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PDX1 regulation of *FABP1* and novel target genes in human intestinal epithelial Caco-2 cells

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ABSTRACT

The transcription factor pancreatic and duodenal homeobox 1 (PDX1) plays an essential role in pancreatic development and in maintaining proper islet function via target gene regulation. Few intestinal PDX1 targets, however, have been described. We sought to define novel PDX1-regulated intestinal genes. Caco-2 human intestinal epithelial cells were engineered to overexpress PDX1 and gene expression profiles relative to control cells were assessed. Expression of 80 genes significantly increased while that of 49 genes significantly decreased more than 4-fold following *PDX1* overexpression in differentiated Caco-2 cells. Analysis of the differentially regulated genes with known functional annotations revealed genes encoding transcription factors, growth factors, kinases, digestive glycosidases, nutrient transporters, nutrient binding proteins, and structural components. The gene for fatty acid binding protein 1, liver, *FABP1*, is repressed by PDX1 in Caco-2 cells. PDX1 overexpression in Caco-2 cells also results in repression of promoter activity driven by the 0.6 kb *FABP1* promoter. PDX1 regulation of promoter activity is consistent with the decrease in *FABP1* RNA abundance resulting from PDX1 overexpression and identifies *FABP1* as a candidate PDX1 target. PDX1 repression of *FABP1*, *LCT*, and *SI* suggests a role for PDX1 in patterning anterior intestinal development.

1. Introduction

Development of the gastrointestinal tract is associated with programmed patterning of epithelial gene expression along the gut A-P axis. Global expression profiling using microarray analysis has revealed distinct gene expression patterns with sharp segmental boundaries for multiple intestine-specific genes along the length of the small intestine [1]. The mechanisms regulating spatial restriction of intestine-specific gene expression along the anterior-posterior axis during gastrointestinal development, however, are largely unknown. Transcription factors expressed in specific regions along the A-P gut axis during gastrointestinal organogenesis have been characterized as spatial regulators. An evolutionarily-conserved group of homeodomain-containing genes that regulate endodermal and mesodermal cell fates during gut ontogeny are grouped in chromosomal regions known as the *Hox* and *Parahox* clusters, reviewed by Krumlauf [2] and Beck [3]. The spatial expression domains for several Parahox transcription factors expressed in endoderm-derived organs have been mapped along the A-P gut axis [4]. The Parahox gene, *PDX1*, pancreatic duodenal homeobox 1, PDX1 (also known as IPF-1, IDX-1 and STF-1) is expressed in the developing pancreas and duodenum. PDX1 is required for pancreas development and the maintenance of functional islet ß cells [5–9]. Specifically, mice null for the *PDX1* gene, $PDX1^{-/-}$, fail to form a pancreas and die in the neonatal period within a week of birth [6,7].

In addition to the pancreas, PDX1 is maximally expressed in the most anterior duodenal region of the intestinal tract with decreased expression in the distal small intestine [10]. In neonatal $PDX1^{-/-}$ null mice, the rostral duodenum shows dilated cystic malformations at the stomach/duodenum junction felt to be abnormal Brunner's glands and areas of local ectopic GLUT2-positive cuboidal epithelium [7]. Just distal of the abnormal epithelium, the numbers of enteroendocrine cells in the villi are greatly reduced. In further support of a role for PDX1 in patterning intestinal development and cell differentiation, mice with misexpression of PDX1 targeted to the large intestine manifest an altered midgut-hindgut union [11] and immature intestinal epithelial rat IEC-6 cells can differentiate into enteroendocrine cells in response to PDX1 overexpression [12]. PDX1 is a known regulator of a number of genes essential for maintaining pancreatic cell identity and function including insulin [13], glucose transporter 2 [14], glucokinase [15], islet amyloid polypeptide [16–18] and somatostatin [8,9]. Both activator [8,9,13–26] and repressor [11,27–31] functions have been described for PDX1. Few genes expressed in the intestine, however, have been identified as intestinal targets capable of being regulated by PDX1. In order to obtain further insights into the role

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of PDX1 in regulating intestinal cell fate determination in epithelial cells, we sought to identify downstream target genes regulated by PDX1. cDNA microarray approaches were used to characterize the effects on gene expression profiles following overexpression of PDX1 in Caco-2 cells, a human adenocarcinoma-derived cell line that mimics a small intestinal enterocyte phenotype with respect to expression of several digestive hydrolases [32].

