarray experiments published between 2003 and 2005 [25–28], and thus updated resources should be checked in order to confirm the expression data of a given gene. The fate of the databases described

above, i.e., cessation of updating after some years of development is unfortunately common in the field, reflecting the lack of interest of financing agencies in supporting these crucial resources on a long-term basis.

The Human Islet Regulome Browser (http://www.isletregulome.org/) is a recently developed tool in which transcripts of human islets, chromatin states, and transcription factor binding sites have been mapped [29]. This very elegant resource enables data downloads and online visualization at desired levels of resolution for islet transcription factor binding sites, chromatin states, motifs, enhancer clusters, and genome-wide significant *p*-values for association with type 2 diabetes and fasting glycemia.

In recent years the advent of next-generation RNA sequencing (RNAseq) has provided an unbiased and high-throughput method for determining the whole transcriptome, allowing the identification of novel transcripts in several cell types and tissues [30–33], including the original RNAseq study of human pancreatic islets [34]. Recent beta cell transcriptome studies based on RNAseq used enriched beta cell preparations (both rodent and human) and include studies establishing the gene expression profile for each islet cell subtype [30, 35, 36] and studies assessing the effect of immune (e.g., cytokine exposure) or metabolic (e.g., palmitate exposure) stress on gene expression patterns in whole human islets [34, 37]. The expression data obtained by these RNA sequencing analyses can be scrutinized to check whether the gene of interest is expressed in pancreatic islets or in beta cells, basally or following exposure to inflammation- or metabolic-mediated stress. Publication of most of these studies were accompanied by depositing the raw data in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) database, an open access resource in which gene expression profiles of array- and sequencing-based experiments can be consulted.

In order to validate the expression data publically available, expression of the gene of interest should be confirmed in both rodent and human pancreatic beta cell samples using real-time PCR, Western blot and histology. In these steps the human islet data is the "golden standard," i.e., if a gene of interest is present in human but not rodent beta cells we go on with the study, but not in the other way around (for instance, caspase-12 was shown to be a key regulator of ER stress-induced apoptosis in rodent cells, including beta cells, but it is not expressed in most human beings). Thus, interspecies variation reinforces the need for human models. Once the expression of a candidate gene is confirmed in human beta cells, the next step involves exposure of human islets or the recently developed human cell line EndoC-βH1 [38] to siRNAs or viral vectors to respectively downregulate or upregulate expression of the candidate gene, and then treatment with pro-inflammatory cytokines, intracellular double stranded RNA (dsRNA is a

by-product generated during replication and transcription of both RNA and DNA viruses, and an efficient inducer of apoptosis, type I interferons, and other cytokines/chemokines important for the host immune response to viral infection) or actual viral infection [6, 7, 11, 12, 39], as described below. We aim to model in vitro and under well-controlled conditions the putative genetic/environmental interactions that may take place in early T1D, with the limitation of addressing only one cell type and not the whole organism.

#### *1.1.3 Identification of Genes with a Potential Relevant Function for T1D Pathogenesis*

In order to select candidate genes with a potential effect on inflammation/innate immunity, apoptosis and/or beta cell metabolism and function [5–12], a systematic bibliographic search using appropriate "search terms" is performed in PUBMED (http://www.ncbi.nlm.nih.gov/pubmed). The goal is to answer three key questions: (1) What is the function of the candidate gene of interest? (2) Are there publications analyzing its role in pancreatic beta cells? (3) Are there publications linking the gene of interest with innate immunity/inflammation, apoptosis or cell metabolism and function in beta cells or other cell types?

#### *1.1.4 Limitations of the Selection Procedure and Selection of the Experimental Approach*

As mentioned above, the genomic regions associated with T1D as defined by GWAS are usually very large and comprise more than one protein-coding gene. Moreover, protein-coding genes in the T1D-associated regions often coexist with other noncoding genes, such as miRNAs and lncRNAs, that may have a role in disease pathogenesis [40–42]. Indeed, a study overlapping islet-expressed miRNAs against T2D association data identified several T2D association signals in target genes of islet-expressed miRNAs [41]. In addition, a recent work has described a set of differentially expressed miRNAs that stratify T1D patients and nondiabetic individuals [42]. Interestingly, the differentially expressed miRNAs are predicted to regulate the expression of several T1D candidate genes that are modulated by pro-inflammatory cytokines in human pancreatic islets [42].

In short, selection of the "right candidate gene" for functional studies is a challenging procedure that risk leading to the selection of a "wrong gene" without a relevant role in the disease. As observed in the T1Dbase (Fig. 2), and independently of the number of genes located in the associated region, one or two causal genes are usually proposed based on their function and/or on the genomic position of the associated polymorphism. In some cases the proposed causal gene is the real etiological gene in the region, with a well-defined functional effect in the pathogenesis of T1D. Good examples of this are the HLA-DRβ1 gene in region 11p15.5 and the IFIH1 gene in region 2q24.2 [6, 43, 44]. In many cases, however, the downstream functional effects of the proposed candidate gene remain to be confirmed. Thus, the other coding and

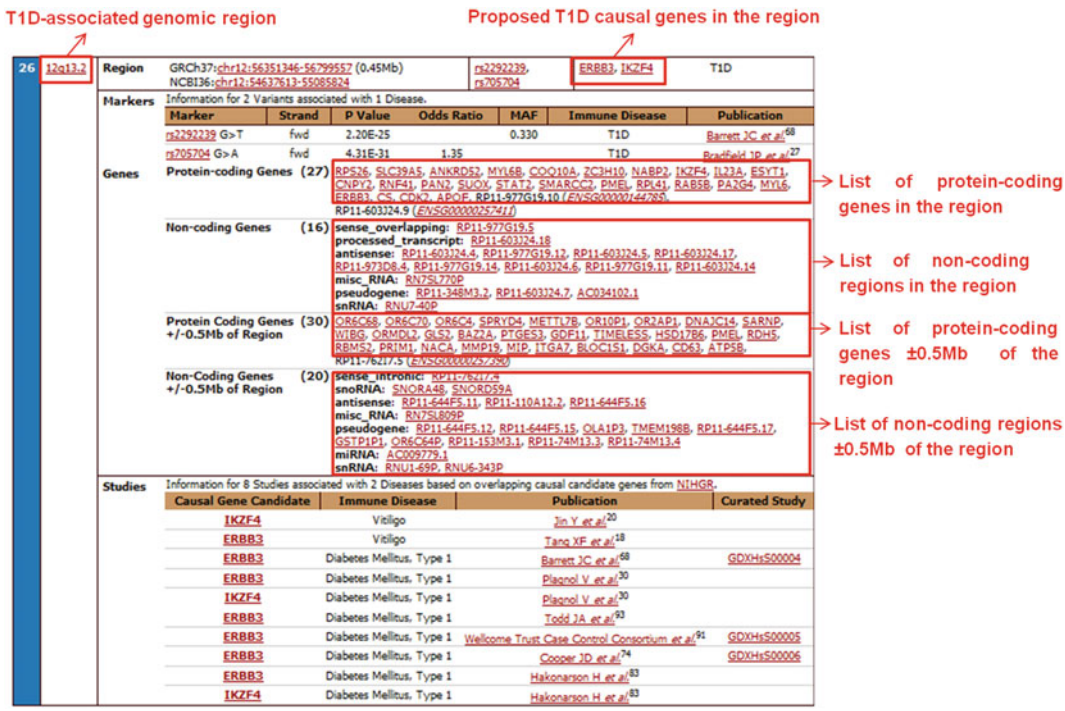


**Fig. 2** Interface of a given T1D-associated genomic region from the table of human T1D *loci* in the Beta Cell Gene Bank database. A table with the human T1D *loci* is available at the Beta Cell Gene Bank database (http://betacellgenebank.ulb.ac.be/cgi-bin/dispatcher.cgi/BCGB_Enter/display). As shown in the example, different information are available for each T1D-associated region: proposed T1D candidate genes (based on both position and function), list of other protein-coding and noncoding genes/regions falling under the association peak, and a list of protein-coding and noncoding genes/regions located at a maximum of ± 0.5 Mb from the association signal

noncoding genes in the region should not be excluded as potential causal genes.

