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Functional genomics of the beta-cell: short-chain 3-hydroxyacyl-coenzyme A dehydrogenase regulates insulin secretion independent of K+ currents

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Functional Genomics of the β-Cell: Short-Chain 3-Hydroxyacyl-Coenzyme A Dehydrogenase Regulates Insulin Secretion Independent of K^+ Currents

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Recent advances in functional genomics afford the opportunity to interrogate the expression profiles of thousands of genes simultaneously and examine the function of these genes in a high-throughput manner. In this study, we describe a rational and efficient approach to identifying novel regulators of insulin secretion by the pancreatic β-cell. Computational analysis of expression profiles of several mouse and cellular models of impaired insulin secretion identified 373 candidate genes involved in regulation of insulin secretion. Using RNA interference, we assessed the requirements of 10 of these candidates and identified four genes (40%) as being essential for normal insulin secretion. Among the genes identified was *Hadhsc*, which encodes short-chain 3-hydroxyacyl-coenzyme A dehydrogenase (SCHAD), an enzyme of mitochondrial β-oxidation of fatty acids whose mutation results in congenital hyperinsulinism. RNA interference-mediated gene suppression of *Hadhsc* in insulinoma cells and primary rodent islets revealed enhanced basal but normal glucose-stimulated insulin secretion. This increase in basal insulin secretion was not attenuated by the opening of the K_{ATP} channel with diazoxide, suggesting that SCHAD regulates insulin secretion through a K_{ATP} channel-independent mechanism. Our results suggest a molecular explanation for the hyperinsulinemia hypoglycemic seen in patients with SCHAD deficiency. (*Molecular Endocrinology* 21: 765–773, 2007)

NUTRIENT SENSING COUPLED to regulated insulin release is required for the pancreatic β-cell to maintain glucose homeostasis. In the fed state, when glucose levels are elevated, insulin secretion is increased to stimulate glucose utilization in muscle, liver, and adipose tissue. During fasting, when glucose levels are low, insulin secretion is down-regulated, thus helping to maintain a minimal level of glucose in the bloodstream. β-Cell dysfunction resulting in dysregulated insulin secretion can have severe consequences and lead to chronic diseases such as diabetes mellitus, characterized by insulin deficiency, or congenital hyperinsulinism, which is due to inappropriate insulin release.

The major pathway of insulin secretion is triggered by glucose uptake into the β-cell, where it is then phosphorylated by glucokinase and further metabolized to ATP. The subsequent increase in the cytoplasmic ATP/ADP ratio closes the ATP-sensitive potassium channel (K_{ATP} channel), leading to depolarization of the plasma membrane and opening of voltage-sensitive Ca^{++} channels. Finally, the resulting rise in intracellular Ca^{++} activates exocytosis of insulin secretory granules (1). This K_{ATP}-dependent pathway is the best characterized mechanism leading to insulin secretion; however, studies of K_{ATP} channel-deficient mice reveal the presence of K_{ATP}-independent pathways to insulin secretion (2). Although the importance of K_{ATP}-dependent and K_{ATP}-independent pathways to insulin secretion is certain, their relative contributions are not clearly defined. The precise mechanisms governing these pathways to insulin secretion remain unknown. Identification of additional components of the insulin secretory apparatus may lead to the development of novel therapeutic regimens for the treatment of diabetes mellitus.

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Abbreviations: Ad-GFP, Adenovirus expressing GFP; Ad-siHadhsc, adenovirus expressing siRNA against Hadhsc; Ad-siScramble, adenovirus expressing a scramble sequence; GFP, green fluorescent protein; HBSS, HEPES balanced salt solution; IAP, inhibitors of apoptosis; InsCre, insulin promoter-driven Cre-recombinase; pfu, plaque-forming units; RAD, RNA Abundance Database; RNAi, RNA interference; SAM, statistical analysis of microarrays; SCHAD, short-chain 3-hydroxyacyl-coenzyme A dehydrogenase; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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In this study, we describe a rational and efficient functional genomics approach to identifying novel regulators of insulin secretion. We began by determining the expression profiles of multiple paradigms of abnormal insulin secretion, including several mouse models of impaired β-cell function, as well as cell culture models of robust or impaired glucose-stimulated insulin release. Computational analysis of these expression profiles identified genes likely to play an important role in insulin secretion. The functional relevance to β-cell function of 10 potential targets identified in this manner were evaluated using RNA interference (RNAi), and several were found to be required for normal insulin secretion. Most notably, the gene encoding short-chain 3-hydroxyacyl-coenzyme A dehydrogenase (SCHAD) was shown to play a crucial role in a K_{ATP} channel-independent mechanism of insulin secretion, explaining the molecular mechanisms of how deficiency for this gene causes congenital hyperinsulinism in humans.