2. Materials and methods

2.1. Plasmid constructs

For use in the transfection experiments, mouse PDX1 cDNA, a gift of C. V. Wright (Vanderbilt University), was subcloned into pAlpha + (Affymax) downstream of a recombinant SV40/HTLV promoter enhancer to generate pmPDX1 as previously described [29]. The 100 bp rat lactase promoter-luciferase reporter plasmid, pgLac100, has been described previously [29]. The 0.6 kb Fabp1 promoter-reporter plasmid, pFABP1luc, was a kind gift of T. Simon, Washington University.

2.2. Transfections and luciferase assays

Caco-2 cells were stably transfected with 1.0 μg of pmPDX1 or empty vector pAlpha + control and selected for G418 resistant pooled or isolated colonies as previously described [29]. The stably transfected cells were maintained in complete DMEM plus G418 and differentiated for 9 days after reaching 100% confluency prior to harvest. For transient transfections, a DNA transfection mixture was prepared consisting of 0.4 pmol of the luciferase reporter construct, 0.05 pmol of pmPDX1 or pAlpha + empty vector, and 7.0 fmol of pRL-TK (Promega) as an internal control. The individual DNA mixtures were transfected into cells (50–80% confluent) with lipofectamine reagent (INTROGEN) according to the protocol of the manufacturer. Cells were harvested 48 h after transfection and luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer, in a Monolight 3010 luminometer. Experimental lactase promoter–reporter activities were normalized to the activity of the pRL-TK internal control and expressed as relative luciferase activity (means + SD, $n = 4$), thereby minimizing experimental variability caused by differences in cell viability or transfection efficiency. Statistical significance (P value) was determined by using Student's unpaired t-test.

2.3. Western blot assays

Caco-2 cell nuclear extracts were prepared as previously described [29]. Twenty micrograms of nuclear extract protein was separated by electrophoresis in 12% SDS polyacrylamide gels. After transfer of the protein to 0.45 micron nitrocellulose membrane, blots were processed using the Fast Western Blot Kit (Pierce) according to the manufacturer's protocol. Rabbit polyclonal antibody against PDX1 (gift of C.V. Wright, Vanderbilt University) was used at a 1:3000 dilution. Blots were subjected to enhanced chemiluminescence detection using the Supersignal West Pico substrate (Pierce) and imaged with the ImageLab software on a ChemiDoc XRS + system (BioRad).

2.4. Gene Expression Profiling

Total RNA was purified from the stably transfected Caco-2 cell populations and processed for DNA microarray analysis as previously described [33]. Briefly, total RNA was labeled with Cy5–deoxyuridine triphosphate and a human reference RNA (Stratagene) was labeled with Cy3–deoxyuridine triphosphate. Hybridizations were performed using human DNA microarrays produced at the Stanford Functional Genomics Facility containing ~41,000 cDNAs of which over 27,000 features represent unique human genes. The microarrays were scanned and the data was deposited in the Stanford Microarray Database (http://genome-www5.stanford.edu). Three preparations of pooled PDX1 transfected cells and two preparations of pooled cells transfected with the pAlpha + vector alone were analyzed. The SAM, Significant Analysis of Microarrays, software program was used to identify genes that are significantly differentially expressed [34]. The software permits the application of a statistical analysis for significance as a means to reduce the number of false positive candidates. Additional analysis of the genes that were significantly elevated >4-fold or significantly decreased >4-fold was performed using software for gene annotation and for the identification of biological processes using DAVID, Database for Annotation, Visualization and Integrated Discovery (http://apps1.niaid.nih.gov/david) [35].

2.5. Real-time quantitative RT-PCR

For quantitative real-time RT-PCR, cDNA was synthesized from total RNA using the High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Expression levels for each specific gene were determined by TaqMan gene expression assays (Applied Biosystems) with human gene-specific, predesigned TaqMan primers and probe sets. PCR amplification and fluorescence data collection were performed with the ABI Prism 7900 HT sequence detection system (Applied Biosystems). Each gene was assayed in quadruplicate. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used to normalize for total RNA amount among samples. Normalized Ct values for each gene (ΔCt_{gene}) were calculated as raw Ct values for the gene (Ct_{gene}) minus corresponding raw Ct values for *Gapdh* (Ct_{Gapdh}): $\Delta Ct_{gene} = Ct_{gene} - Ct_{Gapdh}$. A relative quantification approach was used in this study to calculate relative mRNA abundance for each gene: first, a reference ΔCt value ($\Delta Ct_{reference}$) was calculated by averaging the ΔCt_{gene} values of individual RNA samples from pAlpha + control population. The differences ($\Delta\Delta Ct_{gene}$) of the ΔCt_{gene} values relative to the $\Delta Ct_{reference}$ value were calculated as $\Delta\Delta Ct_{gene} = \Delta Ct_{gene} - \Delta Ct_{reference}$. Relative mRNA abundance values were then calculated using the formula $2^{-\Delta\Delta Ct}{}_{gene}$ [36].

