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The transcription factor HNF1α induces expression of angiotensin-converting enzyme 2 (ACE2) in pancreatic islets from evolutionarily conserved promoter motifs

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Abstract

Pancreatic angiotensin-converting enzyme 2 (ACE2) has previously been shown to be critical for maintaining glycemia and β-cell function. Efforts to maintain or increase ACE2 expression in pancreatic β-cells might therefore have therapeutic potential for treating diabetes. In our study, we investigated the transcriptional role of hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 1β (HNF1β) in induction of ACE2 expression in insulin-secreting cells. A deficient allele of HNF1α or HNF1β causes maturity-onset diabetes of the young (MODY) types 3 and 5, respectively, in humans. We found that ACE2 is primarily transcribed from the proximal part of the ACE2 promoter in the pancreas. In the proximal part of the human ACE2 promoter, we further identified three functional HNF1 binding sites, as they have binding affinity for HNF1α and HNF1β and are required for induction of promoter activity by HNF1β in insulinoma cells. These three sites are well-conserved among mammalian species. Both HNF1α and HNF1β induce expression of ACE2 mRNA and lead to elevated levels of ACE2 protein and ACE2 enzymatic activity in insulinoma cells. Furthermore, HNF1α dose-dependently increases ACE2 expression in primary pancreatic islet cells. We conclude that HNF1α can induce the expression of ACE2 in pancreatic islet cells via evolutionarily conserved HNF1 binding sites in the ACE2 promoter. Potential therapeutics aimed at counteracting functional HNF1α depletion in diabetes and MODY3 will thus have ACE2 induction in pancreatic islets as a likely beneficial effect.

Keywords

Renin angiotensin system; pancreatic islets; promoter; transcriptional regulation; ACE2; HNF1α

1. Introduction

The carboxypeptidase angiotensin-converting enzyme 2 (ACE2) that hydrolyzes the octapeptide angiotensin (Ang)-II to the heptapeptide Ang-(1-7) has an important glycemia-protective role. ACE2 knockout mice have impaired glucose tolerance and impaired insulin

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secretion in response to glucose [1]. Conversely, ACE2 gene therapy delivered to the pancreas improves glycemia and β-cell function in the *db/db* mouse [2], a commonly-used genetic model of obesity-induced diabetes. We have recently demonstrated that deleterious effects of Ang-II on glycemia and beta-cell function of wild-type mice in the absence of obesity can likewise be countered by pancreatic ACE2 gene therapy [3]. Remarkably, these improvements occur with small, less than 3-fold, changes in pancreatic ACE2 activity. Procedures for increasing ACE2 expression might therefore have potential therapeutic value for treatment or prevention of diabetes.

Hepatocyte nuclear factor 1α (HNF1α) and hepatocyte nuclear factor 1β (HNF1 β) are homeodomain transcription factors for which haplo-insufficiency in humans causes MODY3 and MODY5, respectively [4]. HNF1β was previously shown to induce ACE2 promoter activity and ACE2 mRNA expression in a human embryonic kidney cell line [5]. This report led us to investigate whether HNF1α and HNF1β can induce ACE2 in pancreatic β-cells. In this study, we describe the structure of the human ACE2 gene promoter and demonstrate that there are three functional, evolutionarily conserved motifs in the proximal part of the promoter, capable of binding both HNF1α and HNF1β. Both transcription factors induce expression of ACE2 mRNA, leading to elevated levels of ACE2 protein and ACE2 enzymatic activity in insulinoma cells. Finally, we show that overexpression of HNF1α dose-dependently increase ACE2 expression in primary cells from pancreatic islets.

2. Materials and methods

2.1. Cell lines

The rat insulinoma cell line 832/13 [6] was a kind gift from Dr. Christopher B. Newgard, Duke University Medical Center, Durham, NC and maintained as previously described [7]. Mouse βTC3 cells were maintained in DMEM with 4.5 g/l glucose and supplemented with 15 % horse serum, 2.5 % fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human embryonic kidney (HEK) 293T cells (ATCC® CRL-11268) were maintained in DMEM with 4.5 g/l glucose and supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.2 Tissues and islets collection

C56BL/6J mice were used with protocols approved by the Institutional Animal Care and Use Committee at the Louisiana State University Health Sciences Center. Immediately following anesthesia and euthanasia by decapitation, the whole brain, kidney, heart and lung were isolated, snap-frozen, and stored at −80°C until RNA isolation. Whole pancreas was isolated and immediately stored in RNAla® (Ambion/Life Technologies, Grand Island, NY) at −20°C to minimize RNA degradation during subsequentter RNA isolation. Islets were isolated from collagenase-treated pancreata from 25 euthanized female mice, as described [8]. Alzet® micro-osmotic pumps (model 1004, Durect Corporation, Cupertino, CA) containing vehicle (saline) or Ang-II at a flow rate of 600 ng/kg/min were implanted subcutaneously in a subset of mice 15 days before islet isolation. Each islet preparation was from a single mouse. Islets were handpicked and treated with trypsin and repeated pipetting to separate cells. Each resulting suspension of primary islet cells were split into wells of a 12-well plate in 1 ml of islet medium (RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) or, for insulin secretion, into wells of a 48-well plate in 300 μl of islet medium. Assuming an average of 100 cells per islet, the cells were infected with adenovirus at multiplicities of infection (MOI) of 4, 20, or 100 and incubated for up to 48 h. Human islets from normoglycemic individuals were purchased from the National Disease Research Interchange (NDRI), Philadelphia, PA.

2.3 Insulin secretion

After pre-incubation in KRBH buffer (115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 2% w/v BSA) with 2.8 mM glucose, primary islet cells were incubated for 1 h in KRBH with 2.8 or 25 mM glucose. The solution was recovered for determination of secreted insulin by an Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL). The cells were washed with Dulbecco's PBS and lysed in Laemmli buffer for subsequent determination of protein concentration.

2.4 Plasmids and adenovirus

A plasmid, Rc mth HNF1beta, for CMV-driven expression of myc-tagged HNF1β [9] was a kind gift from Dr. G. Ryffel, Universitätsklinikum Essen, Germany. A control plasmid, called pRcNhe, was generated by replacing the HNF1β coding sequence between HindIII and XbaI sites with the sequence CCTGAGCTAGC. An expression plasmid for human HNF1α (cat. no. SC300093) and a corresponding empty control plasmid pCVM6-XL5 were purchased from OriGene Technologies, Inc., Rockville, MD. Mouse −470/−1, −2139/−915 and human −454/−1,−1699/−887, and−-1699/−1 ACE2 promoter sequences (numbers indicate bases upstream of the start codon) were cloned into pGL3-Basic between MluI and XhoI sites. Putative HNF1 binding sites in the human ACE2 promoter were mutated by replacing bases −1195/−1184, −340/−335, − 319/−314 and −249/−244 with NotI, ApaI, SmaI, and XhoI restriction sites, respectively. Amplicons of distal and proximal ACE2 promoter region transcripts identical in sequences to bases 155-267 of sequence NM_001130513.1 and 13-119 of sequence NM_027286.4, respectively, were cloned into the pCR®II-TOPO® plasmid (Invitrogen/Life technologies). The HNF1α coding sequence from the HNF1α expression plasmid was released by EcoRI digestion and cloned into the EcoRI site of plasmid pAd5CMVmscpA which generated an entry clone for subsequent adenovirus production at the Gene Transfer Vector Core at the University of Iowa. The resulting adenovirus, Ad-HNF1α, also expresses enhanced green fluorescent protein (eGFP) from the RSV promoter. A control adenovirus Ad-GFP for CMV-driven expression of eGFP was purchased from the Gene Transfer Vector Core at the University of Iowa.

2.5 Transfection

Cells were seeded in multi-well plates or tissue culture flasks the day before transfection. Cells were transfected with Lipofectamine 2000 in medium DMEM without serum and glucose, as previously described [7, 10]. After incubation for 4 h, the transfection medium was replaced with normal maintenance medium. Cells were incubated for an additional 20 h for nuclear extraction and ChIP and for 44 h for the other assays. Experiments with luciferase reporters were done with 12-well plates, where each well was treated with 1 μg firefly luciferase reporter, 1 μg HNFβ expression plasmid or control plasmid pRcNhe, and 0.25 μg of Renilla luciferase expression plasmid phRL-TK from Promega (control for transfection efficiency). Relative light units (RLU) were calculated as the ratio of firefly and Renilla luciferase activities. Each figure summarizes results of 4 transfection experiments with each treatment given to duplicate wells. Experiments for isolation of RNA, protein, or ACE2 activity were done with cells grown in 6-well plates where each well was treated with 2 μg expression plasmid. For isolation of nuclear extracts, cells grown in 6-well plates were transfected with 2.5 μg expression plasmid per well. For ChIP, cells in a 75 cm^2 tissue culture flask were transfected with 20 μg of the HNF1β expression plasmid.