RESULTS

Combining RNAi with Transcriptional Profiling Identifies Novel Regulators of Insulin Secretion

Large-scale functional genomic approaches have been used successfully to identify novel regulators of multiple biological processes in lower eukaryotic organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster* (3–7). In many of these studies, high-throughput screens using large libraries of double-stranded small interfering RNAs (siRNAs) have facilitated the identification of genes involved in regulatory pathways. One limitation to such an approach in higher organisms is that the increased complexity of physiological processes makes identifying essential regulators less likely, thus making these functional genomic approaches less efficient.

To find essential regulators of insulin secretion, we first identified genes whose expression is altered in models of impaired or enhanced insulin release, thus increasing the likelihood that a given target plays an important role in insulin secretion. Among the systems used are three unique mouse models in which inactivation of *Foxa1*, *Foxa2*, or *Hnf-4α* results in significantly impaired glucose-stimulated insulin release from isolated islets (8–15). In addition, we compared INS1-derived cell lines with robust glucose-stimulated insulin secretion (lines 832/13 and 833/15) *vs.* INS1-derived cells with weak glucose-stimulated insulin secretion (lines 832/1 and 832/2) (16, 17). Finally, we examined the expression profiles of 832/13 cells cultured in the presence or absence of a 0.5-mM oleate: palmitate/albumin, using RPMI 1640 medium that also contains a relatively high glucose concentration (11 mM) to simulate glucolipotoxicity encountered in type 2 diabetes, as we have previously demonstrated that culture of 832/13 cells in the presence of elevated fatty acids and glucose for 48 h causes a striking impairment in glucose-stimulated insulin secretion (Table 1) (18). Through computational analysis of the differentially expressed genes in these five models (see *Materials and Methods*, Fig. 1), we derived a paradigm list of 373 candidate genes that may play a role in regulating insulin secretion (supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org).

To assess the potential contribution of these genes to β-cell function, we employed RNAi for loss of function analysis in insulinoma cells (Table 2, supplemental Table 2). Of 373 genes in our paradigm list, we focused on 59 that changed greater than 1.2-fold in at least two of the paradigms. Of these 59, only 29 corresponded to identifiable genes with characterized reference sequences. For 21 of these genes, siRNA duplexes were commercially available, and we chose 10 at random for further evaluation. Transfection of 832/13 cells with siRNAs against individual genes led to a minimal reduction of expression of 43%, with several genes inhibited by more than 70% (Fig. 2A). Strikingly, glucose-stimulated insulin secretion was affected in four out of 10 genes analyzed (40%), confirming the utility of the functional genomics approach in narrowing the field of targets to be screened (Fig. 2B). Down-regulation of reticulon 4, argininosuccinate synthetase 1, and baculoviral inhibitors of apoptosis (IAP) repeat-containing 5 resulted in a striking decrease in glucose-

Table 1. Models of Impaired β-Cell Function

Models	Description	Refs.
Foxa1$^{-/-}$	Hypoglycemic, impaired glucagon, and insulin secretion	Kaestner *et al.* (11); Shih *et al.* (13); Vatamaniuk *et al.* (15)
Foxa2 $^{loxP/loxP}$, InsCre	Hyperinsulinemic hypoglycemia, abnormal insulin secretion, reduced expression of K_{ATP} channel	Sund *et al.* (14); Lantz *et al.* (9)
Hnf-4α $^{loxP/loxP}$, InsCre	Impaired glucose tolerance due to abnormal glucose-stimulated insulin secretion by β-cell	Gupta *et al.* (8); Miura *et al.* (12)
INS1 model 1	Glucose-responsive *vs.* glucose unresponsive INS1 subclones	Chen *et al.* (16); Hohmeier *et al.* (17)
INS1 model 2: lipotoxicity	Time course of lipid-induced β-cell toxicity	Boucher *et al.* (18)