It must be also taken into account that several T1D-associated variants do not necessarily affect the expression levels of nearby genes (*cis* eQTLs), and that the associated SNP may have an effect in one or more genes located several megabases away from the association signal (*trans* eQTLs) [29, 45]. A sobering example of this is the recent description that the obesity-associated noncoding sequences within FTO, a candidate gene for obesity and T2D [46, 47], are functionally connected with the homeobox gene IRX3, located at a megabase distance [48]. Indeed, functional studies suggest that the obesity-associated interval is related to the regulatory landscape of IRX3 [48], and not to FTO itself, as previously believed. Expression quantitative trait locus (eQTL; genomic regions that regulate RNA expression level) mapping offers a powerful approach to elucidate the genetic components regulating gene expression [45, 49–51]. Most eQTLs studies have been performed with RNA isolated from blood cells due to the

difficulty in obtaining human samples from tissues of difficult access (e.g., pancreatic islets). eQTLs are, however, highly dependent on the specific cell type studied, and eQTL data obtained in short-lived blood circulating cells may not provide adequate information on the impact of the polymorphism in the very long-lived pancreatic beta cells [52]. Moreover, eQTLs are also dependent on the in vitro conditions to which a cell is exposed: e.g., cell exposure to immune activators such as viruses, cytokines, and bacterial products induce major changes in eQTL [53, 54] and these activators should be selected according to the pathogenic mechanism under study. For instance, when studying the impact of the genetic background of an individual in the induction of toxic shock, it may be relevant to determine both basal and lipototoxin-modulated eQTLs in immune cells. Thus, and in the case of diabetes, definition of genetic variants that regulate expression of potential relevant genes for diabetes must rely on eQTL evaluated in human islets and immune cells under basal condition and after exposure to relevant stimuli such as pro-inflammatory cytokines or dsRNA for T1D or free fatty acids or hyperglycemia for T2D (in the case of T2D, human islets and target tissues of insulin, such as liver, muscle and fat, should be evaluated). To reach this aim, a sample size of around 750 preparations is required to enable a statistical power of 80 % to detect *cis* eQTLs with a low minor allele frequency (MAF) [52]; only a global collaborative effort from different human islet-isolating groups around the world can allow the collection and treatment of these large number of human islet preparations under well-standardized conditions.

Another potential issue when performing functional studies of candidate genes for T1D is the risk of selecting the wrong tissue (e.g., pancreatic beta cells or the immune system). T1D is a disease in which the immune system, pancreatic beta cells, and most probably the gut and the microbiome interact [55–57], and it is conceivable that some candidate genes exert composite or even antagonistic effects on these different systems. Deletion/overexpression studies in mouse models may address this issue, but there is an increasing concern regarding the extrapolation of basic mechanisms of inflammation/immunity from mice to humans [58].

Finally, it is important to keep in mind that triggering of complex diseases such as T1D are the consequence of the sum of variations in many genes in cross-talk with multiple environmental factors. Thus, and in order to decipher how a specific genetic background contributes to disease pathogenesis, we need to understand the combined effects of associated genes and their products in the context of interacting functional pathways [10, 59]. For Mendelian disorders in which a mutation in a single gene is sufficient to generate a given pathogenic phenotype, study of a gene-at-a-time is a suitable approach; in complex diseases, however, in which a gene's

effect is usually pleiotropic, context-dependent and contingent on other genes from the same functional pathway, pathway analyses should be performed in order to infer key regulating genes in potential pathogenic pathways [10, 59, 60].

After performing the multistage selection procedure described in Fig. 1, a T1D candidate gene will be chosen for further functional studies in pancreatic beta cell models. As mentioned above, T1D candidate genes may not act as isolated elements in disease pathogenesis and when analyzing the functional effect of a given T1D gene we should consider its potential interaction with other T1D candidate genes [10]. To this aim, there are several online resources that can be used to predict possible interactions between a given T1D candidate gene and other (candidate) genes [61]. One of them is GeneMANIA (http://www.genemania.org), a free online user-friendly web interface for generating hypotheses about gene function, prioritizing genes for functional assays and finding potential interactions between genes based on co-expression, physical interaction, and co-localization data, among others [62] (Fig. 3). Through this online prediction tool we can identify potential interactions between (candidate) genes. This allows the design of experimental procedures to analyze the effect of one gene in combination with others at the pancreatic beta cell level (e.g., by inhibition or overexpression of two or more candidate genes simultaneously). In addition, using this kind of prediction tools we can define potential pathway regulators that allow the analysis of a whole pathogenic pathway via modulation of a single regulatory gene. Indeed, recent works in which analysis of gene networks and protein–protein interaction data were combined with GWAS association data have identified disease-relevant biological gene-networks that are enriched with T1D candidate genes or are directly controlled by one of them [9, 60]. For example, a recent work revealed that a T1D-associated gene network implicated in antiviral responses is partially regulated by the T1D candidate gene EBI2 [60].

To choose an appropriate experimental approach for functional studies (i.e., overexpression or inhibition), it is important to check the predicted functional effect of the associated polymorphism in the expression and function of the gene of interest. To this aim, there are several user-friendly online prediction tools that can be used, such as FastSNP (http://fastsnp.ibms.sinica.edu.tw) and SNPinfo (http://snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm), but unfortunately this crucial information is often not available.

To assess more closely the putative effect of a disease-associated variants using experimental models (e.g., T1D-associated polymorphisms in pancreatic beta cell models), a new technique that allows genome editing in eukaryotic cells has been developed [63]. This technique named CRISPR-Cas9 provides an effective and simple method for making small edits in the genome, such as the