2.6 Quantitative RT-PCR

RNA from pancreas and cell lines was isolated with TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH). RNA from islet cells was isolated with RNeasy® Mini Kit (Qiagen, Valencia, CA). RNA from brain, kidney, heart and lung was isolated with Illustra

RNAspin Mini kit (GE Healthcare, Buckinghamshire. UK). RNA for quantification of ACE2 distal and proximal promoter transcripts (DPT and PPT) was treated with TURBO DNA-free (Life Technologies). DPT and PPT were measured with the Taqman RNA-to-C_T 1-Step Kit (Life Technologies) and Taqman MGB probes with 6-carboxyfluorescein at the 5′ end. All other mRNAs were measured with the Power SYBR Green RNA-to-C_T 1-Step Kit (Life Technologies) and normalized to the concentration of β-actin mRNA. Primer and probe sequences are listed in Table 1. Quantifications of mRNA from cell lines were done using relative standards that were dilution series of RNA from insulinoma cells overexpressing HNF1α or HNF1β. Quantification of mRNA from islet cells were based on values of $\Delta C_t = C_t$ for target mRNA - C_t for β-actin mRNA, where C_t is the cycle-threshold parameter for amplification curves in the real-time RT-PCR assays. The assays were conducted on an Applied Biosystems ABIPRISM 7900HT Sequence Detection System.

2.7 Protein concentration

The protein content of samples was measured with a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL).

2.8 Western blotting

Western blotting of whole cell lysates was conducted as previously described [7]. Antibodies used were anti-ACE2 (sc-20998), anti-HNF1 (sc-8986), and anti-HNF1β (sc-7411) from Santa Cruz Biotechnology, Dallas, TX, and anti-γ-tubulin (T5192) from Sigma-Aldrich, St. Louis, MO. Secondary antibodies were HRP-conjugated anti-rabbit IgG (NA934V) from GE Healthcare Life Sciences, Pittsburgh, PA and HRP-conjugated anti-goat IgG (ab6885) from Abcam, Cambridge, MA.

2.9 ACE2 activity assay

ACE2 was extracted from cells and islets by suspending them in 0.5% (v/v) Triton X-100 in ACE2 reaction buffer (1 M NaCl, 0.5 mM $ZnCl_2$, and 75 mM Tris-HCl, pH 6.5), sonicating the cell suspension on ice, and recovering the supernatant after centrifugation at 20,800 g for 5 min at 4 °C. Assays were conducted as previously described [11], with the ACE2 activity determined as the difference in the hydrolysis rates of 10 μM of the fluorogenic ACE2 substrate (7-Methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-Dinitrophenyl)-OH (Mca-APK(Dnp)) (Enzo Life Sciences, Plymouth Meeting, PA) at 37°C in ACE2 reaction buffer in the absence and presence of 10 μM of the ACE2 inhibitor DX600 (Phoenix Pharmaceuticals, Inc., Burlingame, CA).

2.10 EMSA

Nuclear extracts were generated with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) from un-transfected and transfected 832/13 cells. EMSAs were conducted as previously described [7]. Antibodies for supershifts were anti-HNF1 (Santa Cruz sc-8986), anti-HNF1β (Santa Cruz sc-22840X), and anti-GATA-4 (Santa Cruz sc-25310). Probe sequences are listed in Table 1.

2.11 ChIP

ChIP of untransfected HEK 293T cells or HEK 293T cells transfected with the HNF1β expression plasmid was carried out with the protocol previously described [7] using anti-HNF1β (Santa Cruz sc-22840X) and non-specific IgG (sc-2027). Primer sequences are listed in Table 1.

2.12 Bioinformatics

ACE2 promoter regions upstream of the coding regions from 9 mammalian species representing 8 different mammalian orders were compared. Each region was separately aligned to the human sequence using the blastn algorithm of Basic Local Alignment Search Tool on the NCBI web pages. Repetitive elements were identified with the RepeatMasker web server [12]. Transcription factor binding sites were predicted with the BKL TRANSFAC program from Biobase Gmbh, Wolfenbuettel, Germany.

2.13 Statistics

Experiments were analyzed by analysis of variance, t-tests, or paired-sample t-tests according to the experimental designs. Figures show means and standard error of means. For luciferase and ChIP data, statistics were performed on logarithm-transformed data as previously described [7] with means and standard error of means re-transformed to linear scale for figure presentation. Bonferroni adjustments were made for multiple contrasts of means.

3. Results

3.1 Humans and mice have a bipartite ACE2 gene promoter

The genomic regions upstream of the ACE2 gene were first compared between the following mammalian species: human, mouse (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), dog (*Canis lupus familiaris*), panda (*Ailuropoda melanoleuca*), horse (*Equus caballus*), cow (*Bos taurus*), opossum (*Monodelphis domestica*), and platypus (*Ornithorhynchus anatinus*). As there are at least two transcription start sites for both the human and the mouse ACE2 genes, the bases were numbered relative to the translation start site. Two regions of the human sequence, −1509/−928 and −454/−1, have homologous regions with genomic regions of all the analyzed placental mammals, whereas the more distantly related opossum and platypus only have clear homologies with the human sequence in the downstream region (Fig. 1A, 1E). The homologous sequences were therefore identified as distal and proximal promoter regions. In the human genome, the ACE2 promoter is bipartite with the two promoter regions separated by an Alu element. The mouse ACE2 promoter is likewise bipartite with the promoter regions separated by short interspersed elements, whereas they are contiguous in the cow, horse, dog and rabbit genomes. For both humans and mice, there exist ACE2 mRNAs with transcriptional initiation in the proximal promoter region and mRNAs with initiation in the distal promoter region [13, 14]. These are termed distal promoter transcripts (DPT) and proximal promoter transcripts (PPT), and they encode the same ACE2 protein. The splice sites for the DPT are conserved among humans and mice. According to the UCSC genome browser for the ENCODE project [15], the X-chromosome coordinates for exon 1a, exon 1, and the splice acceptor site in exon 1 are 160577274-160577500, 160578412-160578779, and 160578491 for the mouse genome (July 2007, NCBI37/mm9 assembly) and 15620278-15620079, 15619202-15618849, and 15619137 for the human genome (Feb. 2009, GRCh37/hg19 assembly).

Real-time qRT-PCR assays were designed to allow for direct comparison of the abundance of DPT and PPT in mouse tissues, as the same reverse primer was used for the assays to ensure equal efficiency in the RT-step (Fig. 1B), as the assays were highly specific, and as the fluorescent intensities in the PCR step were very similar (Fig. 1C). While both transcript forms can be detected in the pancreas, the PPT are the most abundant (Fig. 1D). The same pattern was seen in the brain, heart and kidney. Only in the lung were DPT more abundant than PPT.

With the TRANSFAC program, we identified a putative HNF1-binding motif (−346/−330) of the human proximal ACE2 promoter region that also seems conserved as an HNF1-binding motif in the other placental mammals. Two additional putative HNF1-binding motifs (−329/−312 and − 259/−242) seem conserved as HNF1-binding motifs in all the mammals including the marsupial and the monotreme. In the distal ACE2 promoter region, the motif at −1193/−1172 of the human sequence seems conserved as a potential HNF1-binding motif in both humans and mice (Fig. 1E).

3.2 Human pancreatic islets possess enzymatically active ACE2

If islet ACE2 plays a role in the physiologic response of human islets to Ang peptides, the islets should possess ACE2 enzymatic activity. We therefore tested islets from 5 normoglycemic human donors and observed ACE2 activity, as the hydrolysis rate of the substrate Mca-APK(Dnp) was significantly higher in the absence than in the presence of the ACE2-specific inhibitor DX600 (Fig. 2).

