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Defining the transcriptional regulation of the intestinal sodium-glucose cotransporter using RNA-interference mediated gene silencing

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Abstract

Introduction—The sodium glucose co-transporter (SGLT1) is responsible for all active intestinal glucose uptake. Hepatocyte nuclear factors HNF1 α and β activate the SGLT1 promoter, while GATA-5 and CDX2 regulate transcription of other intestinal genes. We investigated SGLT1 regulation by these transcription factors using promoter studies and RNA-interference.

Methods—Chinese Hamster Ovary (CHO) cells were transiently co-transfected with an SGLT1-luciferase promoter construct and combinations of expression vectors for HNF1 α , HNF1 β , CDX2 and GATA-5. Caco-2 cells were stably transfected with knockdown vectors for either HNF1 α or HNF1 β . mRNA levels of HNF1 α , HNF1 β and SGLT1 were determined using qPCR.

Results—HNF1 α , GATA-5, and HNF1 β significantly activated the SGLT1 promoter ($p < 0.05$). Co-transfection of GATA-5 with HNF1 α had an additive effect, while HNF1 β and CDX2 antagonized HNF1 α and GATA-5. SGLT1 expression was significantly reduced in HNF1 α or HNF1 β knockdowns ($p < 0.001$). HNF1 α knockdown significantly reduced HNF1 β expression and vice versa ($p < 0.005$).

Conclusions—HNF1 α and HNF1 β are important transcription factors for endogenous SGLT1 expression by cultured enterocytes. GATA-5 and CDX2 also regulate SGLT1 promoter activity and show cooperativity with the HNF1s. We therefore propose a multifactorial model for SGLT1 regulation, with interactions between HNF1, GATA-5 and CDX2 modulating intestinal glucose absorption.

Keywords

HNF1; GATA-5; CDX2; Intestinal absorption; glucose uptake

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Introduction

The intestinal sodium glucose co-transporter (SGLT1) is located on the brush border of enterocytes and plays a key role in glucose metabolism. SGLT1 is responsible for all active glucose uptake from the intestine, thereby regulating portal and liver glucose concentration^{1, 2}. The transport of sodium and glucose absorbs water from the intestinal lumen, the principle behind the use of oral rehydration therapy^{3, 4}. In addition, recent studies have shown an elevation of SGLT1 expression in the intestine of diabetic rodents and humans and over-expression of SGLT1 is associated with an obese phenotype⁵⁻⁷.

Despite its importance in intestinal function, factors regulating SGLT1 expression in the intestine remain poorly characterized. Cis-regulatory elements for hepatocyte nuclear factors 1 α and β (HNF1 α and HNF1 β) are present in the SGLT1 promoter, and are functional in transient promoter assays^{8, 9}. The ratio of α and β dimerization is particularly important in modulating the transcription of SGLT1 - homodimers of HNF1 α are associated with increased SGLT1 transcription, while heterodimers of HNF1 α / β decrease transcription¹⁰. HNF1 α interacts with caudal-type homeobox protein 2 (CDX2) and GATA-binding protein (GATA) family members to regulate the expression of other intestinal genes, such as sucrase isomaltase and lactase-phloridzin hydrolase^{11, 12}. Cis-binding elements for CDX2 and GATA are present in the SGLT1 promoter, suggesting that these may also co-regulate SGLT1 expression in the intestine.

We hypothesized that HNF1 is a transcription factor for SGLT1, and aimed to confirm this by knockdown of HNF1 α and β in an in vitro enterocyte model. We further hypothesized that CDX2 and GATA-binding proteins co-regulate SGLT1 transcription, and tested this using promoter assays involving co-transfection of combinations of HNF1 α , HNF1 β , CDX2 and GATA-5. We show that HNF1 is an important transcription factor for endogenous SGLT1 expression in CaCo-2 cells. Moreover, co-regulation of SGLT1 promoter activity with CDX2 and GATA-5 suggests a complex, multifactorial control of SGLT1 transcription in the intestine.

