Nkx6.2 synergizes with Cdx-2 in stimulating proglucagon gene expression

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Supported by the Grants from the Canadian Institute of Health Research (CIHR, MOP36398) and Canadian Diabetes Association (1198) to Tianru Jin

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Received: December 23, 2010 Revised: April 11, 2011 Accepted: April 18, 2011 Published online: May 15, 2011

Abstract
AIM: To investigate whether the transactivator of the proglucagon gene (Gcg), Cdx-2, synergizes with other transcription factors in stimulating Gcg expression and the trans-differentiation of Gcg-expressing cells.

METHODS: We conducted affinity chromatography to identify proteins that interact with Cdx-2, using GST-tagged Cdx-2 against cell lysates from pancreatic InR1-G9 and intestinal GLUTag cell lines. This was followed by a mass-spectrometry analysis. From a potential Cdx-2 interaction protein identified, we examined its expression in pancreatic and gut endocrine cells, confirmed its interaction with Cdx-2 by GST-pull down and determined its effect in provoking Gcg expression in cell lines that do not express endogenous Gcg.

RESULTS: We identified 18 potential Cdx-2 interacting proteins. One of them is Nkx6.2. This homeodomain (HD) protein is expressed in pancreatic α and intestinal endocrine L cells but not in insulin producing cell lines, including In111. Nkx6.2, but not Nkx6.1, was shown to interact with Cdx-2, detected by GST-pull down. Furthermore, Nkx6.2 was found to synergize with Cdx-2 in provoking Gcg expression when they were ectopically expressed in the In111 cell line. Finally, when Cdx-2 and Nkx6.2 were co-transfected into the undifferentiated rat intestinal IEC-6 cell line, it produced detectable amount of Gcg mRNA.

CONCLUSION: Cdx-2 recruits Nkx6.2 in exerting its effect in stimulating Gcg expression. Our observations further support the notion that multiple HD proteins, including Cdx-2 and Nkx6.2, are involved in the regulation of Gcg expression and the genesis of Gcg-producing cells.

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Key words: Cdx-2; Nkx6.2; Homeodomain; Proglucagon; Affinity chromatograph

INTRODUCTION
The proglucagon gene (Gcg) is expressed in pancreatic islet α cells, intestinal endocrine L cells and selected neuronal...
cells in the brain[10]. Gcg mRNA encodes three major peptide hormones, glucagon, glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2)[12,13]. These three peptide hormones (proglucagon derived peptides or PGDPs) exert opposite or overlapping physiological functions[14]. Glucagon produced in pancreatic islets β cells is a major counter-regulatory hormone to insulin in blood glucose homeostasis. GLP-1 and GLP-2, however, are synthesized in intestinal endocrine L cells and in selected endocrine neurons in the brain. GLP-1 stimulates insulin secretion, inhibits glucagon release and enhances peripheral insulin sensitivity[15]. GLP-2 was identified as a growth factor for small intestinal epithelia[16].

Great efforts have been made to generate insulin-producing cells *via in vitro* trans-differentiation approaches for therapeutic purposes in diabetes mellitus[17,18]. These investigations have generated very limited success while the currently available cell lines with the potential to produce insulin are derived from fetal bovine serum and oligonucleotides were purchased from Invitrogen Life Technology Inc. (Burlington, Ontario, Canada). Restriction enzymes and DNA modification enzymes were molecular biology grade and were purchased from several sources. Materials for Cdx-2-GST pull down have been described previously[19].

**MATERIALS AND METHODS**

**Materials**

Tissue culture medium, fetal bovine serum and oligonucleotides were purchased from Invitrogen Life Technology Inc. (Burlington, Ontario, Canada). Restriction enzymes and DNA modification enzymes were molecular biology grade and were purchased from several sources. Materials for Cdx-2-GST pull down have been described previously[19].