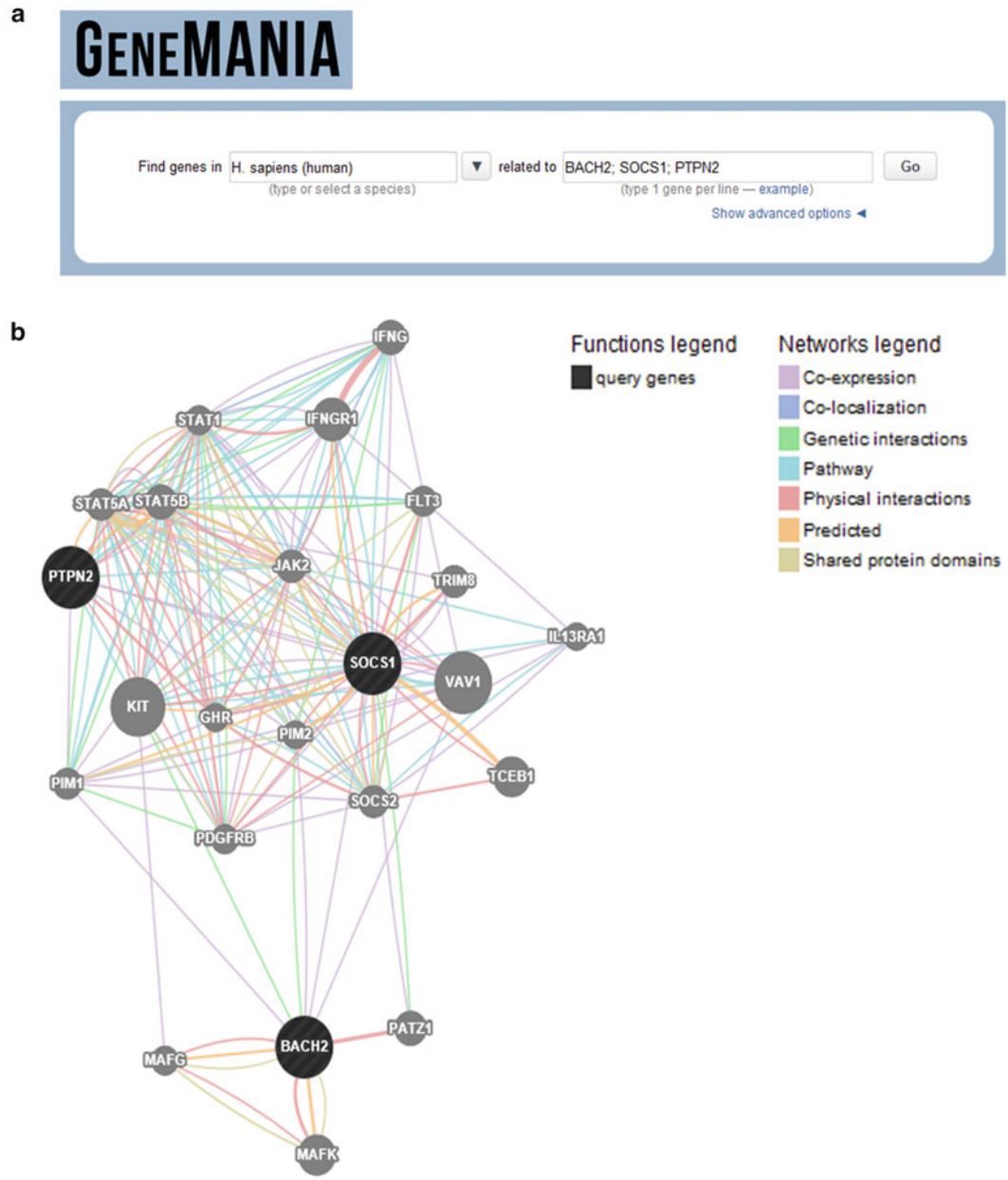


**Fig. 3** The online free bioinformatic tool GeneMania allows the finding of potential interactions between genes. Based on data from co-expression, co-localization, genetic interactions, pathway analysis, physical interaction, in silico prediction, and shared protein domains, GeneMania (http:/www.genemania.org) generates potential interaction nets. (**a**) The interface of GeneMania allows to query several genes selected from different species. (**b**) After analysis, GeneMania provides a schematic interpretation of potential interactions between queried genes and others. As shown in the legend, query genes are represented by *black circles*, other genes in the network in *grey*, while circles and interactions between genes are defined using *colored lines*. Interestingly, the suggested interaction between the T1D candidate genes BACH2 and PTPN2 has been recently experimentally confirmed [12]

introduction of single nucleotide polymorphisms (SNPs) for probing causal genetic variations in cell lines [63]. CRISPR-Cas9 is a microbial adaptive immune system that uses RNA-guided nucleases to cleave foreign genetic elements. Cas9 promotes genome editing by stimulating a DNA double-stranded break (DSB) at a target genomic locus that, upon cleavage of Cas9, undergoes one of the major pathways for DNA damage repair that can be used to achieve a desired editing outcome. This technique is divided in four steps: (a) In silico design; (b) Reagent construction; (c) Functional validation; (d) Clonal expansion of isogenic cells with defined variants. Thus, beginning with target design, gene modifications can be performed within as little as 1–2 weeks, and modified clonal cell lines can be derived within 2–3 weeks [63]. Unfortunately this is presently an expensive technique which is difficult to apply to primary and poorly (or non)-proliferating cells, such as human pancreatic beta cells.

Our laboratory has been studying the role of T1D candidate genes in beta cell dysfunction and death and in the induction of local inflammation. For this purpose, we have established experimental procedures based on gene silencing and overexpression in models of human and rodent pancreatic beta cells [5–7, 11, 12]. We describe below the materials and methods used in our laboratory to select and analyze the effect of T1D candidate genes on key pathways in T1D pathogenesis, i.e., inflammation and innate immune response, apoptosis, and beta cell metabolism and function.

## 2 Materials

Tissue culture reagents should be prepared in an appropriate sterile environment.

### 2.1 Cells

1. The rat insulin-producing INS-1E cell line (kindly provided by Dr. C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland [64]).
2. The human EndoC-βH1 beta cell line (kindly provided by Dr. R. Scharfmann, Centre de Recherche de l'Institut du Cerveau et de la Moelle épinière (CRICM), Paris, France).
3. FACS-purified rat beta and alpha cells from male Wistar rats, housed and used according to the guidelines of the Belgian Regulations for Animal Care. Autofluorescence-activated cell sorting is used to purify rat beta and alpha cells [65–67]. Of note, dispersed islet cells can be used in case FACS sorting facilities are not available. In this case, the procedures for culture and transfection are similar to the rat FACS-purified beta cells.
4. Human islets incubated for 2–3 min in a solution of Dispase (2.5 U/ml in Solution I) in order to disperse the islets.

## 2.2 Reagents and Supplies

### 2.2.1 Culture Media

1. The INS-1E cell line culture media: RPMI 1640 GlutaMAX-I, 5 % fetal bovine serum (FBS), 1 mM Na-pyruvate, 10 mM HEPES, 50 μM 2-mercaptoethanol, and 100 U/ml penicillin and 100 μg/ml streptomycin. The same medium but without antibiotics is used for transfection experiments (*see* **Note 1**).
2. The EndoC-βH1 beta cell line culture media: this cell line is cultured attached to extracellular matrix (ECM)-fibronectin-coated (100 μg/ml and 2 μg/ml, respectively as previously described [38]) (*see* **Note 2**). EndoC-βH1 cells are cultured in DMEM containing 5.6 mM glucose, 5.5 μg/ml transferrin, 6.7 ng/ml selenite, 10 mM nicotinamide, 50 μM 2-mercaptoethanol, 2 % charcoal-absorbed BSA, 100 U/ml penicillin, and 100 μg/ml streptomycin. The same medium but without antibiotics (*see* **Note 1**) and BSA (*see* **Note 3**) is used for transfection experiments. For the post-transfection recovery, 2 % FBS is added in the culture medium.
3. Rat pancreatic islet washing solution 1 (to disperse rat pancreatic islets): (5 mM glucose, 124 mM NaCl, 1 mM $NaH_2PO_4$, 0.71 mM $NaHCO_3$, 5.4 mM KCl, 0.8 mM $H_2SO_4$, and 1 mM EGTA) and incubated for 1–2 min in a solution of Dispase (5 U/ml in Solution I).
4. Dispersed human islet culture medium: Ham's F-10 containing 6.1 mM glucose, 50 μM 3-isobutyl-1-methylxanthine, 2 mM GlutaMAX, 10 % FBS, 1 % BSA, 50 U/ml penicillin, and 50 μg/ml streptomycin [68]. The transfection medium for human dispersed islets is the same used to transfect primary rat beta cells but without glucose. Dispersed islets are cultured on polylysine-coated 96-well plates.
5. FACS-purified rat beta and alpha cells culture medium: Ham's F-10 medium containing 10 mM glucose (beta cells) or 6.1 mM glucose (alpha cells), 50 μM 3-isobutyl-1-methylxanthine, 2 mM GlutaMAX, 5 % FBS (beta cells), or 10 % FBS (alpha cells), 0.5 % charcoal-absorbed BSA, 50 U/ml penicillin, and 50 μg/ml streptomycin [67, 69]. The same medium but without antibiotics (*see* **Note 1**) and BSA (*see* **Note 3**) is used for transfection experiments. Purified rat beta and alpha cells are cultured on polylysine-coated 96-well plates.
6. Phosphate buffered saline (PBS)
7. Solution of trypsin–EDTA at 0.5 mg/ml.

### 2.2.2 RNA Interference

1. AllStars Negative Control siRNA (referred to as siCtrl) is reconstituted at 20 μM using the provided siRNA dilution buffer, aliquoted and stored at −80 °C (*see* **Note 4**). This control siRNA does not affect β-cell gene expression, function, or viability [70, 71].

2. siRNA against the selected candidate gene is reconstituted at 20 μM using the siRNA dilution buffer, aliquoted and stored at −80 °C.
3. The lipid reagent Lipofectamine RNAiMAX is used to transfect the siRNAs [70, 72].
4. Opti-MEM medium without any additive (*see* **Note 5**).

*2.2.3 Recombinant Adenoviral Vectors and Infection Reagents*

1. Recombinant adenovirus vector for the selected candidate gene (SIRION Biotech, Munich, Germany).
2. Recombinant adenovirus encoding Renilla luciferase (Ad-Luc) is used as negative control [73].