Methods

Knockdown vectors and sequences

Knockdown vectors were purchased containing short-hairpin RNA sequences against either HNF1 α or HNF1 β within a lentiviral plasmid vector (pLKO.1-puro, Sigma, St Louis, MO). All shRNA sequences were BLAST searched (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) against all human sequences deposited in the GenBank and RefSeq databases to exclude homology of the HNF1 α shRNA to the HNF1 β gene and vice versa. A scrambled oligonucleotide sequence was used as a negative control (Sigma, St Louis, MO).

Reporter and expression vectors

All reporter and expression vector backbones were purchased from Promega (Madison, WI) and Invitrogen (Carlsbad, CA) respectively. The firefly luciferase-containing reporter vector for the SGLT1 promoter was created by ligation of the -997/-1 fragment of the rat SGLT1 promoter into the pGL3 basic vector backbone. Expression vectors for HNF1 α and HNF1 β were created by ligation of their respective sequences into the pcDNA3.1(+) backbone. CDX2 and GATA-5 expression vectors were prepared in pcDNA3.1 and pRC-CMV respectively^{11, 12}. The pRL-TK reporter vector, with Renilla luciferase fused to the thymidine kinase promoter, was co-transfected into all wells to control for transfection efficiency.

Cell culture and transfections for reporter assays

Chinese Hamster Ovary cells (CHO) purchased from ATCC (Manassas, VA) were maintained in F12K medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). CHO cells have no described endogenous expression of SGLT1, HNF1 α , HNF1 β , GATA-5 or CDX2. Cells between passages 3 and 4 were transfected at 80% confluence with combinations of reporter (SGLT1, pGL3 Basic empty vector) and expression vectors (HNF1 α , HNF1 β , CDX2, GATA-5, empty pcDNA3.1(+) vector) using Effectene (Qiagen, Valencia, CA) according to manufacturer's instructions (n=3 per transfection combination). Luciferase luminescence was quantified at 48 hours post-transfection using the Dual Luciferase Reporter Assay System (Promega, Madison, WI).

Cell culture and transfections for stable knockdowns

Caco-2 cells, which differentiate into small bowel epithelium and express SGLT1 on confluence, were purchased from ATCC (Manassas, VA) and maintained in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA). Cells between passages 10 and 12 were transfected via electroporation (BioRad Gene Pulser, Biorad, Hercules, CA) in Cytomix buffer containing 240mM KCl, 0.3mM CaCl₂, 20mM K₂HPO₄·3H₂O and KH₂PO₄, 50mM HEPES, 4mM EGTA and 10mM MgCl₂, supplemented with 50mM ATP and fresh glutathione (3.08mg/ml). Transfections were carried out at 960uF and 300V. At 48 hours post-transfection antibiotic selection was commenced using 12 μ g/ml puromycin (Invivogen, San Diego, CA).

RNA extraction, reverse transcription and real-time PCR

RNA was extracted from Caco-2 cells at five days post-confluence using the miRVaNA kit (Ambion, Austin, TX). Reverse transcription was carried out with oligoDT priming using the Superscript III kit (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was carried out using SYBR Green master mix (Applied Biosystems, Foster City, CA) and primers for HNF1 α , HNF1 β , SGLT1 and actin (Invitrogen, Carlsbad, CA, Table 1). Primer sequences were BLAST searched (NCBI, <http://www.ncbi.nlm.nih.gov/BLAST/>) against all human sequences deposited in the GenBank and RefSeq databases. No homology of the HNF1 α primer to the HNF1 β gene was detected and vice versa.