**Plasmids, RNA extraction, real time PCR**

GST-Cdx-2 and myc-tagged Cdx-2 have been generated in our previous studies[12,14]. The parental Nkx6 plasmids were kindly gifts from Dr. Johan Ericson (Karolinska Institute, Stockholm, Sweden)[15,16]. GST-Nkx6.1 and GST-Nkx6.2 were constructed by inserting the Bar HI/Eco RI fragment that contains the full length coding region of Nkx6.1 and Nkx6.2 into the PGEX4T-2 vector (Amersham Pharmacia Biotech., Uppsala, Sweden). Myc-tagged Nkx6.1 and myc-tagged Nkx6.2 were made by inserting the corresponding Bam HI/Xho I fragment that contains the full length coding region of Nkx6.1 or Nkx6.2 into the pcDNA3.1-myc-His vector [Invitrogen Life Technology Inc. (Burlington, Ontario, Canada)]. All new plasmids were verified by DNA sequencing for both strains. The oligonucleotide primers used in real time PCR (RT-PCR) and PCR are as follows. A) The cloning primers for making Myc-Tagged Nkx expression plasmids or GST fusion gene constructs. Nkx6.1 Forward: 5'-GCCGCCAACCTGATTGAGTCTGTTG GGGCGATGG-3', Nkx6.1 Reverse: 5'-GCCGCCCT TAGAGGACAGCCCTCGGCTCCGA-3'; Nkx6.2 Forward: 5'-GCCGCCAACCTGATTGAGTCTGTTG GGGCGATGG-3', Nkx6.2 Reverse: 5'-GCCGCCCT AGACAAAGCGGCTCCCGCGCGCGCCCGCCGCGCCCGC-3'. B) RT-PCR primers. Gen Forward: 5'-GCCCAGGACACATCAAG TT-3', Gen Reverse: 5'-TGACGGTGGCGAATGTTG TT-3'. The Primers allow the amplification of Gcg cDNA from rat, mouse and hamster[17]. Nkx6.2 Forward: 5'-CTTGCTACTCTCTGCTCAGCAT-3', Nkx6.2 Reverse: 5'-CGGTCTGTTATTCTCTGATGCTGCT-3', β-actin Forward: 5'-TCATGAGTTGCTAGCTGACTACA-3', β-actin Reverse: 5'-CTTAGAGCATTGCGCTTGGTG-3'.

**Cell lines and transient transfection**

The hamster pancreatic endocrine cell lines InR1-G9 and In111, the mouse pancreatic α cell line α-TC-1, the rat pancreatic β cell line Ins-1, the mouse intestinal endocrine cell lines GLUTag and STC-1, and the rat intestinal non-endocrine cell line IEC-6 have been described in our previous studies[18]. The baby hamster kidney fibroblasts (BHK) were utilized as a naïve cell system to expressed myc-tagged Nkx6.1 and Nkx6.2, also described previously[19,20]. Lipofectamine was utilized for transient transfection and the transfection efficiency in the In111 and IEC-6 cell lines was determined to be above 65% by transfecting these two cell lines with the GFP expressing plasmid, as demonstrated in our previous publication[18]. Methods for fetal rat intestinal cell cultures were described previously[21].

**GST-fusion protein-pull down assay**

The GST-fusion gene plasmids were transformed into the BL-21 strain of *Escherichia coli*. The expression of the GST fusion protein was induced by 0.3 μmol/L IPTG and purified using the glutathione resin (Amersham Pharmacia Biotech.). These proteins were then utilized in the pull-down
assay, either for the screening of Cdx-2 interaction proteins in the InR1-G9 and GLUTag cell lines, or for the verification of the interaction between Cdx-2 and Nkx6 proteins.

Western blotting
Approximately 40 μg of protein from whole cell lysates was utilized for Western blotting, with the method described in our previous studies[18]. The anti-myc tag antibody was the product of Upstate and the β-actin antibody was purchased from Santa Cruz Biotechnology.