3.3 HNF1β responsiveness is mediated by conserved binding motifs in the proximal promoter region

HNF1β induces ACE2 promoter activity in a human embryo kidney cell line [5]. To determine the role of the two promoter regions in the responsiveness to HNF1β, we cloned proximal and distal ACE2 promoter regions from both humans and mice into luciferase reporters. Compared to the control expression plasmid, the HNF1β expression plasmid induces activity from the proximal promoter regions in HEK 293T cells and to an even larger degree in βTC3 and 832/13 insulinoma cells (Fig. 3A). The distal promoter regions gave no promoter activity above the background level from the empty pGL3-Basic luciferase reporter without inserts, indicating that the distal promoter region does not function as an independent promoter in these cell lines.

For the luciferase reporter containing the −454/−1 sequence of the human ACE2 promoter, we mutated the three putative HNF1-motifs. Each of these mutations significantly decreased the HNF1β response in 832/13 cells, while the HNF1β response of the triple mutant was not significantly different from that of empty pGL3-Basic (Fig. 3B). The potential HNF1-binding motif in the distal promoter region was mutated in the context of the −1699/−1 sequence of the human ACE2 promoter which contains both promoter regions. This mutation did not significantly alter the HNF1β response (Fig. 3C). We conclude that HNF1β responsiveness is mediated by three conserved binding motifs in the proximal promoter region. Consistent with these results, ChIP experiments showed significantly stronger binding of HNF1β to the human proximal ACE2 promoter region than to the distal ACE2 promoter region or to exon 18 of the coding region of the ACE2 gene (Fig. 3D).

3.4 HNF1α and HNF1β recognize binding motifs in the ACE2 proximal promoter region

We tested the ability of HNF1β to bind to the putative HNF1 binding motifs of the human ACE2 promoter by EMSA. As a positive control, we included the HNF1 binding motif from the rat β-fibrinogen promoter. Nuclear extracts from 832/13 cells overexpressing HNF1β gave rise to a complex (complex I in Fig. 4A) for the 3 HNF1 motifs in the proximal promoter region and the rat β-fibrinogen HNF1 motif, but not for the HNF1 motif from the distal promoter region (4^{th} motif). For HNF1 motif 2, the complex was supershifted with an antibody against HNF1β, demonstrating that it contains HNF1β. Nuclear extracts from 832/13 cells transfected with the control plasmid did not give rise to complex I. Instead, two slower-migrating complexes occurred for the 3 HNF1 motifs in the proximal promoter and the rat β-fibrinogen HNF1 motif. These complexes (complex II and III) were more pronounced when HNF1α was overexpressed in the 832/13 cells (Fig. 4B). Furthermore, the endogenous complexes were supershifted with an antibody recognizing both HNF1α and

HNF1β. We conclude that both complex II and III contain HNF1α. The putative HNF1 motif in the distal ACE2 promoter region (motif 4) turned out to bind neither HNF1α nor HNF1β. Instead, a fast migrating band (complex IV) was observed for this, as well as for the HNF1 motif 1. TRANSFAC analysis indicated that GATA binding motifs overlapped or were contained within motifs 1 and 4 (Fig. 1E). Supershifts obtained with an antibody against GATA4 (Fig. 4C) demonstrated that complex IV indeed contains a GATA family member. We conclude that conserved HNF1 binding motifs in the proximal, but not the distal, ACE2 promoter region can bind both HNF1α and HNF1β.

3.5 HNF1α and HNF1β induce ACE2 expression in insulinoma cells

We tested whether overexpression of HNF1α and HNF1β would induce expression of endogenous ACE2 in insulinoma cells. Overexpression of both HNF1α and HNF1β in 832/13 cells did indeed strongly induce expression of ACE2 mRNA (Fig. 5A), ACE2 protein (Fig. 5B), and ACE2 enzymatic activity (Fig. 5C). With mouse insulinoma βTC3 cells, overexpression of the two transcription factors increased the concentration of total ACE2 mRNA (Fig. 5A) as well as the concentration of transcripts from both the distal and proximal ACE2 promoter regions (Fig. 5D). The data further indicated that the concentrations of PPT are higher than the concentrations of DPT in the induced cells.

3.6 HNF1α induces ACE2 expression in primary cells from pancreatic islets

Gene therapy with an adenovirus for overexpression of ACE2 delivered to the pancreas was previously used in our laboratory to improve glycemia and β-cell function in animal models of diabetes [2, 3]. A similar approach with an adenovirus for overexpression of HNF1α failed to increase pancreatic ACE2 activity (data not shown), probably due to insufficient infection rate. Instead, we treated primary cells from freshly isolated pancreatic islets *ex vivo* with the adenovirus. With an MOI of 100, an infection rate of > 90 % was achieved, as assessed by observation of cells with green fluorescence.

We measured ACE2 mRNA as well as transcripts for other components of the renin angiotensin system (RAS). Except for Ang-II receptor type 1b, all RAS transcripts could be detected. However, the concentrations of renin, mas (receptor for Ang-(1-7)), and Ang-II receptor type 2 transcripts were so low relative to the assay sensitivities that they were only detected in some of the samples. As expected, treatment with Ad-HNF1α increased the concentration of HNF1α mRNA relative to treatment with the control adenovirus. It also led to a statistically significant 2.1- and 5.0-fold up-regulation of ACE2 mRNA 24 and 48 h after infection, respectively (Fig. 6A-B). The only other significantly increased RAS component was the (pro)renin receptor transcript that was up-regulated 2.3-fold 48 h after infection. Angiotensinogen mRNA was slightly down-regulated (28% after 24 h) by Ad-HNF1α. The high increase of HNF1α mRNA (> 100-fold) achieved by the adenoviral treatment at MOI 100 also led to effects that might impair β-cell function, such as strong depletion (98% after 48 h) of mRNA for the glucose transporter GLUT 2. However, short term (41 h) overexpression of HNF1α had no significant effect on insulin secretion (Fig. 6C). Infusion of Ang-II in mice for 2 weeks caused a significant 30% decrease in the expression of ACE2 mRNA in islet cells. However, the responses to HNF1α overexpression for the saline and Ang-II-infused mice (5.5- and 4.8-fold, respectively) were not significantly different (Fig. 6D), suggesting that the transactivation potential of HNF1α on ACE2 expression is unaffected by Ang-II.

As the overexpression of HNF1α at MOI 100 may have deleterious effects on β-cell function, we also treated primary islet cells with adenovirus at MOI 4 and MOI 20. Both ACE2 and GLUT2 mRNA responded dose-dependently to the abundance of HNF1α mRNA (Fig. 6E-F). Even at MOI 4 when the HNF1α mRNA concentration is only increased by 5.2-

fold, there is still a 40% increase in ACE2 mRNA and only a 27% decrease in GLUT2 mRNA.

The relative change in ACE2 mRNA concentration caused by HNF1α overexpression is smaller in primary islet cells than in the insulinoma cell lines. However, the primary islet cells have a high concentration of ACE2 mRNA in the un-induced state. This is illustrated in Fig. 7 which contrasts the average concentrations of HNF1α and mouse ACE2 mRNA in primary islet cells from the experiments presented in Fig. 6 and βTC3 cells from the experiment presented in Fig. 5A, with calculations based on the observed C_t values. Even after the strong induction of ACE2 mRNA by HNF1α in βTC3 cells, the induced level is still markedly smaller than in un-induced primary cells. This suggests that overexpression of HNF1α, even at MOI 4, in primary islets cells actually results in more ACE2 mRNA molecules being expressed than in βTC3 insulinoma cells. We conclude that HNF1α robustly induces ACE2 expression in primary islet cells.

4. Discussion

Blockade of the RAS, that counteracts the formation or signaling of Ang-II in humans, reduces the frequency of new-onset diabetes of susceptible individuals [16]. ACE2 counteracts Ang-II effects by hydrolyzing Ang-II to Ang-(1-7). Our laboratory has previously reported that the concentration of pancreatic and islet ACE2 tend to become diminished in animal models of type 2 diabetes and that restoration of pancreatic ACE2 expression improves glycemia [2, 3]. As human pancreatic islets furthermore possess enzymatically active ACE2 (Fig. 2), future diabetes therapy based on increasing the expression of ACE2 can be envisioned.