### 2.3 Analysis of Inflammatory Markers

*2.3.1 mRNA Isolation, Reverse Transcriptase Reaction, and Real-Time PCR (RT-PCR)*

1. Dynabeads mRNA DIRECT kit (Invitrogen).
2. Solutions for reverse transcriptase mix: 10× reaction buffer without $MgCl_2$, 50 mM $MgCl_2$ (both from Genecraft, Köln, Germany), 20 mM dNTP mix (Eurogentec, Seraing, Belgium), 2.5 μM random hexamers, 1 U/μl RNase inhibitor, and 2.5 U/μl reverse transcriptase (all from Applied Biosystems, Foster City, California, EUA).
3. iQ SYBR Green Supermix (Bio-Rad, Nazareth Eke, Belgium).
4. Specific primers for the genes of interest (Invitrogen).
5. MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad).

*2.3.2 Enzyme-Linked Immunosorbent Assay (ELISA)*

1. Commercial ELISA kits for rat or human cytokines or chemokines
2. Microplate reader.

### 2.4 Analysis of Cell Viability and Mechanisms Leading to Beta Cell Death

1. A stock solution of Propidium Iodide (PI, Sigma) is prepared in PBS at 1 mg/ml, filtered and used at a final concentration of 5 μg/ml.
2. A stock solution of Hoechst 33342 (HO, Sigma) is prepared in PBS at 1 mg/ml, filtered and used at a final concentration of 5 μg/ml.
3. An inverted microscope (Zeiss, Zaventem, Belgium) with filters for excitation at 358 nM (HO) and 538 nm (PI) is used.

*2.4.1 Expression of Proteins Related to Beta Cell Death*

1. Solutions:
   - To prepare running gel: 30 % acrylamide–bis, 1.5 M Tris–HCl pH 8.8, 10 % SDS, 10 % ammonium persulfate, *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED).
   - To prepare stacking gel: 30 % acrylamide–bis, 0.5 M Tris–HCl pH 6.8, 10 % SDS, 10 % ammonium persulfate, *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED).

- Laemmli buffer: 62 mM Tris–HCl, 100 mM dithiothreitol (DTT), 10 % glycerol, 2 % SDS, 0.2 mg/ml bromophenol blue, 2 % β-mercaptoethanol. Adjust to pH 6.8.
- Running buffer: 25 mM Tris–HCl, 190 mM glycine, 0.1 % sodium dodecyl sulfate (SDS).
- Blotting buffer: 20 mM Tris–HCl, 150 mM glycine, 20 % methanol.
- Tris-buffered saline + Tween 20 (TBS-T): 20 mM Tris–HCl, 150 mM NaCl and 0.05 % Tween 20.

2. Isopropanol.
3. PageRuler prestained protein ladder
4. Supported Nitrocellulose membranes (Bio-Rad).
5. Solution 5 % BSA (fraction V) prepared in TBS-T and filtered.
6. Solution 5 % skimmed milk prepared in TBS-T.
7. Monoclonal or polyclonal primary antibodies against proteins of interest.
8. Secondary antibodies conjugated to horseradish peroxidase (different suppliers).
9. SuperSignal West Femto chemiluminescent substrate (Thermo Scientific).
10. Mini-PROTEAN Tetra Cell (Bio-Rad).
11. PowerPac HC High-Current Power Supply (Bio-Rad).
12. Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad).
13. Molecular Imager ChemiBoc XRS$^+$ (Bio-Rad).
14. ImageLab software (Bio-Rad).

*2.4.2 Nitrite Measurement*

1. A stock solution of sodium nitrite ($NaNO_2$) is prepared in double distilled sterile water ($ddH_2O$) at 0.1 M (69 mg $NaNO_2$ in 10 ml $ddH_2O$) and stored at −20 °C. Protect from light (*see* **Note 6**).
2. A stock solution of *N*-1-napthylethylenediamine dihydrochloride (NED) is prepared in $ddH_2O$ at 1 %. Protect from light (*see* **Note 6**).
3. A stock solution of sulfanilamide is prepared in $ddH_2O$ plus $H_3PO_4$ at 10 %. 1 g of sulfanilamide is solubilized in 4.1 ml $ddH_2O$. Then add 5.9 ml $H_3PO_4$ to the aqueous solution.
4. Culture medium according to the cell type.
5. 96-well flat-bottom enzymatic assay plate (BD Falcon).
6. Microplate reader.

### 2.5 Analysis of Beta Cell Function: Glucose Metabolism and Insulin Release

#### 2.5.1 Glucose Metabolism

1. Krebs-Ringer bicarbonate HEPES buffer (KRBH): 114 mM NaCl, 4.74 mM KCl, 25 mM $NaHCO_3$, 10 mM HEPES, 1.18 mM $MgSO_4$, 1.15 mM $KH_2PO_4$, 4.26 mM NaOH, 1.5 mM $CaCl_2$, and 0.1 % BSA. The medium pH is maintained at 7.4 (*see* **Note 7**).
2. Metabolic poison buffer: 10 ml of citrate buffer (400 mM, pH 4.9), 3 mM KCN, 5 μM antimycin A, 10 μM rotenone.
3. Radioactive D-(U-$^{14}$C) glucose (300 mCi/mmol).
4. A stock solution of glucose monohydrate (Sigma) is prepared in KRBH at 32 mM.
5. Solution of trypsin–EDTA at 0.5 mg/ml.
6. A gas mixture of $O_2$ and $CO_2$ (95 %:5 %).
7. Solution of Hyamine hydroxide 10-×.
8. Small vial tubes made of soda-lime glass with the following dimensions: 6.75 × 35 mm × thickness 0.95 mm (flat bottom).
9. Scintillation liquid or gel.
10. Liquid scintillation counter.

#### 2.5.2 Insulin Secretion and Content

1. Krebs-Ringer solution (KRB): 114 mM NaCl, 4.74 mM KCl, 5 mM $NaHCO_3$, 0.5 mM $NaH_2PO_4$, 0.5 mM $MgCl_2$, 2.54 mM $CaCl_2$, 10 mM HEPES, and 0.1 % BSA (fraction V).
2. Glucose-free complete RPMI: RPMI 1640 without glucose, 5 % heat-inactivated fetal calf serum (FCS), 10 mM HEPES, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol.
3. Stimulation media: The compositions of these media are as followed:
   - 16.7 mM glucose: 49.6 mg glucose monohydrate + 15 ml KRB.
   - 1.67 mM glucose: 500 μl glucose 16.7 mM + 4.5 ml KRB.
   - 10 mM glucose: 3.6 ml glucose 16.7 mM + 2.4 ml KRB.
   - 16.7 glucose + forskolin 10 μM: 6 ml glucose 16.7 mM + 3 μl forskolin (initial stock: 20 mM).
   - 1.67 glucose + 30 mM KCl: 600 μl glucose 16.7 mM + 180 μl KCl (initial stock: 1 M) + 5.22 ml KRB.
4. Acid ethanol solution (95 % ethanol + 5 % 12 M HCl).
5. Ultrasonic probe.
6. Rat or human insulin ELISA kit (Mercodia, Uppsala, Sweden).

## 3 Methods

### 3.1 Preparation of the Cells for Transfection or Infection

#### 3.1.1 INS-1E Cells

1. The rat insulinoma cell line INS-1E is cultured at 37 °C. After reaching a high confluence (around 80 %), cells are washed with PBS and detached with trypsin–EDTA. Trypsin–EDTA is neutralized with INS-1E culture medium and cells are centrifuged at 1400 × *g*/4 min at room temperature. After centrifugation, the pellet is resuspended in fresh culture medium (*see* **Note 1**).
2. After counting the number of cells using a Neubauer chamber, INS-1E cells are plated at $1.0 \times 10^4$ cells/well in a 96-well plate or $1.0 \times 10^5$ cells/well in a 24-well plate depending on the type of experiment (e.g., cell viability and insulin secretion are done in 96-well plates while experiments involving RNA or protein extraction are performed in 24-well plates).
3. The cells are cultured for at least 48 h in antibiotic-free medium prior to transfection/infection.