Affinity chromatography
Bacteria lysate containing over-expressed GST or GST-Cdx-2 was passed through the glutathione column. The columns were washed extensively with PBS buffer and loaded with an equal amount of cell lysate from either GLUTag or InR1-G9 cells. The columns were again washed extensively with PBS buffer. The columns were eluted with elution buffer containing 50 mmol/L glutathione and further eluted with SDS-PAGE Laemmli sample buffer. The various eluates were run on 10% SDS-PAGE and stained with silver nitrate. After visualization, the potentially interested protein bands were excised from the gel and prepared for MALDI-Tof identification. The protein ID was searched against mouse database.

Statistical analysis
The Gcg mRNA expression was measured for calculation of mean values from three independent experiments. Statistical significance of differences among the groups was determined using a two-tailed Student’s T-test. P values less than 0.05 were considered statistically significant.

RESULTS
Nkx6.2 is a candidate Cdx-2-interacting protein, expressed in InR1-G9 and GLUTag cells
Previous studies by our group and by others have shown that Cdx-2 is expressed in pancreatic α cells and stimulates Gcg transcription[10,20-21]. We also demonstrated the synergistic effect between Cdx-2 and other HD proteins including Brn4 and members of Pdx HD protein family[12-13]. To identify additional Cdx-2 interacting proteins in the pancreatic and intestinal Gcg-expressing cells, we expressed GST and GST-Cdx-2 proteins. The fusion proteins were loaded on a glutathione column and incubated with whole cell lysates from InR1-G9 and GLUTag. After elution, the eluates were separated with SDS-PAGE, followed by silver staining (Figure 1). The bands that were shown in the lanes with both GST-Cdx-2 and the GLUTag or InR1-G9 cell lysates, but not shown in the lanes without cell lysates or with the control GST only, were excised out and prepared for MALDI-Tof analysis and potential protein identification. After several rounds of affinity chromatography, we collected 69 protein bands. Of them, 18 proteins were identified (Table 1). Among them, one protein is of particular interest to us. The bands marked with arrows (Figure 1) were show-
n to represent HD protein Nkx6.1 or Nkx6.2. Because the anticipated size of Nkx6.1 is above 30 kDa, we speculated that this candidate Cdx-2 interacting protein is Nkx6.2. Nkx6.2 is expressed in pancreatic α and intestinal endocrine cell lines but not in the pancreatic β cell lines. RT-PCR was then employed to determine Nkx6.2 expression in a battery of pancreatic and intestinal hormone producing cell lines. As shown in Figure 2A, Nkx6.2 mRNA was detected in the mouse and hamster pancreatic α cell line InR1-G9 and α-TC-1, the mouse gut Geg expressing cell lines GLUTag and STC-1, but not in Ins-1 and In111, two pancreatic β cell lines. In the gut, the intestinal endocrine cells accounts for approximately 1%-2% of intestinal epithelia. We then quantitatively compared Nkx6.2 expression in GLUTag and FRIC cultures by real time RT-PCR. As shown in Figure 2B, the expression level of Nkx6.2 in FRIC cultures was approximately 6% of GLUTag. This observation would suggest that in the gut, Nkx6.2 may be mainly expressed in the endocrine cells. This suggestion is consistent with the lack of Nkx6.2 expression in the IEC-6 cell line. Further investigations are needed to determine if it is indeed not expressed in the non-endocrine intestinal epithelia.