Based on homologies with other placental mammals, we describe the human ACE2 promoter as bipartite with the two parts separated by an Alu element. The transcription initiation sites indicated in Fig. 1E are those reported by Itoyama et al. [13], as they used a method for cloning of cDNA from only full-length ACE2 mRNA with a 5′ cap. The mouse ACE2 promoter is likewise bipartite with the majority of ACE2 mRNA molecules in the pancreas originating from the proximal promoter region. We identified three HNF1 binding motifs in the proximal ACE2 promoter region that are well-conserved among mammalian species (Fig. 1). While these and three other sites of the proximal ACE2 promoter region were also indicated as potential HNF1 binding motifs by Senkel et al. [5], our study extended these observations by showing that HNF1β responsiveness of the proximal promoter region is mediated by the combination of the three conserved sites (Fig. 3), and by demonstrating that all three sites have binding affinity for both HNF1α and HNF1β (Fig. 4). A fourth predicted HNF1 motif in the distal promoter turned out not to have affinity for neither HNF1α nor HNF1β but instead to be a GATA binding motif. Both HNF1α and HNF1β strongly induce ACE2 expression in insulinoma cells at the level of mRNA, protein, and enzymatic activity (Fig. 5). The transcription factors induced expression of both the distal and proximal promoter region transcripts (Fig. 5D). As the distal promoter region has no independent promoter activity and no functional HNF1 site in insulinoma cells (Fig. 3), the two promoter regions seem to form a single functional promoter unit.

HNF1β is required for embryonic development of the pancreas [17], but barely detectable in islets after birth [18]. In contrast, HNF1α is well expressed in β-cells [19]. However, responsiveness of target genes to HNF1α may diminish in diabetes due to reduced levels and nuclear exclusion of HNF1α [20]. ACE2 is likewise expressed in the pancreatic β-cells [3, 21]. We demonstrate that overexpression of HNF1α in primary islet cells dose-dependently increase the expression of ACE2 mRNA (Fig 6). While we didn't explore the response of ACE2 to a depletion of HNF1α in our study, ACE2 mRNA was previously

found to be one of the targets down-regulated in islets of HNF1α knock-out mice [22]. It can thus be concluded that ACE2 is a transcriptional target of HNF1α in pancreatic islets.

The fold increase in ACE2 mRNA in response to HNF1α is smaller in primary islet cells (up to 5-fold) than the approximate 100-fold increase observed in the two insulinoma cells lines. However, as the un-induced ACE2 mRNA concentration in the primary islet cells is much higher than in the mouse insulinoma cells, the absolute increase in ACE2 mRNA is larger in the primary cells (Fig. 7). HNF1α overexpression in βTC3 cells is not sufficient to elevate the concentration of ACE2 mRNA to the level existing in un-induced primary cells. This suggests that the high level of ACE2 expression in islets is not solely determined by HNF1α. The content of ACE2 in murine islets originates mainly from the pancreatic β-cells [3, 21]. The well-conserved motifs of the ACE2 promoter outside the HNF1 binding sites (Fig. 1) might therefore very well be binding sites for other transcription factors in β-cells contributing to ACE2 expression. The UCSC genome browser for the ENCODE project [15] indicates that several transcription factors bind to the human distal ACE2 promoter region, including FOXA1 and C/EBPβ in hepatoma HepG2 cells. This indicates that the conserved FOXA and C/EBP motifs indicated in Fig. 1E are functional transcription factor binding sites.

We previously demonstrated that an increase in circulating Ang-II decreases ACE2 expression in the pancreas and the pancreatic islets [3]. Our current study shows that the diminished expression is partly mediated by a decrease in the concentration of ACE2 mRNA in islet cells. It is remarkable that this decrease persists after the cells have been cultured ex vivo for 48 h, indicating that Ang-II has long-term effects on ACE2 expression. Importantly, induction of ACE2 expression by HNF1α completely counters the prior Ang-II-mediated decrease (Fig. 6D). Interestingly, Ang-II was demonstrated to increase ACE2 expression in human cardio-fibroblasts through an Ikaros-like binding site in the −536/−501 (according to our numbering) region of the human ACE2 promoter [23]. However, the −536/−501 region has no apparent homologous counterpart in the mouse ACE2 promoter, so no Ang-II stimulation via this mechanism can be expected in mouse models.

The therapeutic potential of increasing ACE2 via HNF1α should be weighed against effects on other RAS components and against deleterious effects of HNF1α overexpression. The diminished angiotensinogen expression might mean less locally produced RAS substrate, while the increased (pro)renin receptor expression might lead to a more efficient conversion of angiotensinogen to Ang-I by renin. However, it is currently unknown, if the magnitude of these changes in expression of angiotensinogen and (pro)renin receptor is sufficient to alter the local concentration of Ang-II or to affect islet function. A defective HNF1α allele causes diabetes of the MODY3 type in humans. However, excessive overexpression of HNF1α is also deleterious with diminished expression of GLUT2, reduced β-cell mass, impairment of β-cell function, and resulting diabetes in mice [19]. Even though we didn't observe a significant acute effect on insulin secretion (Fig. 6C), the more than 100-fold increase in HNF1α mRNA obtained with an MOI 100 of the HNF1α-encoding adenovirus could possibly have adverse effects, e.g. through the depletion of GLUT2. Considering that small changes in pancreatic ACE2 activity affect whole-animal glucose homeostasis [3], a modest increase in HNF1α expression such as obtained with an MOI of 4 may confer a beneficial balance between increased ACE2 expression and only slightly decreased GLUT2 expression.

From our work, we conclude that HNF1α induces expression of ACE2 in pancreatic islets through evolutionarily conserved motifs in the proximal promoter region. Potential therapeutics aimed at counteracting the diminished concentration of functional HNF1α in patients with type 2 diabetes and MODY3 will thus have ACE2 induction in pancreatic

islets as a likely beneficial effect. In pancreatic islets for transplantation, gene therapeutic approaches to elevate the HNF1α concentration without reaching deleterious levels might likewise result in islets with increased ACE2 expression and hence increased resistance to glycemia-disruptive effects of Ang-II. Additional studies will be needed to explore the feasibility of such approaches.

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Abbreviations

ACE2	angiotensin-converting enzyme 2
HNF1α	hepatocyte nuclear factor 1α
HNF1β	hepatocyte nuclear factor 1β
MODY	maturity-onset diabetes of the young
Ang	angiotensin
MOI	multiplicity of infection
eGFP	enhanced green fluorescent protein
RLU	relative light units
DPT	distal promoter transcripts
PPT	proximal promoter transcripts
Mca-APK(Dnp)	7-(Methoxycoumarin-4-yl)acetyl-Ala-Pro-Lys(2,4-Dinitrophenyl)-OH
RAS	renin-angiotensin system
C_t	cycle-threshold

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HIGHLIGHTS

- Two ACE2 promoter regions are well-conserved in placental mammals
- Three evolutionarily conserved motifs of the ACE2 promoter bind HNF1α and HNF1β
- HNF1α and HNF1β induce ACE2 mRNA, protein, and activity in insulinoma cells
- HNF1α dose-dependently induces ACE2 expression in pancreatic islet cells