#### 3.1.2 Human EndoC-βH1 Cells

1. Plates are coated with extracellular matrix (ECM)-fibronectin coating medium before passage (*see* **Note 8**).
2. The human EndoC-βH1 cells are cultured at 37 °C. After reaching a high confluence (around 80 %), cells are washed with PBS and detached with trypsin–EDTA. Trypsin–EDTA is neutralized with a neutralization solution (80 % PBS + 20 % FCS). After centrifugation (700 × *g*/5 min), the pellet is resuspended in fresh EndoC-βH1 culture medium.
3. After counting the cells using a Neubauer chamber, human EndoC-βH1 cells are plated at $4.0–7.0 \times 10^4$ cells/well in a 96-well plate.
4. The cells are cultured for at least 48 h in antibiotic-free medium prior to transfection/infection.

#### 3.1.3 Rat Primary Beta and Alpha Cells and Human Dispersed Islets

1. FACS-purified rat beta and alpha cells and dispersed human islets are obtained as previously described [66, 67], and plated at $3.0 \times 10^4$ cells/well in 96-well plates with the appropriate medium (*see* Sect. 2.2).
2. After 48 h, the medium is exchanged by fresh medium without antibiotics and BSA. Removal of antibiotics and BSA from the culture medium is recommended for transfection of primary rat beta and alpha cells and dispersed human islets (*see* **Note 9**).

### 3.2 Transfection of siRNAs

This protocol is adapted for the transfection of 30 nM siRNA. For further details regarding the design of efficient siRNAs and adequate controls, *see* Ref. [71].

#### 3.2.1 INS-1E Cells

1. Opti-MEM and antibiotic-free INS-1E culture media are pre-warmed at 37 °C.
2. siRNAs are thawed on ice and Lipofectamine RNAiMAX lipid reagent is kept on ice.
3. siRNAs are diluted at 300 nM in Opti-MEM. From an initial stock of 20 μM, it means a 66.6× dilution.
4. The Lipofectamine RNAiMAX lipid reagent dilution differs between transfection in a 96- or a 24-well plate (*see* **Note 10**).
   - For 96-well plates: 0.2 μl/well is used (0.2 μl of Lipofectamine RNAiMAX is diluted in 9.8 μl of Opti-MEM).
   - For 24-well plates: 1 μl/well is used (1 μl of Lipofectamine RNAiMAX is diluted in 49 μl of Opti-MEM).
5. The diluted siRNA and Lipofectamine RNAiMAX are incubated separately for 5 min at room temperature and then mixed at a ratio of 1:1.
   - For 96-well plates: 10 μl of each diluted solution (20 μl final volume).
   - For 24-well plates: 50 μl of each diluted solution (100 μl final volume).
6. This new solution is gently mixed and incubated for 20 min at room temperature without agitation.
7. After 20 min, the siRNA/lipid complex is diluted 1:5 with antibiotic-free INS-1E medium.
   - For 96-well plates: 80 μl of antibiotic-free medium is added to the 20 μl of siRNA/lipid mix (100 μl final volume)
   - For 24-well plates: 400 μl of antibiotic-free medium is added to the 100 μl of siRNA/lipid mix (500 μl final volume)
8. The culture medium is removed and the transfection mix (100 μl for a 96-well and 500 μl for a 24-well) is added for overnight incubation at 37 °C (*see* **Note 11**).
9. The next day, the transfection mix is exchanged by fresh INS-1E medium for a given recovery period (24–96 h) (*see* **Note 12**).

#### 3.2.2 Primary Rat Beta and Alpha Cells, Human EndoC-βH1 Cells, and Human Dispersed Islets

The protocol used to transfect primary rat beta and alpha cells, human EndoC-βH1 cells and human dispersed islets is similar to the one used for transfection of INS-1E cells in 96-well plates but with a different concentration of Lipofectamine RNAiMAX.

1. Primary rat beta and alpha cells, human EndoC-βH1 cells and human dispersed islets require a higher concentration of

Lipofectamine RNAiMAX than INS1-E cells to be efficiently transfected (*see* **Note 10**).

- For primary rat beta and alpha cells: 0.25 μl/well is used (0.25 μl of Lipofectamine RNAiMAX is diluted in 9.75 μl of Opti-MEM).
- For human EndoC-βH1 cells and dispersed human islet cells: 0.4 μl/well is used (0.4 μl of Lipofectamine RNAiMAX is diluted in 9.6 μl of Opti-MEM).

2. The siRNA/lipid complexes are diluted 1:5 with the antibiotic- and BSA-free transfection medium described in Sect. 2.1.
3. After overnight incubation period, the transfection medium is exchanged by culture medium specific for each cell type (*see* Sect. 2.1).

### 3.3 Adenoviral-Mediated Expression of Proteins

#### 3.3.1 Use of Adenoviral Vectors

The delivery of cloned DNA into cells using adenovirus as vectors is a tool widely used in molecular biology. Two main advantages of this method are the absence of major phenotypic changes in infected cells and the fact that adenoviral infections take place in both dividing and nondividing cells at a very high efficiency without integration into the host cell's genome [74].

Generation of recombinant adenoviruses is made by deletion of the early transcription units E1 (involved in the viral replication) and E3 (involved in host immune suppression) from the adenoviral genome. These changes create a virus with insufficient viral replication ability but still able to replicate inside packaging cell lines (e.g., human kidney embryo cell 293, HEK 293) [75, 76].

To generate a recombinant adenovirus expressing a specific gene (e.g., a T1D candidate gene of interest), first the coding region of the gene must be amplified by PCR and then cloned into a shuttle vector under the control of the human cytomegalovirus (CMV) promoter (e.g., pO6-A5-CMV). Afterwards the region of interest is transferred by recombination into a plasmid containing the genome of a recombinant adenoviral vector in which the E1 and E3 transcription units have been deleted. In order to verify the presence and accuracy of the gene-open reading frame (ORF) in the resulting vector, DNA sequencing is recommended.

We describe below a protocol of infection using a recombinant adenovirus encoding Renilla luciferase (Ad-Luc) as an example. The titer of this adenovirus is $3.0 \times 10^9$ IU/ml and we use a multiplicity of infection (MOI) of 1. Of note, INS-1E cells and rat beta and alpha cells are very sensitive to toxicity secondary to adenoviral infection, and rarely survive infections with MOIs >10. Human and mouse beta cells are, however, more resistant and can be infected at higher MOIs.

#### 3.3.2 Infection of INS-1E Cells

1. INS-1E media (with and without antibiotics) is warmed at 37 °C.
2. Adenoviral vectors are thawed on ice.
3. The number of cells is counted in an extra well (*see* **Note 13**).
   - For 96-well plates: Wells are washed with 100 μl PBS, 20 μl trypsin are added and then the reaction is stopped with 80 μl PBS.
   - For 24-well plates: Wells are washed with 500 μl PBS, 100 μl trypsin are added and then the reaction is stopped with 400 μl PBS.
4. The volume of adenovirus needed to reach the desired MOI is calculated (*see* **Note 14**). For this purpose, the following equation is used: Volume of virus from stock (μl) = number of cells in the well × (MOI/titer of Adenovirus in PFU (plate forming units)) × 1000. In our case:
   - For 96-well plates: Volume of virus from stock (μl) = $10{,}000 \times (1/3.0 \times 10^9) \times 1000 = 0.003$ μl for one well.
   - For 24-well plates: Volume of virus from stock (μl) = $100{,}000 \times (1/3.0 \times 10^9) \times 1000 = 0.033$ μl for one well.
5. Adenovirus is diluted in antibiotic-free INS-1E medium taking into account the number of wells to be infected.
6. The culture medium is removed and the infection medium is added (100 μl for 96-well plates and 500 μl for a 24-well plates).
7. Cells are incubated during 3 h at 37 °C.
8. After the incubation period, the infection medium is removed and replaced by fresh INS-1E medium.

#### 3.3.3 Primary Rat Beta and Alpha Cells, Human EndoC-βH1 Cells and Human Dispersed Islets

The protocol used to infect primary rat beta and alpha cells, human EndoC-βH1 cells and human dispersed islets is similar to the one used to infect INS-1E cells in 96-well plates. However, in these cell types the media used to dilute the adenovirus are BSA- and FBS-free (*see* **Note 15**).