Table 1  Potential Cdx-2 interaction proteins in Gcg-expressing cell lines identified by affinity chromatography

| Number | Category                        | Name                        | Accession ID | Occurrence |
|--------|---------------------------------|-----------------------------|--------------|------------|
| 18     | Heterogeneous nuclear ribonucleoprotein (hnRNP) | HnRNP F | NP_004957.1 | 1          |
|        |                                  | HnRNP A1 | XP_123260.1 | 6          |
|        |                                  | HnRNP U | AAC26866.1 | 4          |
|        |                                  | HnRNP A3 | AAF83646.1 | 1          |
|        |                                  | HnRNP2/B1/B0 | AAN16352.1 | 6          |
| 4      | Zinc finger protein              | Zinc finger protein 224 | NP_71,155.1 | 2          |
|        |                                  | Zinc finger protein 2      | AAI51902.1 | 1          |
|        |                                  | Zinc finger protein 422    | AAH43067.1 | 1          |
| 4      | Signaling pathway                | Ras-GTPase-activating domain | 2780994 | 1          |
|        |                                  | Disheveled associated activator | QBBFMO | 1          |
|        |                                  | Soluble adenyl cyclase      | AA038673.1 | 1          |
| 5      | Transcription factor             | Nkx6.2 (HD protein)         | NP_795974.1 | 3          |
|        |                                  | Repressor of GATA          | AAF61244.1 | 1          |
|        |                                  | Transcription coactivator Sp110b | AAF99318.1 | 1          |
| 1      | DNA binding protein              | WD repeat and HMG-box DNA binding protein 1 | P99328 | 1          |
| 1      | House-keeping protein            | Gamma actin                | NP_033739.1 | 1          |
| 1      | Ion channel                      | Potassium channel          | AAC79846.1 | 1          |
| 1      | Splicing factor                  | PTB associated splicing factor | AAK60397.1 | 1          |
| 1      | Unnamed protein                  | Unnamed protein            | CAA43620.1 | 1          |
| 1      | Contaminated protein             | Keratin 1                  | NP_00612.2 | 1          |
| 25     | Not able to define               |                             |             | 25         |
| 7      | GST                              |                             |             | 69         |

Figure 2  Detection of Nkx6.2 expression. A: Detection of Nkx6.2 mRNA expression by RT-PCR in pancreatic and intestinal endocrine cell lines. Detection of actin expression serves as the control for the quality of cDNA generated from each sample; B: Comparison of the expression of Nkx6.2 by real time RT-PCR in GLUTag cell line and the FRIC culture (n = 4).
Verification of the interaction between Cdx-2 and Nkx6.2 by GST-pull down

We then made the GST-Nkx6.2 fusion protein. Figure 3A shows the generation of control GST (approximately 26 kDa) and GST-Nkx6.2. The main band is approximately 62 kDa. We, however, cannot avoid the generation of two minor bands which could be due to the degradation of the fusion protein. Both GST and GST-Cdx-2 were utilized in the pull down assay for cell lysates of BHK transfected with myc-tagged Nkx6.1 or Nkx6.2. As shown in Figure 3B left panel, both GST and GST-Cdx-2 did not pull down Nkx6.2 but not Nkx6.1. GST-Cdx-2 column was incubated with lysate either from pcDNA3-Nkx6.1-myc transfected BHK cells (lanes 1, 2 and 3) or from pcDNA3-Nkx6.2-myc transfected cells (lanes 4, 5 and 6). Myc tag antibody was utilized in Western blotting. B2: Right panel. GST-Nkx6.2 pulls down Cdx-2. GST-Nkx6.2 column was incubated with cell lysate from pcDNA3-Cdx-2-myc transfected BHK cells.

Cdx-2 synergizes with Nkx6.2 in provoking ectopic Gcg expression in the pancreatic β cell line In111