All samples underwent reverse transcription and real-time PCR simultaneously to minimize variations in reaction efficiencies. PCR reaction mixtures contained 2ul experimental cDNA and 12.5pmol each of forward and reverse primers in a total volume of 12.5ul. Amplification was carried out for 2 minutes at 50°C, 10 minutes at 95°C, then 40 cycles of 15 seconds 95°C and 1 minute at 60°C. Fluorescence was measured at the end of each 60°C step. Expression of HNF1 α , HNF1 β and SGLT1 was expressed as a ratio to the stably expressed housekeeping gene actin. Melting curve analysis confirmed that all samples were free of non-specific products, multiple amplicons or contaminants.

Statistical analysis

Data are presented as means \pm standard error of the mean. p-values were estimated using ANOVA with post-hoc Tukey's HSD test for comparisons between multiple groups and Student's t-test for comparisons between 2 groups, with p<0.05 taken as significant. The effect of co-transfection of two expression vectors was compared to the effect of individual expression vectors by a previously published method termed "interaction response"^{11, 12}. This was determined by the mean logarithm of the ratio of the effect observed on co-transfection to the sum of effects observed on single transfection (e.g. $\log \{ (GATA-5 + HNF-1 \alpha) / [(GATA-5) + (HNF-1 \alpha)] \}$). Interaction values from -0.1 to +0.1 were defined as additive, with values >+0.1 considered synergistic and values <-0.1 considered antagonistic.

Computations were performed using a commercially available statistical package (Statistica V4.3, StatSoft, Tulsa, OK).

Results

Knockdown of HNF1 α and HNF1 β significantly decreases SGLT1 expression in vitro

Real-time PCR results confirmed that controls bearing scrambled shRNA vectors expressed 2.1-fold higher HNF1 α mRNA expression compared to HNF1 α knockdown cells ($p < 0.05$, Fig 1). Similarly, controls expressed 2.7-fold higher HNF1 β mRNA expression compared to HNF1 β knockdown cells ($p < 0.05$, Fig 1). SGLT1 mRNA expression was 2.0-fold and 2.5-fold higher in negative controls compared to HNF1 α knockdowns and HNF1 β knockdowns respectively ($p < 0.001$, Fig 1).

Interdependence of HNF1 α and HNF1 β expression

Knockdown of HNF1 α reduced expression of HNF1 β and vice versa. Controls expressed 1.9-fold greater HNF1 β mRNA expression than HNF1 α knockdown cells, and 2.5-fold greater HNF1 α mRNA expression than HNF1 β knockdown cells ($p < 0.005$, Fig 1).

GATA-5 stimulates SGLT1 promoter activity in vitro

GATA-5 and HNF1 α had stimulatory effects on the SGLT1 promoter, producing luciferase levels 2.3-times and 6.4-times that of the empty vector respectively ($p < 0.05$, Fig 2). HNF1 β produced a much smaller increase in SGLT1 promoter activity, with a 1.9-fold increase in luciferase expression compared to the empty vector ($p < 0.05$, Fig 2).

Multiple transcription factors co-regulate SGLT1 promoter activity in vitro

We also sought to determine the effects of co-transfection of multiple expression vectors transcription factors on SGLT1 promoter activity. GATA-5 had an additive effect on the SGLT1 promoter when co-transfected with HNF1 α (interaction response of 0.05, Fig 2 and 3) but an antagonistic effect with HNF1 β and CDX2 (interaction response of -0.10 and -0.23 respectively, Fig 2 and 3). CDX2 had antagonistic effects on the SGLT1 promoter on co-transfection with either HNF1 α or HNF1 β (interaction response of -0.27 and -0.80 respectively, Fig 2 and 3). We confirmed previous reports showing that HNF1 β antagonizes the stimulatory effects of HNF1 α on the SGLT1 promoter (interaction response -0.34 , Fig 2 and 3)⁹.

Discussion

We show for the first time that HNF1 α and HNF1 β are important transcription factors for the expression of endogenous SGLT1 in CaCo2 enterocytes. Moreover, we found activation of the SGLT1 promoter by GATA-5 and CDX2, which revealed a functional cooperativity between HNF1, GATA-5 and CDX2.