### 3.4 Posttransfection/ Postinfection Recovery Period

In experiments involving transfection of siRNAs or adenoviral-mediated expression of proteins it is important to allow an adequate recovery period after manipulating the cells for the following reasons: (a) cells need time to recover from the stressful process of transfection/infection before being challenged with another potential stressful treatment (e.g., cytokine exposure); (b) achievement of a better inhibition (by siRNAs) or overexpression (by adenoviral vectors) of target genes/proteins requires a proper

recovery period. Under our experimental conditions, a recovery period of 24 h is usually sufficient to observe a clear modulation of the target protein. However, in some cases a higher recovery time (48–96 h) is necessary to observe changes in protein expression (*see* **Note 16**).

### 3.5 Evaluation of Efficiency

The best way to evaluate whether the expression of the gene/protein of interest has been efficiently modulated is through the measurement of its expression at mRNA and protein levels. To this aim, real-time PCR and Western blot can be used, respectively. In the case of secreted proteins (e.g., chemokines) the use of ELISA is recommended. If the target gene is a transcription factor, modulation of downstream target genes can be additionally evaluated.

In siRNA experiments, an inhibition of >50 % of the target gene expression is usually considered as efficient, but in some cases (e.g., kinases) it is necessary to reach >80 % inhibition to observe clear biological effects. Adenoviral-mediated gene overexpression based on CMV promoter usually lead to a five to tenfold increase in protein expression. In case more nuanced changes in gene/protein expression is required, an alternative is to use one approach called tetracycline-controlled transcriptional activation, in which the expression of a given gene of interest is turned on or off—Tet-On and Tet-Off, respectively—in the presence of tetracycline or doxycycline (TET-inducible adenovirus platform, SIRION Biotech). In the Tet-On system, cells are transfected with a system constituted by the tetracycline-controlled transactivator (composed by fusing the tetracycline repressor of *E. coli* with the activating domain of the herpes simplex virus VP16 protein) and the Tet-Responsive Element expression vector (formed by repeats of the tetracycline operator, a minimal CMV promoter and the gene of interest). The presence of doxycycline induces the binding of the tetracycline-controlled transactivator to the Tet-Responsive Element expression vector, thus leading to activation of gene transcription [77, 78].

### 3.6 Analysis of Inflammatory Markers

Proinflammatory cytokines, synthetic viral double stranded RNA (PIC; Polyinosinic–polycytidylic acid) and viral infections stimulate expression and secretion of several cytokines and chemokines in rat beta cells and in human pancreatic islets [25, 28, 34, 70, 79–82]. Our group has previously shown that the T1D candidate genes *MDA5* and *PTPN2*, and *USP18*, a member of the T1D-associated interferon regulatory factor 7-driven inflammatory network (IDIN), regulate expression of several chemokines in pancreatic beta cells [6, 10]. Among others, the following pro-inflammatory chemokines can be evaluated to assess a possible effect of a given candidate gene in pancreatic beta cell inflammation: CCL2 (MCP-1), CCL3 (MIP-1α), CCL5 (RANTES), CCL20 (MIP-3α), CXCL1 (GROα), CXCL2 (GROβ), CXCL9 (MIG), CXCL10 (IP-10), and CXCL11 (I-TAC).

In order to study the impact of T1D candidate genes in the modulation of chemokine/cytokine expression and release by beta cells real-time PCR and ELISA are respectively used.

#### 3.6.1 mRNA Isolation, Reverse Transcriptase Reaction, and Real-Time PCR

1. After harvesting the cells with an appropriate Lysis/Binding buffer, Poly(A)$^+$ mRNA is isolated from the crude lysate using oligo $(dT)_{25}$-coated Dynabeads according to the manufacturer's instructions.
2. After mRNA isolation, cDNA is obtained by reverse transcription reaction, which is performed as follows:
   - 12 μl mRNA are added to 28 μl reverse transcriptase mix containing 1× reaction buffer without $MgCl_2$, 5 mM $MgCl_2$, 2 mM dNTP mix, 2.5 μM random hexamers, 1 U/μl RNase inhibitor, and 2.5 U/μl reverse transcriptase.
   - The mix is incubated at room temperature for 5–10 min and then at 42 °C for 1 h.
   - To stop the reaction, the samples are incubated at 99 °C for 5 min. After this step, cDNA samples can be immediately used or kept at −20 °C until assay.
3. The RT-PCR amplification reactions are performed in 20 μl containing 10 μl iQ SYBR Green Supermix, 0.5 μM of each primer (forward and reverse), 3 mM $MgCl_2$, and 2 μl template cDNA [67, 83]. The number of copies of the gene of interest is calculated by comparison with a standard curve as previously described [84] (*see* **Note 17**).

#### 3.6.2 Enzyme-Linked Immunosorbent Assay (ELISA)

The ELISA assay is a quantitative technique based on the adsorption of some components of a reaction mixture (e.g., chemokines in the cell culture supernatant) to a stationary solid phase with special binding properties. Using the chemokines as examples, monoclonal antibodies specific for a given chemokine are pre-coated onto the microplate wells. The liquid sample, i.e., the cell culture supernatant, is added into the wells and any chemokine present binds to the antibody. After the washing steps to remove unbound substances, an enzyme-linked polyclonal antibody specific for the chemokine is added, washed and followed by color development by the product of an enzymatic reaction. The intensity of the color, which is proportional to the amount of chemokine in the sample, is measured spectrophotometrically.

Cytokines and chemokines released by pancreatic beta cells are measured by commercial ELISA kits in the cell culture supernatant according to the instructions provided by the manufacturers.

### 3.7 Analysis of Cell Viability and Mechanisms Leading to Beta Cell Death

Accumulating evidence suggest that a "dialogue" between invading immune cells and the target beta cells triggers beta cell death. In this context, pro-inflammatory cytokines—IL-1β, IFN-γ, and TNF-α—produced by T cells and infiltrating macrophages induce

beta cell death through apoptosis [55]. Our recent findings showing that expression of several candidate genes is modulated by pro-inflammatory cytokines in pancreatic human islets and that some candidate genes play a direct role in beta cell apoptosis indicate that these genes may be important at the beta cell level as regulators of beta cell survival, and thus contribute to T1D pathogenesis [5–7, 10–12, 34].

To better understand the molecular mechanisms by which T1D candidate genes contribute to beta cell survival, in addition to measure cell viability using DNA-binding dyes, several proteins involved in different apoptotic pathways can be evaluated by RT-PCR and Western blot.

### 3.7.1 Cell Viability

The use of DNA-binding dyes, Hoechst 33342 (HO) and propidium iodide (PI) provides a simpler approach to evaluate cell viability in transfected/infected cells [26, 67, 85]. While HO has free passage across the plasma membrane and enters cells with preserved or damaged membranes, PI is impermeable to cells with intact membranes due to its high polarity.

1. Cells are plated and prepared for transfection/infection as described above (Sects. 3.2. and 3.3).
2. After treatment (e.g., cytokines), half of the medium is carefully removed and replaced by the same volume of culture medium containing nuclear dyes HO and PI (final concentration of 5 μg/ml).
3. After 15-min incubation, half of the staining medium is carefully removed and replaced by the same volume of fresh medium.
4. Cells are visualized and counted under an inverted microscope with filters for excitation wavelengths at:
   - 358 nm (HO, blue emission): viable and early apoptotic cells.
   - 538 nm (PI, red emission): dead cells (apoptotic + necrotic).
5. Cell viability is calculated as percentage of cell death (or apoptosis): number of dead (or apoptotic) cells/total number of cells (living + dead cells) × 100 (*see* **Note 18**).

### 3.7.2 Evaluation of Proteins Related to Beta Cell Death

In order to evaluate the molecular mechanisms by which a given T1D candidate gene modulates pancreatic beta cell death, we usually evaluate the intrinsic or mitochondrial pathway of cell death, the endoplasmic reticulum (ER) stress pathway and the nitric oxide (NO)-driven pathway.

### 3.7.3 Mitochondrial Pathway of Cell Death: Activation of Caspases 9 and 3

In mammalian cells there are two main pathways of apoptosis that lead to the activation of the final effectors, the caspases: (a) intrinsic pathway, which is initiated by events such as DNA damage, growth factor withdrawal, or cytotoxic insults. This pathway is also known as mitochondrial pathway due the key role of this organelle in the process; (b) extrinsic pathway, which is induced upon stimulation of cell surface death receptors belonging to the TNFR family (e.g., TNF-RI, Fas/CD95, and TRAIL R2/DR5) and caspase-8-cleavage/activation [86, 87].