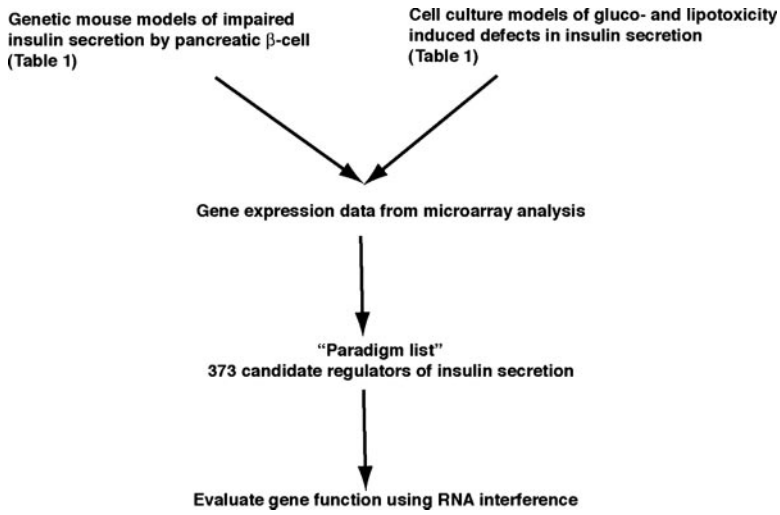

Fig. 1. Combining Expression Profiling with RNAi

Gene expression profiling was performed on isolated islets of three mouse models of perturbed β-cell function (*Foxa1*$^{-/-}$, *Foxa2*$^{loxP/loxP}$, InsCre; *Hnf-4*$\alpha^{loxP/loxP}$, InsCre) (8, 11, 14), compared in each case to littermate controls. Expression profiles were also determined for INS1-derived cell lines with robust glucose-stimulated insulin secretion (cell lines 832/13 and 833/15) *vs.* INS1-derived cells with weak glucose-stimulated insulin secretion (lines 832/1 and 832/2) (16, 17), as well as for 832/13 cells cultured in the presence or absence of a 0.5 mM oleate:palmitate/albumin. Computational analysis of all expression profiles led to a paradigm list of 373 possible regulators of insulin secretion. The full list is published as supplemental Table 1 at http://mend.endojournals.org.

stimulated insulin secretion. Conversely, reducing the expression of *Hadhsc* encoding SCHAD caused a marked increase in basal insulin secretion. Together, these results confirm that the functional genomics approach described here provides a rational and efficient method for identifying novel regulators of insulin secretion.

SCHAD Regulates Basal Insulin Secretion Independent of K_{ATP} Channels

Among the genes validated from the paradigm list is *Hadhsc*, encoding SCHAD, the mitochondrial enzyme that catalyzes the conversion of 3-hydroxyacyl-coenzyme A to 3-ketoacyl-coenzyme A, the penultimate reaction in the β-oxidation of fatty acids (19). Notably, patients with mutations in *HADHSC* leading to functional SCHAD deficiency develop congenital hyperinsulinism (19–22). However, it remains unclear whether hypersulinism in these patients is to due to a primary defect in β-cell function or is a secondary consequence of SCHAD deficiency in other tissues. In addition, there is much controversy regarding whether or not reduced β-oxidation of fatty acids regulates insulin secretion through K_{ATP} dependent or independent pathways. Thus, we sought to investigate the role of Hadhsc in insulin secretion in further detail.

Table 2. Expression Changes of Candidate Genes in Various Paradigms

Gene name	Locus symbol	*Foxa1*$^{-/-}$	*Foxa2*$^{loxP/loxP}$, InsCre	*HNF-4*$\alpha^{loxP/loxP}$, InsCre	INS1 model 1	INS1 model 2
Chromogranin B	Chgb	−N.C.	3.2	2.1	2.1	1.3
Transforming growth factor α	Tgra	1.2	−N.C.	7.8	−N.C.	1.2
Sulfotransferase family 1D	Sult1d1	1.8	−N.C.	−3.7	−N.C.	−N.C.
L-3-hydroxyacyl-coenzyme A dehydrogenase, short chain	Hadhsc	−1.3	−2.8	−N.C.	−N.C.	−N.C.
Pyruvate kinase, muscle	Pkm2	−1.2	1.2	−N.C.	−N.C.	−N.C.
Aromatic L-amino acid decarboxylase	Aadc	−N.C.	−2.1	−2.5	−N.C.	−N.C.
Baculoviral IAP repeat-containing 5	Birc5	−1.6	−1.2	−N.C.	−N.C.	−N.C.
Argininosuccinate synthetase 1	Ass1	1.3	1.3	−N.C.	−N.C.	−N.C.
FK506 binding protein 11	Fkbp11	−N.C.	−N.C.	1.4	−N.C.	1.2
Reticulon 4	Rtn4	−1.2	−1.7	−1.2	−3.8	N.C.

Fold changes listed above are relative to littermate control groups used for each individual experiment. N.C., No significant difference compared with controls.