The In111 cell line is known to express pro-insulin mRNA but not Gcg mRNA\textsuperscript{13}. To examine whether ectopic expression of Nkx6.2 in In111 can provoke it to express Gcg, we transiently transfected this cell line with PCDNA-3 (as control) or Cdx-2 or Nkx6.1 or Nkx6.2 or Cdx-2 plus one of the Nkx6 expression plasmids. The transfection efficiency reached 65%, assessed by transfecting the same In111 cell line with an EGFP expression plasmid (data not shown), which is consistent with our previous study in InR-G9 cell line\textsuperscript{18}. Successful transfection with the Nkx6 plasmids or Cdx-2 was verified by Western blotting, using the antimyc tag antibody (Figure 4A). RNAs from In111 cell line transfected with those plasmids were extracted and utilized in real time RT-PCR for detecting Gcg expression. As shown in Figure 4B, In111 cells that received Cdx-2 or Nkx6.2 transfection increased Gcg expression approximately 2.4 and 1.9 fold respectively. Nkx6.1 transfection generated no
notable stimulation and Nkx6.1 did not enhance the stimulatory effect of Cdx-2. Nkx6.2 transfection, however, generated a robust synergistic effect with Cdx-2 (Figure 4B).

Cdx-2 and Nkx6.2 transfection provoked Gcg expression in the intestinal IEC-6 cell line

The undifferentiated intestinal cell line IEC-6 does not express Gcg. Previous studies have shown that ectopic expression of Pdx-1 in this cell provoked it to express detectable level of pro-insulin. We found when Cdx-2 and Nkx6.2 were transfected into this cell line (Figure 4C); it expressed detectable amount of Gcg mRNA by RT-PCR. Real time RT-PCR detected a 3.9 fold activation of Gcg expression (Figure 4D).

DISCUSSION

Pancreatic islet differentiation and gene expression require the functions of both the basic loop helix transcription factors such as neurogenin-3, β2/NeuroD, foxa proteins, as well as HD proteins from different families. One important phenomenon is that HD protein transcription factors, like many other so called “tissue specific” transcription factors, are never expressed as specific as their downstream target genes. In pancreatic islets, Pdx-1 was found to regulate the expression of both insulin and somatostatin genes, as well as several other β cell specific genes. The paired HD protein Pax6, however, is expressed in three major types of hormone producing endocrine cells in pancreatic islets and could be involved in regulating the expression of insulin, Gcg and somatostatin genes.

More recent studies have shown that Nkx6.1 and Nkx6.2 are critically involved in the genesis and differentiation of pancreatic α and β cells and the expression of both pro-insulin and Gcg genes. Enormous efforts have been made to generate insulin-producing “β-cells” for treating diabetes mellitus in the past few decades with very limited success. Although we are able to make pancreatic duct cells, such as AR42J cell line, the intestinal cell line IEC-6 and liver cells to express insulin, those differentiated or trans-differentiated cells cannot express sufficient amount of insulin for therapeutic purpose and their insulin secretion cannot be regulated by glucose. Within the last few years, intensive attention has been given to utilize human and mouse stem cell for differentiating islet-like structure for improving glucose disposal in diabetic animal models. These islet-like structures contain not only the insulin-producing cells but also the glucagon-producing cells, as well as cells that express other endocrine hormones. It is possible that intra-islet interactions are essential for the maintenance of functional islets in blood glucose homeostasis. Furthermore, we have learned that pancreatic α cells can be trans-differentiated into insulin producing β cell after the ablation of β cell.
We therefore suggest that to study mechanisms underlying the trans-differentiation of Gcg-expressing cells will not only deepen our knowledge of pancreatic α cell genesis and Gcg expression but also add to our understanding of the generation of islet-like structures for therapeutic purposes.

We and others have shown previously that both Cdx-2 and Brn-4 are able to bind to the G1 enhancer element of the Gcg promoter and activate its transcription. Members of the PBX HD protein family were also shown to regulate Gcg expression\cite{20, 21, 24}. Here we identified additional Cdx-2 interacting proteins, including Nkx6.2, a few heterogenous nuclear ribonucleoproteins, Zinc finger proteins, the WD repeat and HMG-box DNA binding proteins 1, as well as proteins belonging to other functional categories (Table 1). The involvement of these proteins in the function of Cdx-2 as well as the genesis of pancreatic α and intestinal L cells should be further investigated. No investigation has been conducted in determining whether Nkx6.2 is able to interact with Gcg promoter directly or if it only interacts with Cdx-2 to exert its stimulatory effect on Gcg transcription. This is mainly due to the lack of a proper Nkx6.2 antibody for conducting either electrophoretic mobility shift assay (EMSA) or chromatin immunoprecipitation (ChIP) (data not shown).