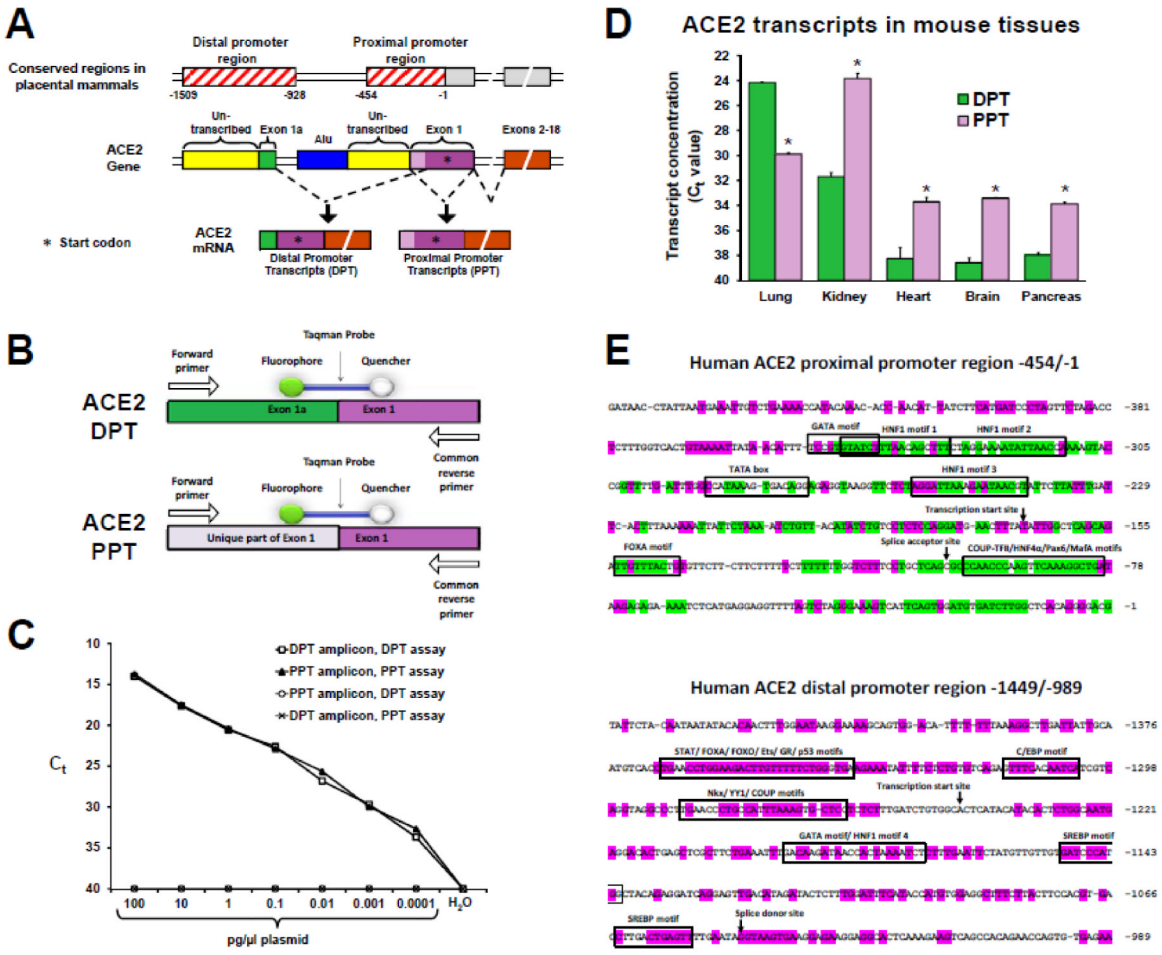


Fig. 1.

The ACE2 gene promoter is bipartite in humans and mice. (A) Diagram of the human ACE2 promoter with two conserved regions separated by a repetitive Alu element giving rise to two transcript forms encoding the same protein. (B) Design of DPT and PPT assays involves a common reverse primer, unique forward primers, and unique Taqman probes. (C) The DPT and PPT assays were run with different concentration of pCRII-TOPO plasmids containing cloned DPT and PPT amplicons as samples. A water sample was run as a no-template control. The cycle-threshold values (Ct) were recorded. (D) Comparisons of the concentrations of DPT and PPT in mouse tissues. Concentrations are shown as the C_t values obtained for RNA samples diluted to 20 ng/µl. Samples giving no amplification were assigned a Ct value of 40 which is the highest cycle number in the assays. The lung, kidney, heart, and brain were harvested from 3 female mice and the pancreas from 5 male mice. *: $p < 0.05$ vs. DPT. (E) Sequences of the conserved distal and proximal promoter regions with potential transcription factor binding motifs. Green shading indicates bases conserved among all tested mammalian species. Pink shading indicates bases conserved among the tested placental mammals. Hyphens indicate positions, where other species have additional bases.

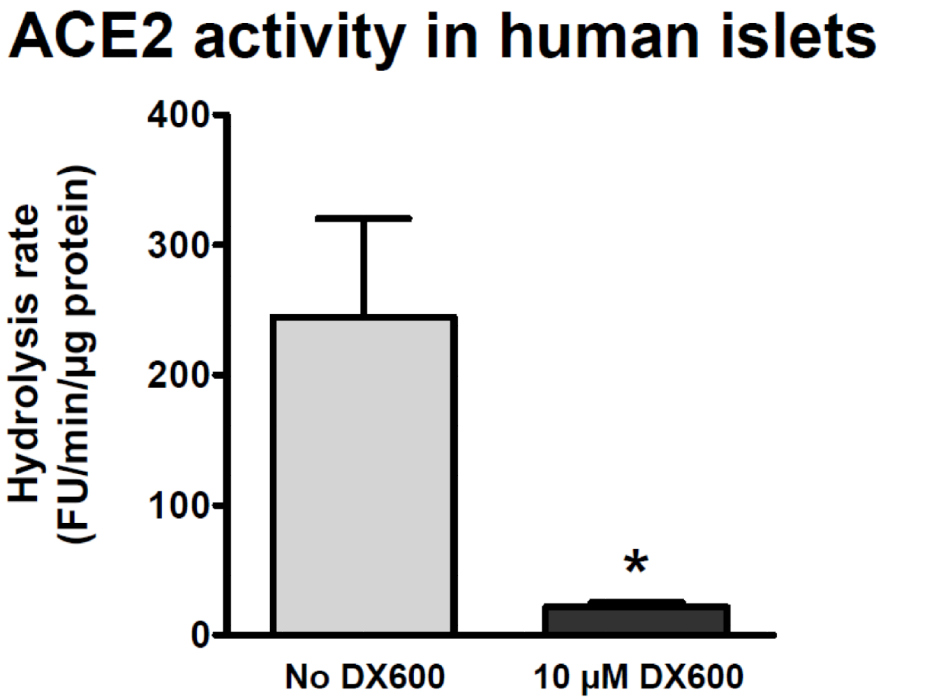


Fig. 2.

Pancreatic islets from humans possess ACE2 activity. The hydrolysis rate of the ACE2 substrate Mca-APK(Dnp) was measured for pancreatic islets from 5 normoglycemic individuals in the absence and presence of the ACE2-specific inhibitor DX600. *: $p < 0.05$ vs. no DX600.

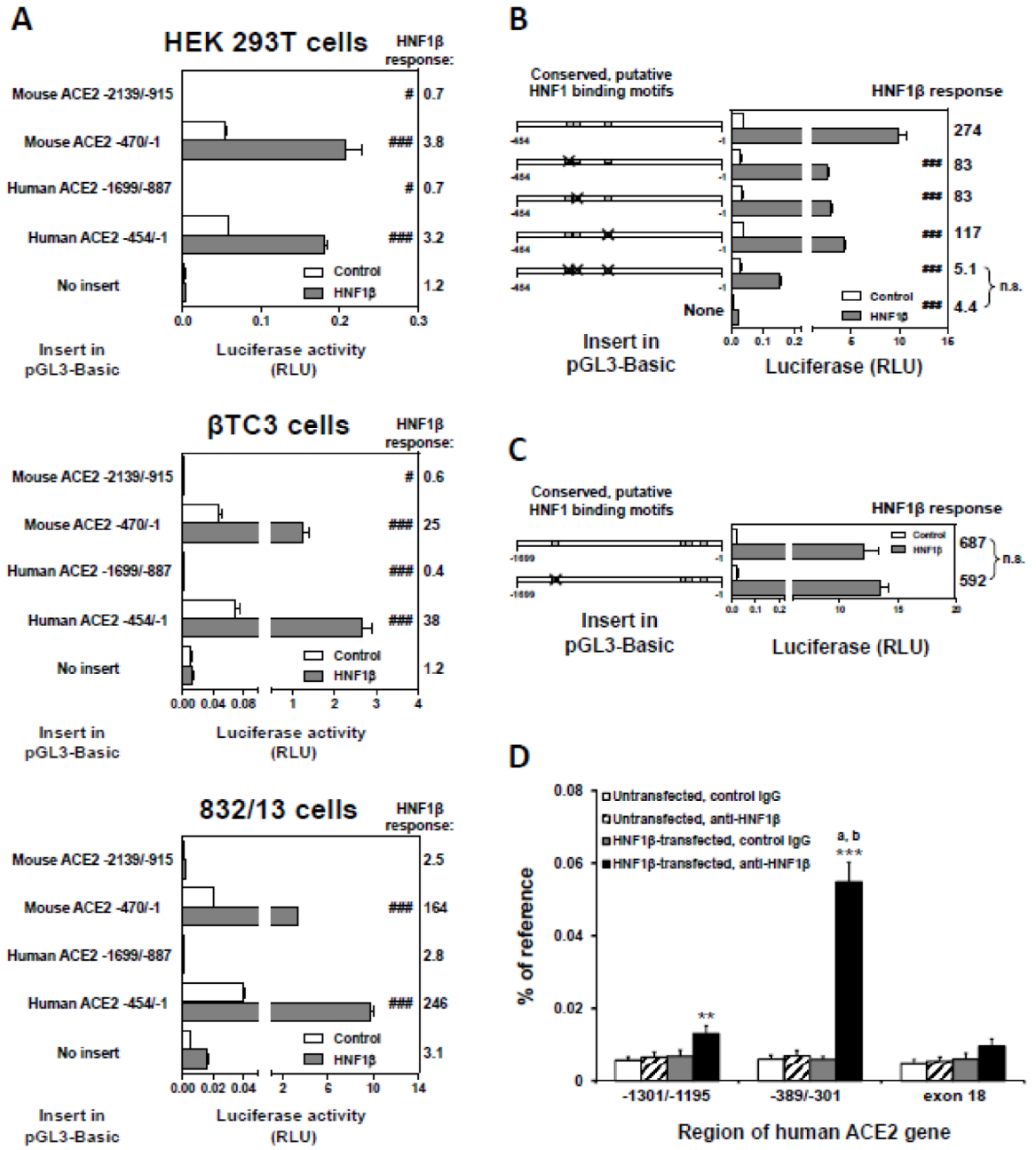


Fig. 3.