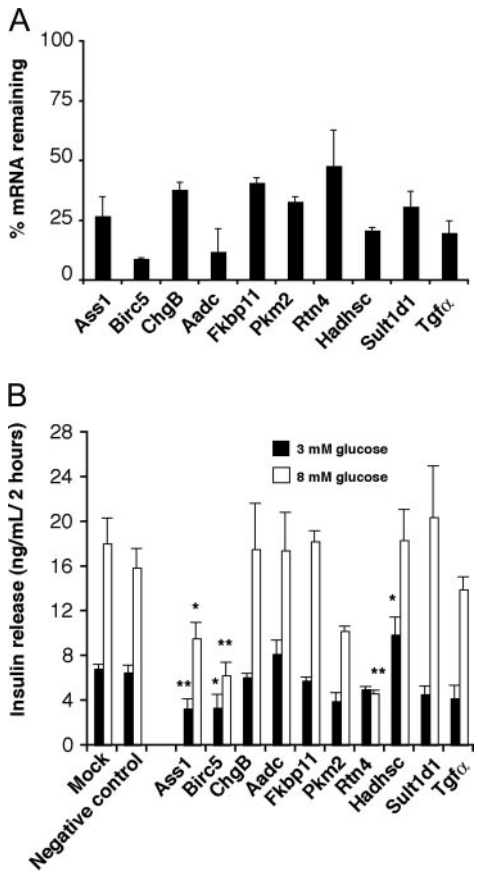

Fig. 2. Validation of Functional Genomics Approach

A, Real-time PCR analysis of mRNA levels after RNAi-mediated gene suppression of 10 candidate regulators of insulin secretion. B, Of 10 candidate genes chosen, down-regulation of four genes (40%) results in dysregulated insulin release. Suppression of SCHAD expression increases basal insulin release at 3 mM glucose, whereas reduction in argininosuccinate synthetase 1 (Ass1), baculoviral IAP repeat containing 5 (Birc5), and reticulon 4 (Rtn4) results in attenuated glucose-stimulated insulin secretion. *Bars* represent mean + SEM of three to 11 independent assays. *, $P < 0.05$; **, $P < 0.01$ by Student's *t* test.

Although nucleofection of RNAi oligonucleotides allows for rapid screening of multiple candidate genes in insulinoma cell lines, we have not found this method to be efficient for gene transfer into primary pancreatic islets. For further analysis of *Hadhsc* function in β-cell, we employed adenoviral-mediated gene transduction in primary islets in addition to insulinoma cells (23, 24). We constructed a recombinant adenovirus containing a short hairpin RNA (shRNA) sequence specific to this gene [adenovirus expressing siRNA against Hadhsc (Ad-siHadhsc)]. To test the ability of Ad-siHadhsc to suppress Hadhsc expression, we assayed Hadhsc mRNA levels in INS1 cells after transduction with varying multiplicities of infection of Ad-siHadhsc for 24 or 48 h. Treatment of 832/13 cells with Ad-siHadhsc caused decreases in Hadhsc transcript levels in as little as 24 h after transduction, with maximal suppression (86%) at 48 h (Fig. 3A). Increasing the multiplicity of infection had no further effect on the efficiency of the shRNA approach (Fig. 3A).

Next we measured glucose-stimulated insulin secretion in insulinoma cells with reduced levels of Hadhsc. We used Ad-siHadhsc at a dose of 100 plaque-forming units (pfu) per cell for 48 h to suppress Hadhsc mRNA levels by 83% in 832/13 cells. Suppression of Hadhsc expression caused a significant increase in basal insulin secretion compared with untreated cells and cells transduced with an adenovirus expressing a scramble sequence (Ad-siScramble) (Fig.

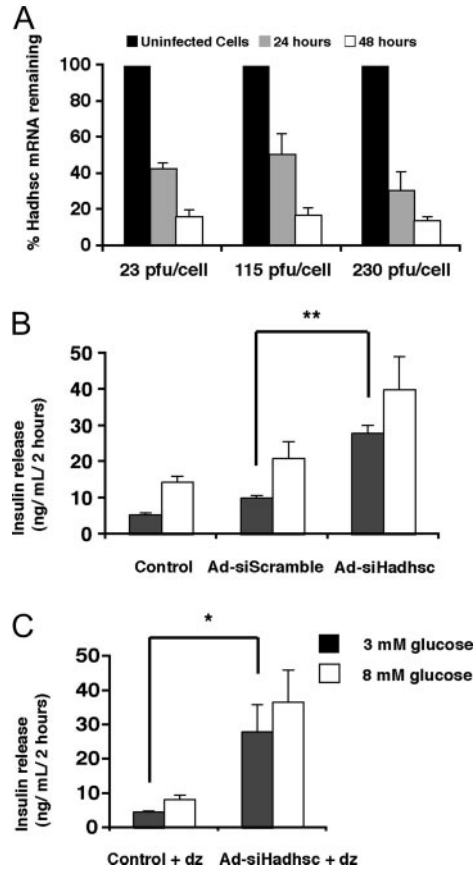

Fig. 3. SCHAD Is Required for K_{ATP}-Independent Basal Insulin Secretion

A, Real-time PCR analysis of Hadhsc mRNA levels in 832/13 cells after Ad-siHadhsc with varying time exposure and multiplicity of infection. B, Transduction of nonspecific shRNA (Ad-siScramble) had no significant effect on basal or glucose-stimulated insulin secretion from 832/13 cells, whereas transduction of Ad-siHadhsc significantly increased basal but not glucose-stimulated insulin secretion (Note: INS1 832/13 cells saturate at 8 mM glucose). C, Whereas glucose-stimulated insulin secretion is blunted as expected by diazoxide (dz), the increased basal rate of insulin secretion caused by suppression of Hadhsc expression is maintained in the presence of the drug, indicating that the increased basal insulin from SCHAD-deficient β-cells is not dependent on K_{ATP}-dependent pathway activation. Data represent the mean + SEM for three independent assays. *, $P < 0.05$; **, $P < 0.01$ by Student's *t* test.