As discussed above, pro-inflammatory cytokines induce apoptosis in beta cells. Several in vitro studies suggest that combinations of cytokines (e.g., TNF-α + IFN-γ or IL-1β + IFN-γ or IL-1β + IFN-γ + TNF-α) activate different cell death pathways [88–91]. One of the main features of cytokine-induced beta cell apoptosis is Bax translocation to the mitochondria, cytochrome *c* release and activation of the initiator caspase 9. Once activated, caspase 9 cleaves and activates the downstream effector caspase 3, which is essential for the execution of apoptosis. These events characterize the intrinsic pathway of apoptosis, which seems to be the main cell death pathway induced by cytokines in beta cells [90, 92, 93].

To investigate whether candidate genes are modulating the mitochondrial pathway of cell death, we measure protein levels of cleaved caspases 9 and 3 by Western blot (see below).

### 3.7.4 Mitochondrial Cell Death: Modulation of Bcl-2 Family Proteins

The balance between anti- and pro-apoptotic B-cell lymphoma 2 (Bcl-2) family proteins regulates the mitochondrial apoptotic pathway in beta cells [94]. This family consists of three protein groups: anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1, Bcl-W, and A1), pro-apoptotic (Bax, Bak, and Bok) and BH3-only proteins. This latter group of proteins can be further subdivided in BH3-only sensitizers (DP5, Bad, Bik, Bnip3, Bmf, Noxa) and BH3-only activators (Puma, Bim, tBid) [95]. The BH3-only sensitizers are able to bind and inactivate anti-apoptotic Bcl-2 proteins, which, in turn, are displaced from their original partners (BH3-only activators). Once released, BH3-only activators are free to interact and activate pro-apoptotic Bcl-2 members, leading to formation of pores in the mitochondria, cytochrome *c* release and, ultimately, apoptosis [96–98].

Pro-inflammatory cytokines regulate Bcl-2 protein expression in beta cells. This regulation may occur at different levels (transcriptional or posttranscriptional) and directions (upregulation or downregulation), being dependent on the cytokine combination and time of exposure used [11, 12, 90, 99, 100]. We usually evaluate the expression of the anti-apoptotic Bcl-2, Bcl-XL, Mcl-1, A1 and of the pro-apoptotic Bax, DP5, Puma, and Bim mRNA/proteins by RT-PCR (Sect. 3.6.1) and Western blot (see below), respectively. Selection of these proteins for study is based

on our previous work indicating their relevant role in cytokine-induced beta cell apoptosis [89–91, 94, 100]. As Bim has three main isoforms generated by alternative splicing, namely BimEL, BimL, and BimS [101], we evaluate all splice variants using both approaches mentioned above. Moreover, phosphorylation of Bim at serine 65 enhances its pro-apoptotic capacity [7] and thus evaluation of Bim phosphorylation should be additionally determined by Western blot.

### 3.7.5 Mitochondrial Cell Death: SDS Polyacrylamide Gel Electrophoresis and Western Blot

As SDS Polyacrylamide gel and Western blot techniques have been developed in detail in a previous volume (for more details, *see* Ref. [102]), in the present chapter we will briefly describe these two techniques.

1. Heat samples previously harvested in Laemmli buffer at 99 °C for 10 min and centrifuge to bring down the condensate.
2. Prepare running and stacking gels in the adequate percentage based on the molecular mass of the protein of interest.
3. Cast the running gel and gently overlay with isopropanol or water to accelerate polymerization. After polymerization, dry the isopropanol or water, add the stacking gel and insert a gel comb without introducing air bubbles.
4. When the gel is ready, load samples and protein ladder.
5. Start the electrophoresis at 80 V during 10–15 min to allow samples to stack and then continue at 100–150 V until the dye front reaches the bottom of the gel.
6. Following the electrophoresis, remove the gel carefully and transfer it to a nitrocellulose membrane previously cut to the size of the gel.
7. Perform electrophoretic transfer. We transfer gels using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the instructions provided by the manufacturer. The run is carried out at 80 V during 90 min.
8. After electrophoretic transfer, remove membrane from the transfer unit and block it with 5 % skimmed milk for 1–2 h. Wash the membrane three times with TBS-T (10 min per wash).
9. Incubate overnight with primary antibody (*see* **Note 19**).
10. Following overnight incubation, remove primary antibody and wash the membrane three times with TBS-T (10 min per wash).
11. Incubate with secondary antibody for 1 h and then wash the membrane three times with TBS-T (10 min per wash).
12. Proceed with the development of the membrane. In our case we use the SuperSignal West Femto chemiluminescent

substrate. Immunoreactive bands are detected using a Molecular Imager ChemiBoc XRS$^+$ and the densitometry of the bands is evaluated using Image Laboratory software.

### 3.7.6 ER-Stress Driven Cell Death

The correct functioning of the endoplasmic reticulum (ER) is essential for the cell. Perturbation of ER homeostasis may lead to accumulation of unfolded proteins and activation of a specific stress response known as ER stress and its consequent adaptive response, unfolded protein response (UPR) [103]. The main goal of this cellular response is to restore ER homeostasis by increasing the folding capacity and degradation of misfolded protein. When the changes mediated by the UPR do not solve the ER stress, the apoptosis pathway is activated [104].

The UPR signaling is mediated by three main ER-resident transmembrane proteins: activating transcription factor 6 (ATF6), inositol requiring ER-to-nucleus signal kinase 1α (IRE1α), and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK). These proteins are activated by accumulation of unfolded proteins in the ER lumen and transduce signals that modulate expression of key genes and proteins [105].

In beta cells, an extensive body of evidences shows that pro-inflammatory cytokines and PIC induce ER stress and apoptosis [104, 106, 107]. There is activation of several ER stress components, such as increased IRE1α expression, XBP1 splicing, PERK phosphorylation and ATF4 and CHOP induction, which triggers the mitochondrial pathway of apoptosis [106, 108]. Of note, cytokines inhibit ATF6 and BiP expression, hampering beta cell defenses against ER stress [106].

Analysis of the three UPR branches and their signaling pathways is critical to understand whether the mechanisms underlying beta cell death induced upon different stimuli (e.g., cytokines) are via ER stress activation. For this purpose we use RT-PCR and/or Western blot to measure, respectively, mRNA and/or protein levels of the three UPR mediators (ATF6, IRE1α, and PERK) as well as some key proteins related to ER stress (BiP, CHOP, spliced form of XBP1 (XBP1s) and ATF4). As PERK and eIF2α are activated by phosphorylation, the phosphorylated forms of these proteins are also evaluated by Western blot.

### 3.7.7 NO-Induced Cell Death

The NFκB-stimulated iNOS expression is responsible for the increase in NO production observed in beta cells exposed to cytokines [5, 109, 110]. Our group has previously shown that around 50 % of the genes modified by late (8–24 h) cytokine exposure are NO dependent, indicating a key role of this radical for the effects of cytokines and the beta cell fate [26].

Thus, in order to analyze whether a given T1D candidate gene modulates beta cell death via NO production, NO formation and

secretion can be measured as describe below. It may be also of interest to determine iNOS mRNA and protein expression.

#### 3.7.8 NO-Induced Cell Death: Nitrite Measurement

Of note, this is an adaptation of the protocol from Green et al. [111].

1. Prepare a 100 μM $NaNO_2$ solution by diluting 0.1 M $NaNO_2$ stock solution 1:1000 in the same medium used for culture. From this new solution (100 μM), prepare different dilutions (using the same culture medium) for standards: 20, 10, 5, 2.5, 1, 0.5, and 0.25 μM (*see* **Note 20**).
2. In a 96-well plate, add 200 μl of medium alone (Blank), each dilution of the standard curve and the samples. Put the Blank and standard curve samples in triplicate.
3. Prepare the Griess reagent mixing the same amounts of the sulfanilamide (stock solution at 10 %) and NED (stock solution at 1 %) solutions.
4. Add 20 μl Griess reagent in each well and incubate the plate at 65 °C for 2 min, protected from light.
5. Incubate at room temperature for 10–15 min, protected from light.
6. Measure absorbance in a microplate reader (wavelength = 546 nm) (*see* **Note 21**).