HNF1β stimulates ACE2 promoter activity. (A) HEK 293T, βTC3, and 832/13 cells were transfected with pGL3-Basic with or without distal and proximal ACE2 promoter region sequences. An HNF1β expression plasmid or the control plasmid pRcNhe was co-transfected. The HNF1β response is calculated as the luciferase activity for the HNF1β expression plasmid divided by the luciferase activity for the control plasmid. #, ###: $p < 0.05$, $p < 0.001$ vs. the HNF1β response for the pGL3-Basic plasmid without inserts. (B) The HNF1β response was determined for 832/13 cells transfected with pGL3-Basic containing the human ACE2 −454/−1 promoter sequence with or without mutations of three putative HNF1 binding sites in the proximal promoter region. ###: $p < 0.001$ vs. the HNF1β response of the unmutated promoter sequence. n.s.: No significant difference. (C) The HNF1β

response was determined for 832/13 cells transfected with pGL3-Basic containing the human ACE2 −1699/−1 promoter sequence with or without mutation of the putative HNF1 binding site in the distal promoter region. (D) Five individual ChIP experiments of untransfected HEK 293T cells and HEK 293T cells transfected with the HNF1β expression plasmid were conducted. **, ***: $p < 0.01$, $p < 0.001$ vs. transfected cells with control IgG; a: $p < 0.001$ vs. the −1301/−1195 amplicon; b: $p < 0.001$ vs. the exon 18 amplicon.

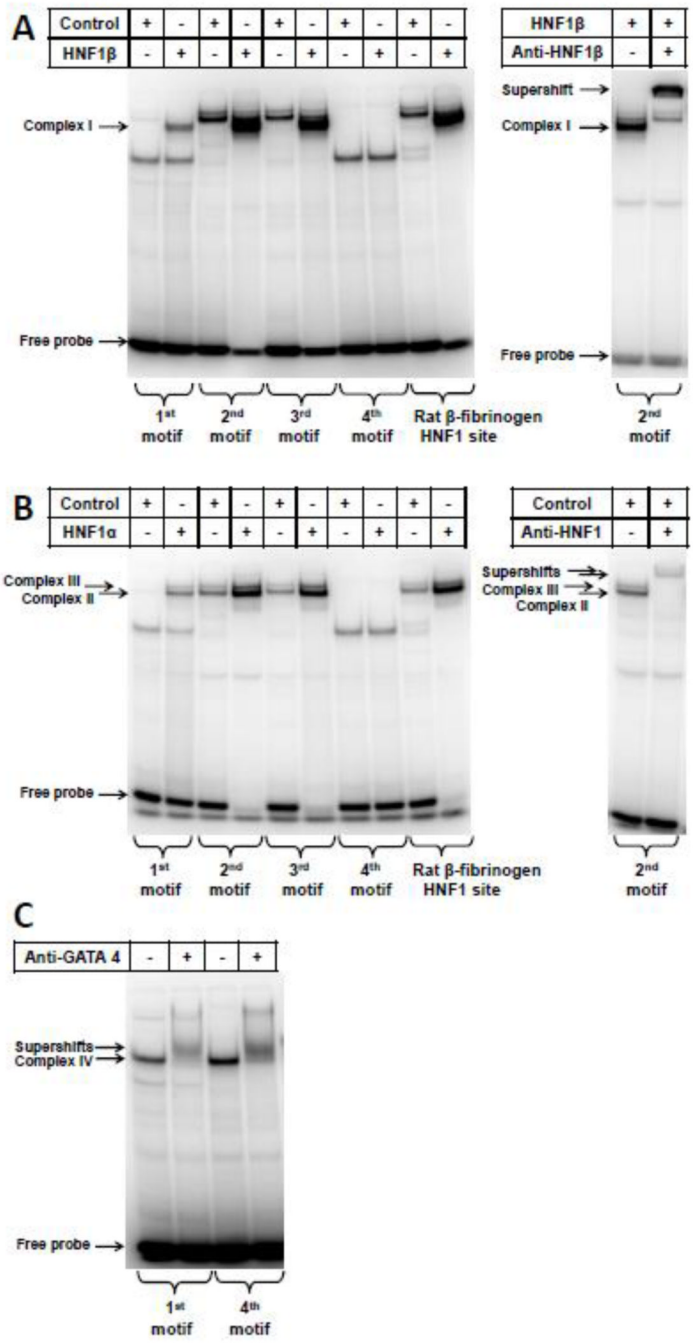


Fig. 4.

HNF1α and HNF1β bind to three motifs in the proximal ACE2 promoter region. (A) EMSA with nuclear extracts from 832/13 cells transfected with the HNF1β expression plasmid or the control plasmid pRcNhe. The probes represent the 3 potential HNF1 binding sites in the proximal promoter region (1st, 2nd, and 3rd motif), the potential HNF1binding site in the distal promoter region (4th motif), and the HNF1 binding site from the rat β-fibrinogen promoter. Complex I is a complex generated for cells transfected with the HNF1β expression plasmid. An HNF1β antibody supershifts complex I (right panel). (B) EMSA with nuclear extracts from 832/13 cells transfected with the HNF1α expression plasmid or the control plasmid pCVM6-XL5. Complex II and II are complexes whose intensities

increase for extracts from cells transfected with the HNF1α expression plasmid. The endogenous complexes II and III are supershifted with an HNF1 antibody (right panel). (C) EMSA with nuclear extracts from untransfected 832/13 cells. Complex IV is a fast-migrating complex binding to the 1st and 4th motif that can be supershifted with a GATA4 antibody.