3B). To determine if this increase in basal insulin secretion is mediated through a K_{ATP} channel-dependent mechanism, we performed glucose-stimulated insulin secretion assays in the presence of diazoxide, which functions to keep K_{ATP} channels open, thus suppressing the K_{ATP}-dependent pathway to insulin release. Treatment of the cells with diazoxide did not alter the enhanced basal insulin secretion caused by suppression of Hadhsc, although the same dose of diazoxide completely abrogated glucose-stimulated insulin secretion in Ad-siScramble-treated cells (Fig. 3C). Together these results indicate that SCHAD functions directly in β-cells to regulate a K_{ATP}-independent pathway to insulin secretion.

SCHAD Is Required for Basal Insulin Secretion in Primary Mouse Islets

Next, we examined the impact of SCHAD deficiency on basal insulin secretion in primary islets because insulinoma cells do not always replicate all aspects of normal β-cell biology. To test the efficiency of recombinant adenovirus to transduce islets, we assayed green fluorescent protein (GFP) expression in islets after transduction with an adenovirus expressing GFP (Ad-GFP). Treatment of mouse islets with Ad-GFP at a viral dose of 1.3×10^6 pfu per islet for 24 h followed by culture for 2 more days resulted in efficient GFP expression within the islets. In addition, when we treated islets with Ad-siHadhsc, Hadhsc transcript levels were reduced by 80% 4 d after transduction (Fig. 4A), consistent with our experiences in other studies (24, 25) employing adenovirus vectors for knock-down of gene expression in primary rodent islets.

Insulin secretion from isolated islets was determined at 3 or 16.7 mM glucose in a 2-h static incubation assay. Adenovirus-mediated silencing of Hadhsc increased basal insulin secretion in islets, confirming our findings in insulinoma cells; however, there was no difference in glucose-stimulated insulin secretion (Fig. 4B). Thus, we conclude that *Hadhsc* has a primary function in pancreatic islets for the regulation of basal insulin secretion.

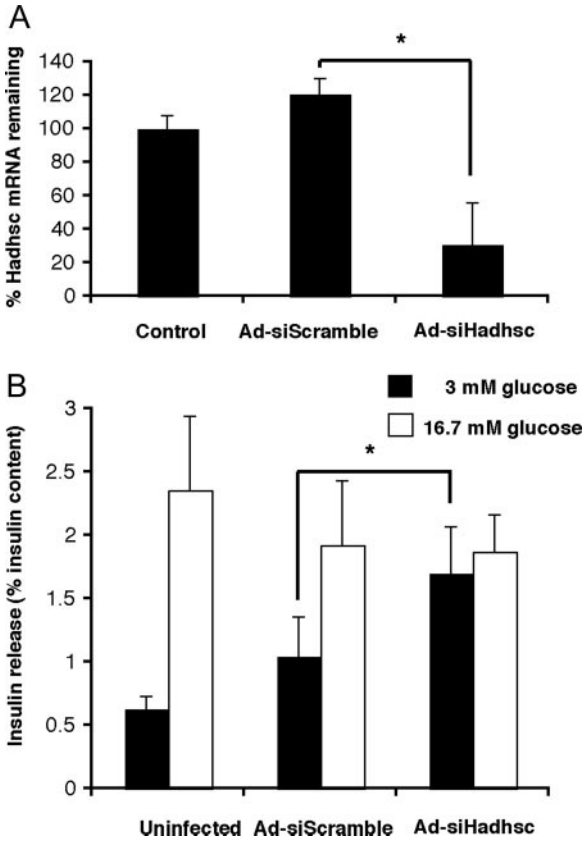

Fig. 4. Glucose-Stimulated Insulin Secretion from Mouse Islets

A, Transduction of isolated mouse islets with Ad-siHadhsc results in reduced Hadhsc mRNA levels, whereas Ad-siScramble has no significant effect. B, Insulin secretion from isolated islets cultured in either 3 mM or 16.7 mM glucose for 2 h. Adenovirus-mediated silencing of Hadhsc increases basal insulin secretion from mouse islets treated with 3 mM glucose, but does not effect the response to high glucose. Data for secretion assay represent the mean + SEM for sixteen independent experiments with data normalized to total insulin content. *, $P < 0.05$ by Student's *t* test.

DISCUSSION

Although there has been substantial progress in understanding β-cell biology and the contributions made by β-cell dysfunction to the development of diabetes, treatment options remain less than optimal. Thus, it is important to search for genes that regulate β-cell function, growth, and survival. Such genes could be targets for development of more effective drugs for the treatment of diabetes. Previous large-scale screens to identify regulators of signaling cascades and physiological processes have used large siRNA libraries containing over 20,000 siRNA duplexes. Although these studies have identified hundreds of regulators, these approaches have a relatively low success rate, with as little as 2% of the siRNAs producing a measurable phenotype (3, 7).