3. Results

3.1. Caco-2 cells engineered to overexpress PDX1

Few gut-specific target genes capable of being regulated by PDX1 have been identified. In order to characterize global gene expression patterns regulated by PDX1 and to identify novel PDX1 intestinal target genes, Caco-2 human intestinal epithelial cells were engineered to overexpress PDX1 for use in characterization of gene expression analyses. Specifically, Caco-2 cells were stably transfected with the PDX1 expression construct, pmPDX1, or the empty expression vector to generate pooled cell populations. PDX1 overexpression was confirmed by Western blot (Fig. 1). Anti-PDX1 antibody detects increased abundance of the roughly 45-kilodalton PDX1 protein in Caco-2 cells stably transfected with pmPDX1 compared to cells transfected with the empty vector.

3.2. Gene expression profiles for Caco-2 cells overexpressing PDX1

To identify novel PDX1 intestinal target genes, gene expression profiles for the Caco-2 cells engineered to overexpress PDX1 were assessed using cDNA microarrays. Total RNA was purified from the

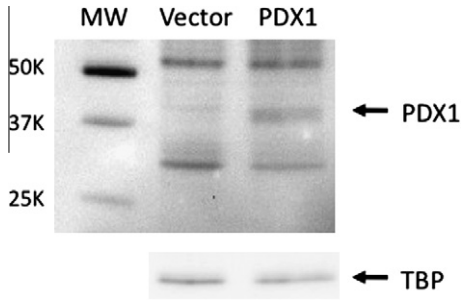

Fig. 1. Western blot analysis of PDX1 protein expression in stably transfected Caco-2 cells. A rabbit polyclonal anti-PDX1 antibody detects the roughly 40-kilodalton PDX1 protein in Caco-2 cells stably transfected with pmPDX1 (PDX1) but not in cells transfected with the empty vector pAlpha + (vector). The membrane was stripped and probed with antibody against TATA binding protein (TBP) to verify loading and transfer.

stably transfected cell populations (PDX1 or empty vector) described above and was processed for DNA microarray analysis. The SAM, Significant Analysis of Microarrays, software program was used to identify genes that are significantly differentially expressed. 806 genes were found to be significantly differentially expressed at least twofold in the PDX1 expressing cells with a median number of falsely significant genes of 1. A total of 129 genes were found to be significantly elevated or depressed at least 4-fold. The results are graphically displayed using pseudocoloring in Supplementary data 1.

Additional analysis of the 80 genes that were significantly elevated >4-fold and the 49 genes that were significantly decreased >4-fold was performed using software for gene annotation and for the identification of biological processes that are enhanced with PDX1 expression. Annotation of the 129 genes using DAVID, Database for Annotation, Visualization and Integrated Discovery and limited to classifications with 3 or more hits revealed genes that include transcription factors, growth factors, kinases, digestive glycosidases, nutrient transporters, nutrient binding proteins, and structural components.

To validate our initial microarray analysis, we assayed by real-time RT-PCR 13 genes with a >4-fold change in microarray expression levels in pooled PDX1 transfected cells vs. empty vector transfected cells. PDX1 regulation was confirmed by RT-PCR in all 13 genes. In order to investigate variability between different PDX1 overexpression cell populations, two additional distinct clonal PDX1 stably transfected populations were characterized. Additional gene expression analysis was performed for 18 genes (13 genes validated above plus PDX1, ESR1, MAPK8, LCT and SI) in order to compare expression profiles in the three different preparations (pooled and two clonal) of PDX1 expressing cells. Of the 18 genes regulated in the PDX1 pooled population, 11 were similarly regulated in both clonal populations, 6 were similarly regulated in one clone with less than a 2-fold change in the other, and 1 had less than a 2-fold change in both clones (Fig. 2). With respect to regulation of the previously reported intestine-specific target genes sucrase–isomaltase (*SI*) and lactase-phlorizin hydrolase (*LCT*) quantitative RT-PCR confirmed PDX1-mediated repression of both gene transcripts in the three different populations. While it is presumed that several of the differentially expressed genes identified are candidate targets (direct or indirect) of PDX1, it is possible that differences in gene expression may result from effects independent of PDX1 overexpression. Varying proliferation rates for the cells stably transfected with PDX1 or the control empty vector might affect cell differentiation stage and thus gene expression profiles. In order to determine whether a specific gene could be regulated by PDX1, we therefore proceeded to investigate promoter regulation of a candidate target gene in cells transiently transfected with PDX1.