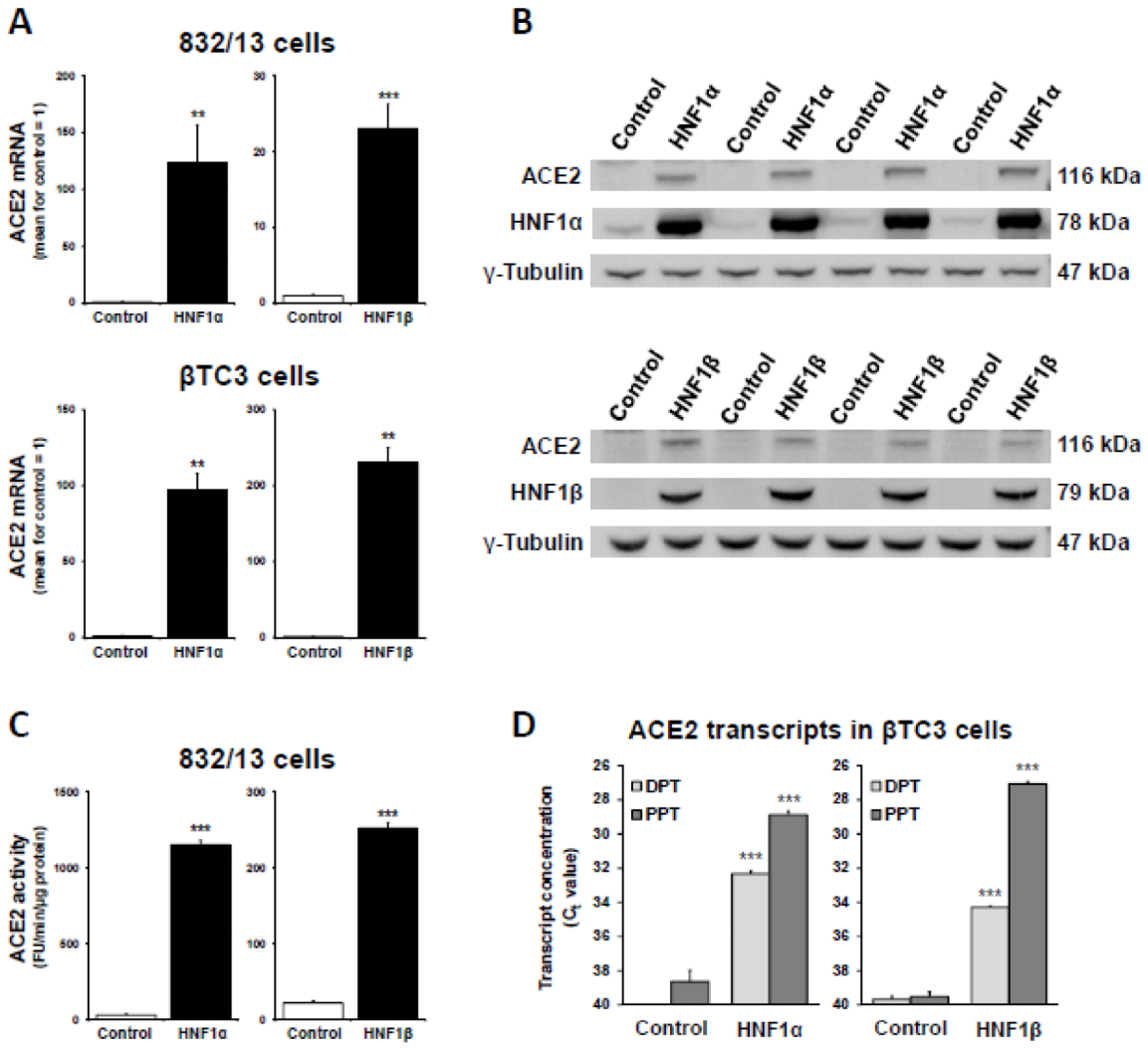
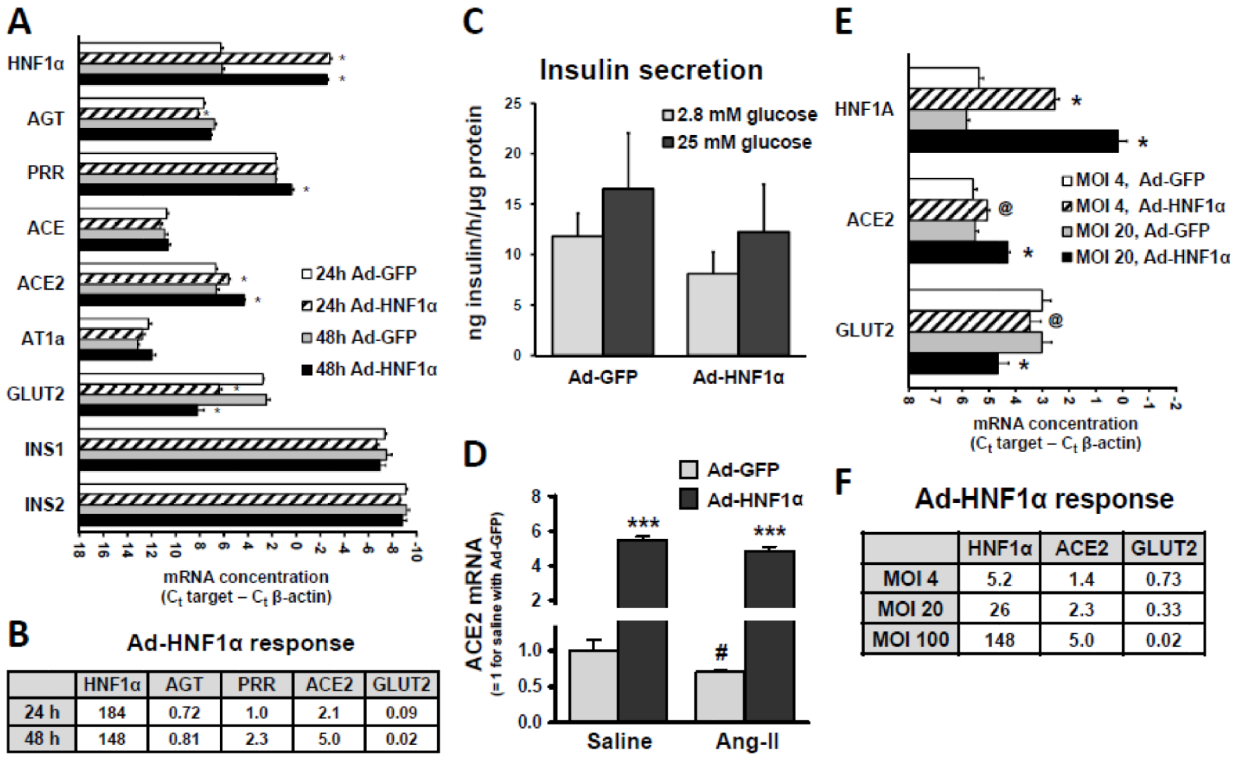


Fig. 5.

HNF1α and HNF1β induce ACE2 expression in insulinoma cells. (A) Concentrations of ACE2 mRNA normalized to β-actin mRNA were determined for 832/13 cells and βTC3 cells transfected with the HNF1α expression plasmid, the HNF1β expression plasmid, or the corresponding control plasmids. Each panel summarizes results for four independent transfection experiments. (B) Western blotting was performed for 832/13 cells transfected with the HNF1α expression plasmid, the HNF1β expression plasmid, or the corresponding control plasmids. Each blot shows expression of ACE2 and the loading control γ-tubulin in cell lysates from four independent transfection experiments with 50 μg protein loaded per lane for the upper blot and 100 μg protein loaded per lane for the lower blot. To verify expression of HNF1α and HNF1β, membranes were stripped and re-probed with an antibody recognizing both HNF1α and HNF1β (upper blot) and with an antibody against HNF1β (lower blot). (C) ACE2 enzymatic activity was measured in extracts of 832/13 cells transfected with the HNF1α expression plasmid, the HNF1β expression plasmid, or the corresponding control plasmids. Each panel summarizes results for four independent transfection experiments. (D) Concentrations of ACE2 DPT and PPT were determined for βTC3 cells transfected with the HNF1α expression plasmid, the HNF1β expression plasmid, or the corresponding control plasmids. Samples giving no amplification were assigned a C_t

value of 40 which is the highest cycle number in the assays. Each panel summarizes results for four independent transfections. **, ***: $p < 0.01$, $p < 0.001$ vs. control plasmid.



	HNF1α	AGT	PRR	ACE2	GLUT2
24 h	184	0.72	1.0	2.1	0.09
48 h	148	0.81	2.3	5.0	0.02

	HNF1α	ACE2	GLUT2
MOI 4	5.2	1.4	0.73
MOI 20	26	2.3	0.33
MOI 100	148	5.0	0.02

Fig. 6.

HNF1α induces ACE2 expression in pancreatic islet cells. (A) Concentrations of mRNA for RAS components and proteins involved in β-cell function relative to β-actin mRNA were determined for primary islet cells 24 and 48 h after being infected with the HNF1α-encoding adenovirus Ad-HNF1α or the control adenovirus Ad-GFP at MOI 100. AGT = angiotensinogen, PRR = (pro)renin receptor, ACE = angiotensin-converting enzyme, AT1a = angiotensin receptor type 1a, GLUT2 = glucose transporter 2, INS1 = insulin 1, INS2 = insulin 2. Concentrations are shown as the cycle-threshold (C_t) values for the target mRNA minus C_t for the normalizer β-actin mRNA obtained for RNA samples diluted to 0.5 ng/µl. Each data set was obtained from islet cells from 4-7 mice. *: $p < 0.05$ vs. Ad-GFP by pair-wise t-tests with p-values Bonferroni-adjusted by the number of comparisons. (B) For the mRNA targets that were significantly affected by infection with Ad-HNF1α, the Ad-HNF1α responses are calculated as the mRNA concentration for cells infected by Ad-HNF1α divided by the mRNA concentration for cells infected by Ad-GFP. (C) Insulin secretion at 2.8 and 25 mM glucose was determined for primary islet cells 41 h after being infected with Ad-HNF1α or Ad-GFP at MOI 100. Islet cells were from 3 mice, with each treatment combination of an adenovirus and a glucose concentration given to duplicate cell aliquots. (D) The concentration of ACE2 mRNA relative to β-actin mRNA was determined for primary islet cells 48h after being infected with Ad-HNF1α or Ad-GFP at MOI 100. Islet cells were from 3 mice infused with Ang II and 3 mice infused with saline. ***: $p < 0.001$ vs. Ad-GFP; #: $p < 0.05$ vs. saline. (E) Concentrations of mRNA for HNF1α, ACE2, and GLUT2 relative to β-actin mRNA were determined for primary islet cells 48 h after being infected with Ad-HNF1α or Ad-GFP at MOI 4 or 20. *: $p < 0.05$ vs. Ad-GFP by pair-wise t-tests with p-values Bonferroni-adjusted by the number of comparisons; @: $p < 0.05$ by pair-wise t-tests without adjustment for multiple comparisons. (F) Calculations of the Ad-HNF1α responses of HNF1α, ACE2, and GLUT2 for cells treated with adenovirus at MOI 4, 20, and 100.