In this study, we describe a rational and efficient approach to identifying regulators of insulin secretion. By combining computational analysis with expression profiling, we have derived a list of potential new target genes that affect the function of the β-cell. Screening of 10 of these candidate genes identified four targets (40%) that are involved in regulating insulin secretion. Blindly screening genes without using the guidance of the expression data would have likely required an order of magnitude more genes to reach the same number of positive results. In addition, the paradigm gene list of 373 genes provides a valuable resource to be exploited by β-cell researchers in the future.

Among the genes identified as essential regulators of glucose-stimulated insulin release are *Argininosuc-*

cinate synthetase 1, Baculoviral IAP repeat containing 5, and *Reticulon 4*. Argininosuucinate synthetase catalyzes the synthesis of argininosuccinate, the immediate precursor of arginine, from citrulline and aspartate. First identified in the liver, argininosuucinate synthetase is now recognized as a ubiquitous enzyme in mammalian tissues whose regulation is dependent on arginine utilization in the tissue of interest (26). Previous studies have shown that L-arginine stimulates insulin release from pancreatic β-cells. One hypothesis is that the L-arginine potentiation of glucose-induced insulin secretion is mediated by L-arginine-derived nitrogen oxides (27), whereas another group proposes that it occurs via membrane depolarization, which stimulates insulin secretion through protein kinase A- and C-sensitive mechanisms (28). In this study, down-regulation of argininosuccinate synthetase expression by RNAi results in reduced insulin secretion, indicating that argininosuccinate synthetase is a potential activator of insulin secretion.

Baculoviral IAP repeat containing 5 (survivin) is a member of the mammalian IAP family, along with baculoviral IAP repeat-containing 4, which encodes negative regulatory proteins that prevent apoptotic cell death. Previous studies have shown that overexpression of baculoviral IAP repeat-containing 4 in β-cell lines and human islets enhances β-cell survival, possibly by inhibiting TNF-related apoptosis-inducing ligand mediated pathways (29–31). In this study, down-regulation of baculoviral IAP repeat containing 5 expression by RNAi results in reduced basal and glucose-stimulated insulin release. Further studies are needed to determine how proteins in the IAP family regulate insulin secretion.

Likewise, very little is known about the function of reticulons in insulin secretion. In neuroendocrine cells, reticulons are localized primarily to the endoplasmic reticulum and can immunoprecipitate with soluble N-ethylmaledimide-sensitive factor attachment protein receptors, which are essential for secretory granule release from β-cells (32). Therefore, it is tempting to speculate that reticulon 4 functions to control the priming or release of insulin secretory granules. Indeed, down-regulation of reticulon 4 results in a dramatic reduction of basal and glucose-stimulated insulin secretion. The INS1 cells with reduced reticulon 4 expression described in this study provide novel tools to examine the exocytotic machinery involved in regulating insulin secretion.

Most notable among the genes validated from the paradigm list is *Hadhsc. Hadhsc* encodes SCHAD, the mitochondrial enzyme that catalyzes the conversion of 3-hydroxyacyl-coenzyme A to 3-ketoacyl-coenzyme A, the penultimate reaction in the β-oxidation of fatty acids. Several cases of hyperinsulinism in children associated with mutations in *HADHSC* have been described (19–22). However, until now it has remained unclear whether hyperinsulinism in these patients is due directly to the loss of SCHAD in pancreatic β-cells, or occurs secondary to the metabolic stress initiated by altered lipid metabolism in other tissues. Our *in vitro* model of reduced SCHAD expression demonstrates for the first time that SCHAD is required directly in β-cells for the regulation of basal insulin release.

There is much speculation regarding the mechanism of increased insulin release from SCHAD-deficient β-cells. Molven *et al.* (22) proposed that the L-form of 3-hydroxybutyryl-carnitine that accumulates in these patients may interfere with potassium channel function or with the ATP-independent and lipid-sensitive mechanism of insulin secretion. We observed that increased basal insulin secretion from cells with suppressed SCHAD expression is sustained in the presence of diazoxide, supporting a potassium channel-independent pathway, thus making it unlikely that human SCHAD deficiency manifests in impaired regulation of insulin secretion via effects on K_{ATP} channels. Clayton *et al.* (19) suggested that the accumulation of short-chain acyl-coenzyme A esters in the mitochondrion causes insulin secretion by inhibition of carnitine palmitoyltransferase I. Fatty acids are also known to increase insulin secretion by stimulation of G-protein-coupled receptors and by activation of L-type Ca^{2+} channels (21, 33, 34). Future studies with this model of SCHAD deficiency will be used to identify mechanisms by which fatty acid and fatty acid metabolites modulate insulin release.

In summary, by combining expression profiling with RNAi, we provide a rational and efficient approach to identifying novel regulators of insulin secretion by the β-cell. This functional genomics approach can be applied to other mammalian systems and may someday lead to the development of novel therapeutic regimens for the treatment of diseases such as diabetes mellitus.