Previous studies have identified HNF1-mediated stimulation of the SGLT1 promoter in vitro⁸⁻¹⁰. There have been no data to date on the effect of HNF1 on SGLT1 expression in the native (endogenous) state. The significant reduction in SGLT1 expression on knockdown of HNF1 α or HNF1 β shows that HNF1 regulates enterocyte SGLT1 transcription in vitro. Homodimers of HNF1 α stimulate SGLT1 transcription, while HNF1 α / β heterodimers reduce SGLT1 transcription¹⁰. This is supported by our reporter assays which show a significant increase in SGLT1 promoter activity by HNF1 α , an effect that decreases on addition of both HNF1 α and HNF1 β .

Knockdown of either HNF1 α or HNF1 β reduced SGLT1 expression. However, knockdown of HNF1 β reduced HNF1 α expression and vice versa, making it difficult to distinguish the relative importance of HNF1 β vs. HNF1 α in vitro. Our results suggest that the transcription of each isoform is dependent on expression of the other. Choice of non-conserved regions in the HNF1s as shRNA or PCR primer targets (also confirmed by BLAST searches) makes it unlikely that cross-reactivity produced non-specific knockdown.

Cooperative regulation of brush border hydrolases sucrase isomaltase and lactase phloridzin hydrolase by HNF1, CDX2 and GATA-5 in vitro has been previously described. We demonstrate that GATA-5 and CDX2 also regulate SGLT1 promoter activity. GATA-4, 5 and 6 regulate the expression of several intestinal genes – intestinal fatty acid binding protein (IFABP), lactase phloridzin hydrolase (LPH) and sucrose isomaltase (SI)^{11, 13–15}. GATA-4 and 6 null mice exhibit early embryonic lethality^{16, 17}. Female GATA-5 null mutant mice display severe genitourinary abnormalities; however intestinal gene expression in this model has not been determined¹⁸. CDX2 is a homolog of the *Caudal* gene in *Drosophila*, and plays a key role in intestinal epithelial development and maintenance¹⁹.

The SGLT1 promoter contains several putative binding sites for each of these transcription factors; however their exact functional binding sites on the SGLT1 promoter remain to be determined. Physical interaction between HNF1 α and CDX2 or GATA-5 mediates the cooperative regulation of the LPH gene promoter. These proteins also act in concert with co-factors such as CBP to drive sucrase transcription^{11, 20, 21}. Physical interaction and co-factors may similarly mediate the regulation of SGLT1.

These findings may be relevant in modulating the change in SGLT1 expression as enterocytes mature along the crypt-villus axis. HNF1 α and HNF1 β are expressed at high levels in the crypt and at low levels at the villus tips²². CDX2 is expressed all along the crypt-villus axis²³, while GATA-5 is localized to the villus tip¹³. SGLT1 mRNA expression increases with distance from the crypt, with the highest level of expression at the villus tips where nutrient exposure is highest²⁴. We hypothesize that HNF1 α and β initiate SGLT1 transcription in the lower villus, while GATA-5 maintains SGLT1 expression in differentiated cells at the villus tip. CDX2 may negatively modulate SGLT1 expression along the length of the crypt-villus axis.

In summary our data show that HNF1 α and HNF1 β are essential transcription factors for SGLT1 expression in vitro. We also identify activation of the SGLT1 promoter by GATA-5 and CDX2, and determine functional cooperativity between HNF1, GATA-5 and CDX2 on SGLT1 promoter activity. Our findings suggest complex regulation of SGLT1 transcription by multiple transcription factors and raise the possibility that a group of intestine-specific transcription factors interact to regulate the expression of numerous transporters and enzymes expressed by differentiated enterocytes. Understanding the exact mechanisms underlying this may reveal new treatments for the modulation of SGLT1 expression in diseases such as malabsorption, diabetes and obesity.