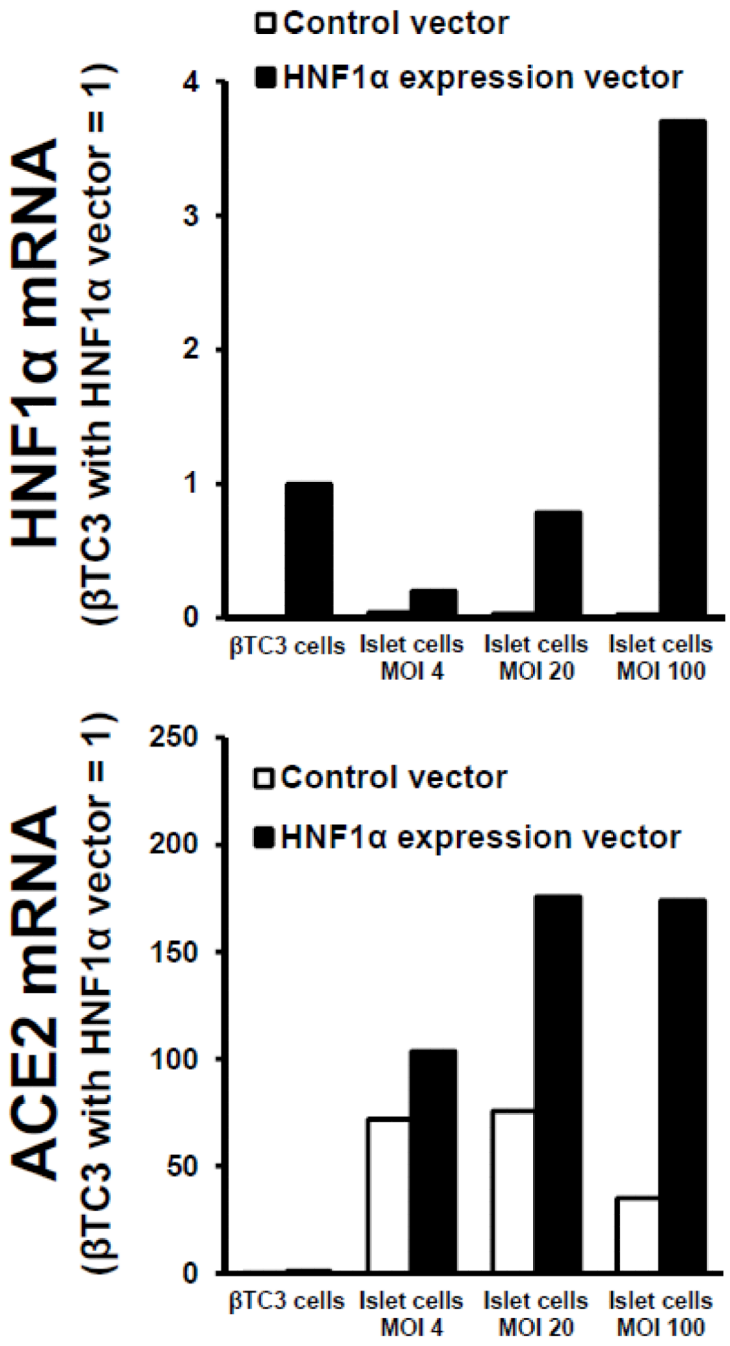


Fig. 7.

Comparison of the induction of ACE2 by HNF1α in islet cells and insulinoma cells. The mean concentrations of ACE2 mRNA and HNF1α mRNA relative to β-actin determined for islet cells infected for 48h with Ad-HNF1α or Ad-GFP and βTC3 cells transfected with the HNF1α expression plasmid or the control plasmid pCVM6-XL5 were compared. The calculations were based on the C_t values obtained in the quantitative RT-PCR experiments for Figs. 5 and 6.

Table 1
Sequences of oligonucleotides for qRT-PCR and EMSA

Sequences are listed in the 5′ ∧ 3′ direction

Primers and Taqman probe sequences for DPT and PPT of mouse ACE2			
mRNA	**Forward primer**	**Reverse primer**	**Taqman probe**
DPT	gat gcg ctt tgg att tca taa tg	agt ttt tct ctc tca tca gcc ttt g	tgt aag cag tgc cca ac
PPT	ctc ttc tgt ttc ttc ttc tgc ttt ttt	agt ttt tct ctc tca tca gcc ttt g	tca gtg ccc aac cca

Primers for SYBRgreen based qRT-PCR assays		
mRNA	**Forward primer**	**Reverse primer**
Rat ACE2	atg ccg acc aaa gca tta aag t	atg atc gga ata ggt aca ttt cgt t
Rat p-actin	aga tga ccc aga tca tgt ttg aga	cca gag gca tac agg gac aac
Mouse and human HNF1a	aaa gag ctg gag aac ctc agc	gac agg tgg gac tgg ttg a
Mouse angiotensinogen	agt ggg aga ggt tct caa tag ca	gac gtg gtc ggc tgt tcc t
Mouse renin	gtg act gtg ggt gga atc act gt	gcc agc atg aaa ggg atc ag
Mouse prorenin receptor	tct ctc cga act gca agt gct aca	cca aac ctg cca gct cca atg aat
Mouse ACE	ttt gct aca caa atg gca ctt gt	cat gcc cat agc aat tct tca tat
Mouse ACE2	gag gat aag cct aaa atc agc tct tg	tcg gaa cag gaa cat ttc gtt
Mouse AT_{1a} receptor	tca cca gat caa gtg cat ttt ga	aga gtt aag ggc cat ttt gct tt
Mouse AT_{1b} receptor	agc caa gag gcg tga aag aa	acc aca aag atg atg ctg tag aga gt
Mouse AT_2 receptor	ggg agc tcg gaa ctg aaa gc	cct tca tat tga act gca gca act
Mouse Mas	ggt tcc cac cgc tgt gtt c	gat gtc ata ttt gac tgg tcc atg a
Mouse GLUT2	gct ggt cag cta ttc atc cac at	ccg gtg atc ttg tct tct gac at
Mouse insulin 1	ttc aga cct tgg cgt tgg a	gtc gag gtg ggc ctt agt tg
Mouse insulin 2	gag cag gtg acc ttc aga cct t	ctg ggt agt ggt ggg tct agt tg
Mouse p-actin	tgt gat ggt ggg aat ggg tca gaa	tgt ggt gcc aga tct tct cca tgt

Primers for ChIP		
Site	**Forward primer**	**Reverse primer**
ACE2 -1301/-1195	cgt cag gta ggc cct tga ac	aat ttc aga agc gag ctc agt gt
ACE2 -389/-301	ttc tag acc tct ttg gtc act gta aaa tt	acc ggt act ttt ggt taa tat ttt cc
ACE2 exon 18	aat cct tat gcc tcc atc gat att	ata caa caa aat cac ctc aag agg aaa

Oligonucleotides for EMSA		
Site	**Forward oligonucleotide**	**Reverse oligonucleotide**
Rat beta-fibrinogen HNF1 site	cca aac tgt caa ata tta act aaa ggg	ccc ttt agt taa tat ttg aca gtt tgg
1st HNF1 motif	tcc gtg tat ctt taa cag ctt tct agg	cct aga aag ctg tta aag ata cac gga
2nd HNF1 motif	ctt tct agg aaa ata tta acc aaa agt	act ttt ggt taa tat ttt cct aga aag
3rd HNF1 motif	ctc tag gat taa aga ata acg tat tct	aga ata cgt tat tct tta atc cta gag
4th HNF1 motif	ttg aca aga taa cca cta aaa tct ctt	aag aga ttt tag tgg tta tct tgt caa