MATERIALS AND METHODS

Expression Profiling

Gene expression profiling was performed on multiple two-state models of islet function/dysfunction. This included a comparison of gene expression in isolated islets of three mouse models of perturbed β-cell function [*Foxa1*$^{-/-}$, *Foxa2*$^{loxP/loxP}$, insulin promoter-driven Cre-recombinase (InsCre); *Hnf-4α*$^{loxP/loxP}$, InsCre] (8, 11, 14), compared in each case to littermate controls. We also included a comparison of INS1-derived cell lines with robust glucose-stimulated insulin secretion (lines 832/13 and 833/15) *vs.* INS1-derived cells with weak glucose-stimulated insulin secretion (lines 832/1 and 832/2) (16, 17). Finally, we cultured a robustly glucose-responsive INS1-derived cell line (832/13) in the presence or absence of a 0.5 mM oleate: palmitate/albumin, using RPMI medium that also contains a relatively high glucose concentration (11 mM) to simulate conditions encountered in type 2 diabetes. Boucher *et al.* (18) have previously demonstrated that culture of 832/13 cells in the presence of elevated fatty acids and glucose for 48 h causes a striking impairment in glucose-stimulated insulin secretion. Replicate RNA samples (three to five per condition) were collected from all of these two state models and used for microarray analysis on PancChip cDNA microarrays (version 4.0 for the

Foxa2$^{loxP/loxP}$ InsCre study, and version 5.0 for the others) (35). Over 100 two-channel hybridization assays for the five independent paradigms were performed, collecting more than 10 million data points. All studies, including Minimum Information about a Microarray Experiment (MIAME)-compliant (www.mged.org) detailed biomaterial and protocol annotation and raw and processed data, have been deposited into the RNA Abundance Database (RAD) (36) and are available for querying and downloading at http://www.cbil.upenn.edu/RAD. Further information on the PancChip array is available at http://www.cbil.upenn.edu/EPConDB/ Chips/pancChip.shtml.

All arrays were scanned using an Agilent DNA Microarray Scanner Model No. G2565BA (Agilent Technologies, Wilmington, DE), and the images were quantified using GenePix Pro, version 5 (Molecular Devices). Scanning and quantification parameters used for each assay are available at the RAD website described above. The GenePix foreground mean intensities were used for each spot and each channel. No background subtraction was performed because these methods add to data variability and lead to the occurrence of negative values (37). For each assay performed, Cy3 anchors, blanks, yeast and dilution controls, and Stratagene PCR controls were removed from the analyses (thus, the total number of spots analyzed was 11,400 for PancChip 4.0 and 13,008 for PancChip 5.0 experiments). For each assay, M values were computed for each spot considered, where the M value for two conditions, C1 and C2, is defined as $\log_2(C1)-\log_2(C2)$. The M values were normalized on each assay with the print-tip loess approach (38) using the implementation provided by the R (http://cran.r-project.org) marrayNorm package from Bioconductor (http://www.bioconductor.org), with default parameter settings (R version 1.8.1, marrayNorm version 1.1.6). After normalization, the M values for each available pair of dye-swaps were combined $[(M_1-M_2)/2]$.

For each of the five paradigms described above, differentially expressed transcripts between the conditions of interest were identified using a combination of approaches. For the three mouse models of impaired β-cell function, the conditions of interest were mutant *vs.* littermate controls. For each model we ranked all spots according to LOD scores (39) and performed statistical analysis of microarrays [SAM, version 2.0 (http://www-stat.stanford.edu/~tibs/SAM)]. The same tools were used for the comparison of the robustly *vs.* poorly glucose responsive INS1-derived cell lines. Finally, for the study of lipid-induced impairment of glucose-stimulated insulin secretion, we analyzed a time course of exposure to oleate/palmitate (0, 12, 24, and 48 h), which required a different computational approach. First, we compared treated to untreated cells at each time point separately in a pair-wise (by biological sample) mode. Secondly, we compared all of the treated *vs.* all of the control assays using SAM with an unpaired block design with three blocks, one per time point.

Computational Analysis of Expression Profiles

After analyzing each study individually, the results were combined to generate a master list of candidate targets to be considered for further validation. This paradigm list was compiled using multiple criteria. First, we computed a "top" list (based on LOD scores) for each study, after removing spots with PCR failure flags and spots flagged by GenePix in greater than 40% of the arrays. The number of "top" spots for each paradigm was established according to a suitable weight given to each of the five studies and to each of the comparisons within such studies. The weight of each paradigm took into account the number of replicates available in that study and the number of differentially expressed genes identified by the SAM analyses. The union of these lists yielded 373 distinct transcripts, which were annotated according to their additional lines of evidence for differential expression coming from the SAM analyses. The complete paradigm list is available at http://mend.endojournals.org/ (supplemental Table 1).