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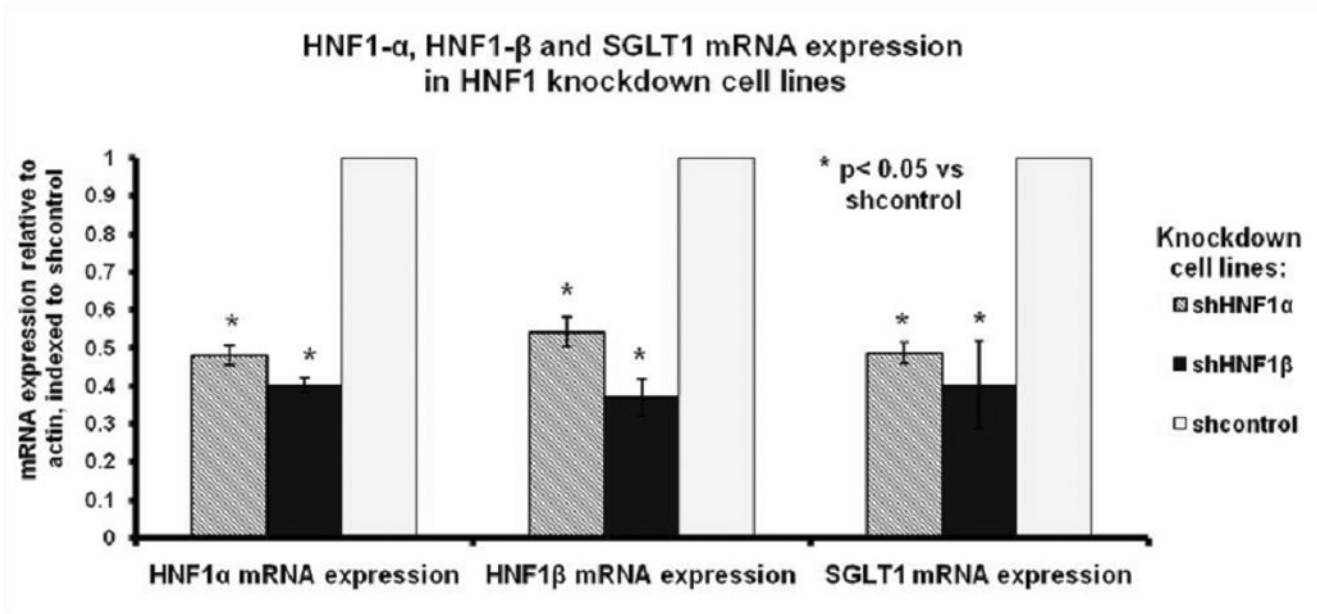
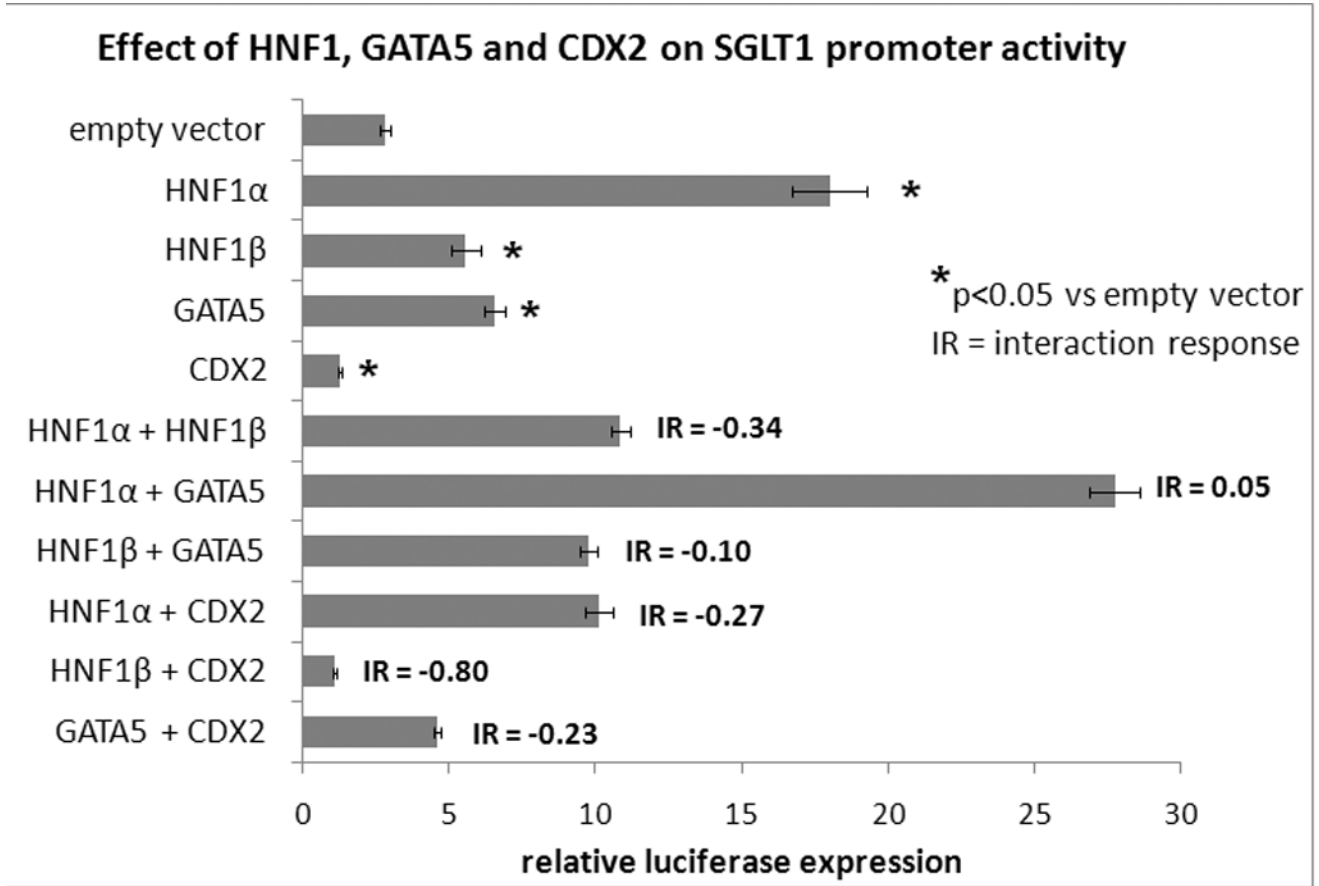