Nkx6 and Nkx2 proteins are known to be involved in the establishment of motoneuron identity in the hindbrain\cite{23, 24}. The expression and function of Nkx6 proteins in both human and rodent pancreatic islets were then clearly demonstrated by different laboratories\cite{24, 25, 26}. Nkx6.1 can serve as a developmental marker for β cells. Its role in stimulating β cell replication was also presented. Schisler et al found that Nkx6.1 repressed Gcg expression and controls glucose-induced insulin secretion in a subclass of the pancreatic β cell line Ins-1\cite{23}. Furthermore, Gauthier et al demonstrated that in the BHK naïve cell system, Nkx6.1 repressed 93% Pax6-mediated activation of the Gcg promoter, while the stimulations mediated by Cdx-2 and Maf cannot be altered by Nkx6.1 transcription\cite{24}. Consistently, we did not find the interaction between Cdx-2 and Nkx6.1 in our GST-pull down experiment.

Sander and colleagues demonstrated that in Nkx6.1 mutant mice, β cell numbers were selectively reduced while other islet cell types develop normally. We show here that Cdx-2 selectively recruited Nkx6.2, but not Nkx6.1, in regulating Gcg expression. A study by Pedersen et al showed that the expression of Nkx61 but not Nkx6.2, requires the expression of the β cell specific HD transcription factor Pdx-1\cite{27}. These observations collectively suggest that Nkx6.1 and Pdx-1 are essential HD transcription factors in β cells, while in pancreatic α cells, Nkx6.2 works with the α cell counterpart of Pdx-1, Cdx-2, to exert their biological functions.

In conclusion, by conducting GST-pull down and a number of in vitro experimentations, we have identified a number of potential Cdx-2 interaction proteins, including the HD protein Nkx6.2, which synergizes the effect of Cdx-2 in stimulating Gcg expression as well as the trans-differentiation of Gcg-expression cells.

**ACKNOWLEDGEMENTS**

We thank Dr. Johan Ericson for providing the Nkx6.1 and Nkx6.2 cDNAs.

**COMMENTS**

**Background**

Proglucagon (Gcg) expressing cells are among islet-like structures generated from stem cell differentiation in the attempt for diabetes treatment. Those cells may contribute to the maturation of islet-like structures. We learned that a number of homeodomain (HD) proteins, including Cdx-2, stimulate Gcg expression and the genesis of Gcg-expressing cells. We do not know whether Cdx-2 recruits other HD transcription factors via protein-protein interactions, in stimulating Gcg expression and the trans-differentiation of Gcg-expressing cells.

**Research frontiers**

To understand mechanisms underlying the trans-differentiation of Gcg-expression cells may help improving the protocols for the genesis of islet-like structures in the transplantation for diabetes, especially Type 1 diabetes treatment.

**Innovations and breakthroughs**

Utilizing the powerful proteomic approaches, we have identified a battery of novel Cdx-2 interaction proteins, including Nkx6.2. We show here that Nkx6.2 indeed interacts with Cdx-2 and these two HD proteins synergistically stimulate Gcg expression and the trans-differentiation of Gcg-expressing cells.

**Applications**

Knowledge generated from this study can be incorporated in the study of trans-differentiation of islet-like structures for treating Type 1 diabetes in animal models.

**Terminology**

The methods utilized in this study are useful in studying protein–protein interactions in other systems.

**Peer review**

Overall, this study is interesting, nicely done and could potentially enhance our understanding of the mechanisms underlying Gcg expression.

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S- Editor Zhang HN L- Editor Roemmele A E- Editor Zhang L