Gene Silencing in Insulinoma Cells with siRNA Duplexes

The rat insulinoma line 832/13 was cultured as described (17). Transfection of siRNA duplexes (Ambion, Austin, TX) (supplemental Table 2) was performed using the Amaxa nucleofection system (Amaxa, Gaithersburg, MD) as described (42). Briefly, 2 μg of siRNA duplexes was transfected into 1.5×10^6 832/13 cells using program no. T-27 of the nucleofector device. After 48 h, cell RNA was extracted for quantitative RT-PCR analysis. Primers used for analysis of gene expression are available upon request.

Construction of an Adenoviral Vector Producing a shRNA against Hadhsc

Two shRNA adenoviruses were constructed to target rat Hadhsc and mouse Hadhsc mRNAs. The 5′ end of the target corresponds to rat Hadhsc (accession no. NM_057186) nucleotides 840–858 (AGT TCA TCT TAG ACG GGT G) and mouse Hadhsc (accession no. NM_008212) nucleotides 401–419 (GAA CGA GCT GTT CCA GAG G). Ad-GFP and Ad-siScramble (GAG ACC CTA TCC GTG ATT A) were used as controls (23). Sense and antisense oligonucleotides were designed and synthesized as described (40). Oligonucleotides were annealed in STE buffer (10 mmol/liter Tris, 1 mmol/liter EDTA, 50 mmol/liter NaCl, pH 8.0) and ligated into *Bgl*II and *Hind*III-linearized pSUPER (Oligo-Engine, Seattle, WA). The shRNA expression cassette was excised from the pSUPER-based plasmid using *Eco*RI-*Hind*III and ligated into *Eco*RI-*Hind*III linearized adenoviral shuttle vector EH006 (23). Adenoviruses were created by homologous recombination as previously described (23). Titers of tertiary viral lysates were $2.0 \times 10^8 - 1.7 \times 10^{12}$ pfu/ml. For adenoviral transduction, cells were cultured in six-well plates at 60,000 cells/cm^2 and the following day transduced with Ad-siHadhsc or Ad-siScramble at a viral dose of 100 pfu per cell for 24 h. Virus was removed, fresh medium was added, and cells were cultured for an additional 24 h. Hadhsc transcript levels were assayed by real-time PCR analysis with the following primers: (5′-CGTGCCTGGGAACATTTGTA-3′ and 5′-AAGATGG-GCCAAGAACCAAG-3′).

Glucose-Stimulated Insulin Secretion in Insulinoma Cells

Insulin secretion was assayed in HEPES balanced salt solution (HBSS) (114 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.2 mmol/liter KH_2PO_4, 1.16 mmol/liter $MgSO_4$, 20 mmol/liter HEPES, 2.5 mmol/liter $CaCl_2$, 25.5 mmol/liter $NaHCO_3$, and 0.2% BSA, pH 7.2). Cells were washed in 1 ml HBSS with 3 mmol/liter glucose followed by a 2-hr preincubation in 2 ml of the same buffer. Insulin secretion was then measured by static incubation for a 2-h period in 1 ml HBSS containing various glucose concentrations. For studies of K_{ATP} channel-independent insulin secretion, assays were performed in the presence of 250 μM diazoxide. Insulin levels were determined by ELISA using Crystal Chem Ultra Sensitive Rat Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL).

Gene Silencing in Mouse Islets of Langerhans

Under a protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), pancreatic islets of Langerhans were isolated from 6-wk-old female CD1 mice using standard collagenase digestion followed by purification through a Ficoll gradient (41). RPMI culture medium was supplemented with penicillin, strepto-

mycin, 10% fetal bovine serum, and 8 mM glucose. Islets in aliquots of 150 per well of a 12-well plate in 800 μl medium were transduced with Ad-siHadhsc or Ad-siScramble for 24 h at a dose of 2.6×10^6 pfu per islet. After transduction, islets were cultured for 4 d before measurement of Hadhsc transcript levels as described above with the following primers (5′-AATGCCACCACCAGACAAGA and 5′-CGGTGTTTTG-ATGACCTCCA).

Glucose-Stimulated Insulin Secretion in Mouse Islets

Four days after viral treatment, glucose-stimulated insulin secretion was assessed. Insulin secretion was assayed in HBSS (114 mmol/liter NaCl, 4.7 mmol/liter KCl, 1.2 mmol/liter KH_2PO_4, 1.16 mmol/liter $MgSO_4$, 20 mmol/liter HEPES, 2.5 mmol/liter $CaCl_2$, 25.5 mmol/liter $NaHCO_3$, and 1% BSA, pH 7.4). Islets were pre-incubated for 1 h in 1 ml HBSS with 2.8 mmol/liter glucose. Insulin secretion was then measured by static incubation for a 2-h period in 0.5 ml HBSS containing various glucose concentrations. Insulin levels were determined by ELISA.

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