3.3. PDX1 transcriptional regulation of the FABP1 target gene promoter

Fatty acid binding protein 1, liver, *FABP1*, a candidate gene with known intestinal cell expression, was identified by the microarray screen described above. *FABP1* mRNA was decreased ~4-fold in pooled PDX1 expressing Caco-2 cells. *In silico* analysis of the proximal promoter for the *FABP1* gene using the MatInspector

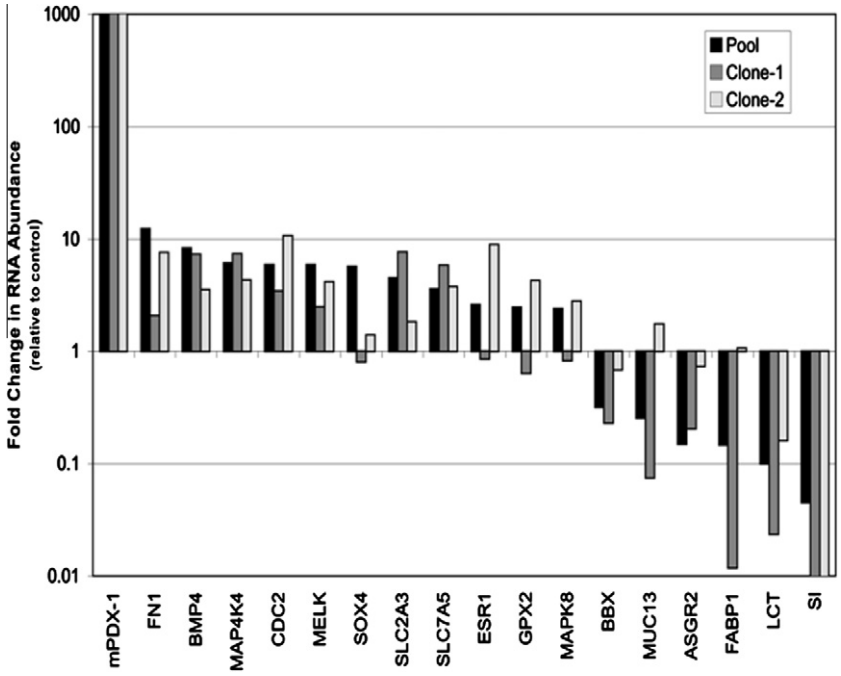

Fig. 2. PDX1 regulation of gene expression in stable transfected Caco-2 cells. Total RNA was isolated from cell populations stably transfected with PDX1 expression construct or empty vector. Transcript abundance detected by real time RT-PCR for various genes is plotted as fold change relative to the empty vector control population. Means +/− SD. $n = 4$ technical replicates (error bars not detectable at figure resolution).