### 3.8 Analysis of Beta Cell Function: Glucose Metabolism and Insulin Secretion

Some candidate genes for T1D play important roles in the regulation of beta cell metabolism and function [8, 11]. The T1D susceptibility gene *GLIS3* interacts with other beta cell transcription factors (e.g., Pdx1, MafA, and NeuroD1) to increase insulin promoter activity [112]. Inhibition of *GLIS3* affects beta cell function, decreasing glucose metabolism and insulin release [11].

The protocols described below allow the measurement of glucose oxidation and insulin secretion in beta cells under different stimuli.

#### 3.8.1 Glucose Metabolism

This protocol has been adapted from Andersson and Sandler [113].

1. Add 15 μl of D-(U-$^{14}$C) glucose (300 mCi/mmol; Perkin Elmer, Waltham, Massachusetts, USA) in two tubes and dry under nitrogen.
2. Add 1.6 ml of the 3.2 or 32 mM glucose solutions prepared in KRBH (one solution per tube) and mix well.
3. For each sample to be measured is necessary (work in triplicate):
   - Vials with 20 μl of the KRBH solution containing 3.2 mM glucose + D-(U-$^{14}$C) glucose (prepared in **step 2**).
   - Vials with 20 μl of the KRBH solution containing 32 mM glucose + D-(U-$^{14}$C) glucose (prepared in **step 2**).

4. Prepare the blanks (work in triplicate):
   - Blank 1.6 mM: Mix 20 µl KRBH 3.2 mM glucose + radioactive glucose with 20 µl of glucose-free KRBH.
   - Blank 16 mM: Mix 20 µl KRBH 32 mM glucose + radioactive glucose with 20 µl of glucose-free KRBH.
5. Trypsinize cells and resuspended in glucose-free KRBH solution to a density of $5.0 \times 10^6$ cells/ml.
6. Add 20 µl of the cell suspension to the tubes prepared in **step 3**. At this point, glucose concentrations in the tubes will be 1.6 or 16 mM of nonradioactive glucose and 0.19 µCi D-(U-$^{14}$C) glucose. Place each vial in a Packard flask, cover with rubber cap and seal.
7. Prepare the Max (tubes with the highest level of radioactivity) (work in triplicate):
   - Max 1.6 mM: add 10 µl of the solution 1.6 mM glucose prepared in the **step 2** directly in the Packard flask, cover with rubber cap and seal.
   - Max 16 mM: add 10 µl of the solution 16 mM glucose prepared in the **step 2** directly in the Packard flask, cover with rubber cap and seal.
8. Gas for 5 min with a mixture of $O_2$ and $CO_2$ (95 %:5 %). Then keep the flasks for 2 h in a water bath at 37 °C with slight agitation.
9. Inject 10 µl of metabolic poison solution into the glass vials containing samples or blanks (but not in Max). This metabolic poison solution will stop glucose oxidation.
10. Inject 250 µl of Hyamine hydroxide 10-× in the bottom of all vials (*see* **Note 22**) and incubate for 1 h at 37 °C with slight agitation.
11. Open the Packard flasks, add 6 ml of scintillation liquid or gel, mix well and close with a normal cap (*see* **Note 23**).
12. Read in a liquid scintillation counter.

### *3.8.2 Insulin Secretion in INS-1E Cells*

1. All media are pre-warmed at 37 °C.
2. Remove the medium and replace by 500 µl glucose-free complete RPMI. Incubate for 1 h at 37 °C.
3. Remove the medium and wash cells with 500 µl KRB.
4. Add 500 µl KRB in the wells and incubate for 30 min at 37 °C.
5. Remove the medium and wash cells again with 500 µl KRB.
6. Treat cells with the different glucose stimulation media (*see* Sect. 2.5) (500 µl per well) for 30 min at 37 °C.

7. After incubation, remove 400 μl of the media, centrifuge at 300 × *g*/5 min, collect 300–350 μl supernatant and freeze at −20 °C (*insulin secretion* is measured in these samples).
8. To lyse the cells, wash with PBS, add 100 μl sterile $ddH_2O$, harvest the cells and collect in a tube. Do one additional wash with 100 μl sterile $ddH_2O$, add to the tube and freeze at −20 °C (*insulin content* is measured in these samples).
9. For insulin content measurements, sonicate samples collected in the previous step (twice for 10 s).
10. Mix 50 μl sonicated lysate with 125 μl acid ethanol solution. Freeze again at −20 °C until assay.
11. Insulin is measured by ELISA kits according to the manufacturer's instructions (Mercodia, Uppsala, Sweden).

## 4 Notes

1. The use of antibiotic-free medium before and during transfection is recommended to avoid cell death. This is an advice of the Lipofectamine's manufacturer (Invitrogen).
2. This medium should be prepared at 4 °C and avoid returning the ECM to room temperature (thaw it at 4 °C).
3. Previous experiments by our group showed that BSA induces aggregation of the lipid reagent, which impairs transfection efficiency.
4. Once frozen, do not thaw/freeze more than two times to avoid siRNA degradation.
5. Opti-MEM must be used free of additives (e.g., BSA and serum).
6. $NaNO_2$ and NED solutions must be protected from light. NED solution may change its color in contact with light. However, NED performance is not significantly affected by this change in color.
7. KRBH solution must be freshly prepared on the day of the experiment. If necessary, the pH can be adjusted using a gas mixture of $O_2$ and $CO_2$ (95 %:5 %) after dissolving the salts.
8. At least 1 h before starting the cell passage, plates or flasks must be coated and then placed in an incubator at 37 °C. The volume of coating medium should be added in accordance with the culture support size (e.g., ~100 μl per well for 96-well plates). Make sure that the coating medium is covering the whole surface of the culture support.
9. This step aims to remove antibiotics and BSA. As described above, these two additives may interfere with the transfection.

10. We have observed that the ratio between the number of cells and the concentration of the lipid carrier is not linear. Moreover, the cell type used affects this ratio. Thus, when starting experiments in new cell types, it is crucial to try different transfection conditions by testing different concentrations of lipid reagent and siRNA.
11. We tested shorter periods of 14–18 h without noticing significant changes in efficiency of transfection or toxicity.
12. We did not observed toxicity when using medium with antibiotics at this step.
13. The number of cells per well must be counted to calculate the MOI as accurately as possible. The counting is not necessary for cells that do not replicate, such as primary rat beta cells and dispersed human islets. In these cases, the number of cells originally plated must be used as an estimation of the number of cells per well.
14. To prepare aliquots with different MOIs, start by preparing the one with the highest MOI and make serial dilutions.
15. When setting the presently described experimental conditions, we observed that these cells were better infected in BSA- and FBS-free medium.
16. It is strongly recommended to perform a time course of 24–96 h of recovery after transfection/infection in order to establish the time point in which the maximum inhibition/overexpression of the protein is assessed. Cell viability should be evaluated in parallel to exclude nonspecific toxicity; for this purpose, non-transfected/infected cells are used as controls.
17. Standard curves are prepared for each gene of interest. To this aim a fragment of the gene is amplified by conventional PCR using specific primers. Afterwards, the PCR products are purified and serial dilutions performed in order to get the different points of the curve.
18. A minimum of 600 cells is counted in each experimental condition. Viability is evaluated by two independent observers, one of them unaware of sample identity, and the agreement between findings obtained by the two observers must be higher than 90 %.
19. Primary antibodies are diluted in 5 % BSA (fraction V) while secondary antibodies are diluted in 5 % skimmed milk in TBS-T.
20. To obtain an accurate quantitation of $NO_2$-levels, a nitrite standard reference curve must be prepared for each assay. This standard curve must be prepared in the same buffer used for the samples.

21. It is crucial to measure absorbance within 30 min. After this time, color may disappear.
22. Hyamine hydroxide 10-× is a $^{14}CO_2$ trapping agent that will trap all the $^{14}CO_2$ formed during glucose metabolism.
23. At this step the samples can be left in a cold room (at 4 °C) up to 24 h before reading.

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