Fig 1. HNF1 α , HNF1 β and SGLT1 mRNA expression in HNF1 α , HNF1 β and negative control knockdown cell lines (labeled shHNF1 α , shHNF1 β and shcontrol respectively). mRNA was extracted from Caco-2 cells at 5- days post confluence and quantified by real-time PCR. Values are expressed as means \pm SEM, normalized to actin and indexed to the negative control (n=5 per group).

**Fig 2.**

SGLT 1 promoter activity on co-transfection of the SGLT1-luciferase promoter with individual or multiple expression vectors. Firefly luciferase expression was used as a surrogate measure of SGLT1 promoter activity and normalized to Renilla luciferase to correct for variability in transfection efficiency. Results are expressed as means \pm SEM, n=3 per group, and are representative of 2 individual experiments. Transfection of the SGLT1-luciferase promoter with the pcDNA3.1 empty vector served as the control.

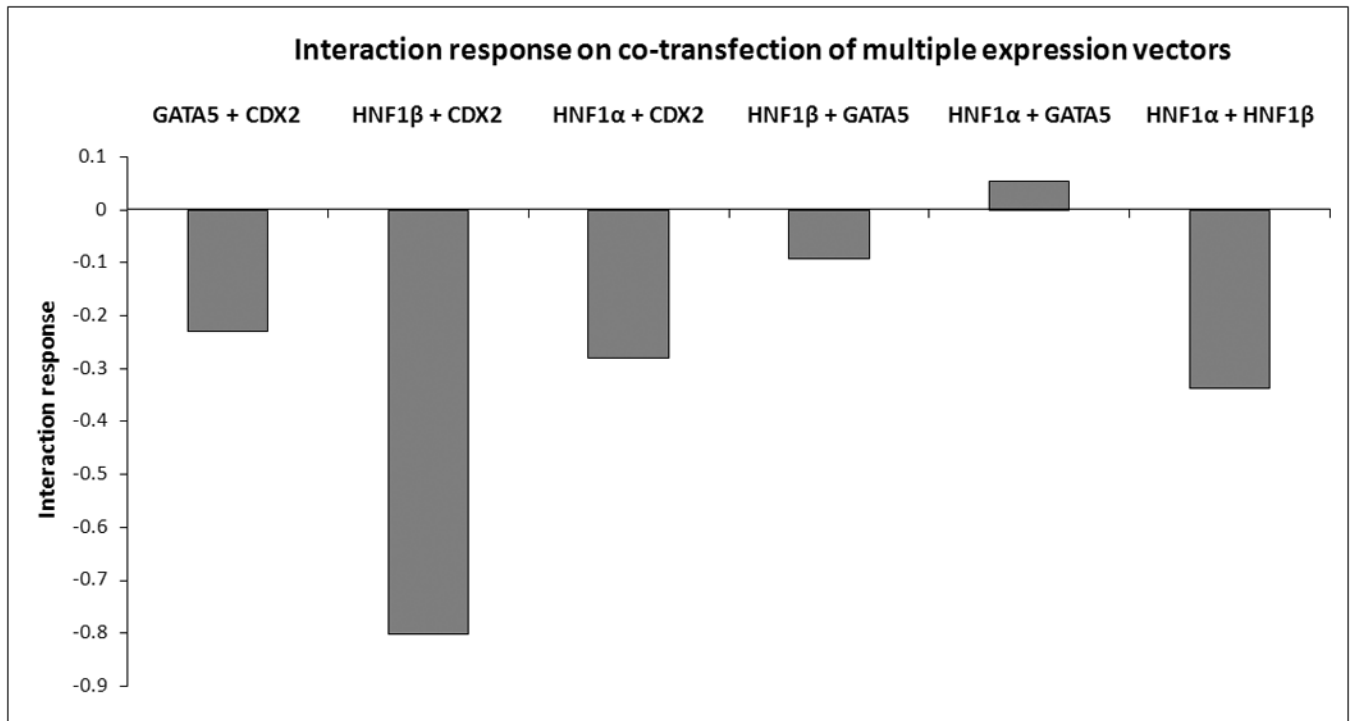


Fig 3.

An overview of the interaction response on co-transfection of multiple expression vectors. An additive interaction was noted between HNF1 α and GATA-5, while antagonistic interactions were observed in all other co-transfections. Additive interactions are defined as values between 0.1 and -0.1, and antagonistic interactions as values <-0.1 .

Table 1

Primer sequences used for real-time PCR

Primer	Sequence
HNF1 α forward	5'- ACGCCCTCTACAGCCACAAGC
HNF1 α reverse	5'- GGTGATGAGCATAGTCTGCG
HNF1 β forward	5'- CAGCGGGCGGAGGTGGACCGG
HNF1 β reverse	5'- CCCTTGATCATTTTAGCAGCC
SGLT1 forward	5'- CCTCTTCGCCATTTCTTTCATC
SGLT1 reverse	5' - ATGCACATCCGGAATGGGT
β -actin forward	5' - AGCAGGAGTATGACGAGTCCG
β -actin reverse	5' - AGTCATAGTCCGCCTAGAAGCA