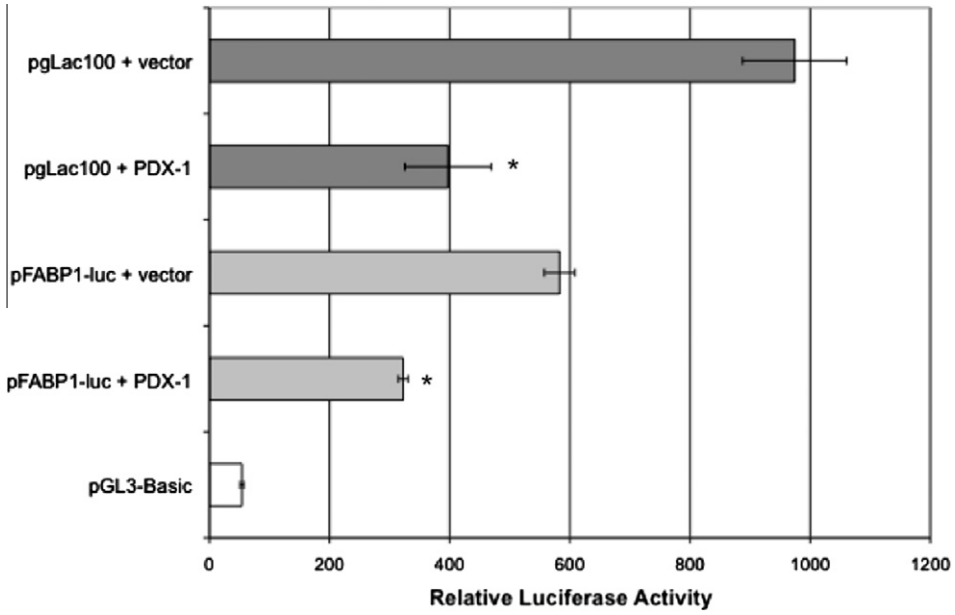

Fig. 3. PDX1 regulation of *FABP1* promoter activity. Caco-2 cells were co-transfected with fatty acid binding protein 1 or lactase gene promoter-luciferase constructs (pFABP1-luc, pgLac100, pGL3Basic respectively) along with the pmPDX1 expression construct (PDX1) or pAlpha + (empty vector). Transfection efficiencies were normalized to renilla luciferase expression of a cotransfected pRL-TK vector and expressed as relative luciferase activity (means +/− SD, $n = 3$). *Significant decrease compared with empty vector ($P < 0.05$).

(Genomatix, Inc.) transcription factor binding site software, reveals multiple PDX1 consensus binding sites located 5′ to the transcription start-sites (not shown). To determine whether the *FABP1* promoter is capable of being regulated by PDX1, Caco-2 cells were co-transfected with a 0.6-kb *FABP1* promoter-luciferase construct (pFABP1-luc), or a lactase promoter-luciferase reporter construct (control) and the PDX1 expression construct, pmPDX1, or empty vector. Caco-2 cell extracts were assayed for relative luciferase activity 48 h after transfection as shown in Fig. 3. PDX1 overexpression results in repression of promoter activity driven by the 0.6 kb *FABP1* promoter relative to the empty vector control. PDX1 regulation of promoter activity is consistent with the decrease in *FABP1* RNA abundance resulting from PDX1 overexpression and identifies *FABP1* as a candidate PDX1 target.

4. Discussion

While PDX1 is a known regulator of a number of genes essential for maintaining pancreatic cell identity and function, few genes expressed in the intestine, including adenosine deaminase (*ADA*) [19], sucrase–isomaltase (*SI*) [11] and lactase-phlorizin hydrolase (*LCT*) [30], have been identified as intestinal target genes capable of being regulated by PDX1. The adenosine deaminase gene is expressed along a defined spatiotemporal pattern in the developing mammalian small intestine. High-level expression of *ADA* is limited to the villous epithelium of the duodenum similar to the expression profile of PDX1. Dusing et al. have shown that PDX1 can interact with a small duodenal enhancer region in the *ADA* gene [19,37,38]. Loss of PDX1 binding, via a PDX1 mutated enhancer transgenic construct, resulted in complete loss of high-level activation in the duodenum. Sucrase–isomaltase gene expression is maximal in the middle segments of the small intestine and decreased in the proximal duodenum. Heller et al. have reported that PDX1 is capable of inhibiting transactivation of the *SI* promoter by the transcription factor CDX2 [11]. In addition, PDX1 was shown to be capable of physical interaction with CDX2. Similar to sucrase–isomaltase, lactase-phlorizin hydrolase gene expression is maximal in the middle segments of the small intestine and decreased in the proximal duodenum. PDX1 can similarly repress activation of the *LCT* promoter in intestinal cell culture [30]. In support of a role for PDX1 in specifying spatial restriction during intestinal development, Grapin-Botton et al. have reported that ectopic expression of PDX1 in chick embryo intestinal epithelial cells extinguishes markers for other non-pancreatoduodenal regions of gut endoderm [39]. Specifically, PDX1 expression in the small intestine between the duodenum and yolk stalk turns *CdxA* off, down-regulates *CdxC*, and turns off *Hex* in the bile duct.

In the present study, several candidate PDX1 target genes have been identified by gene expression profiling of intestinal Caco-2 cells engineered to overexpress PDX1. Multiple genes identified were classified as transcription factors, growth factors, kinases, digestive glycosidases, nutrient transporters, nutrient binding proteins, and structural components. The gene encoding fatty acid binding protein 1, liver, *FABP1*, was among the genes significantly repressed greater than 4-fold by PDX1 overexpression. Fatty acid binding proteins are small cytoplasmic lipid binding proteins involved in intracellular lipid transport. FABP1 binds free fatty acids and their co-enzyme A derivatives. The mouse *FABP1* gene homolog, *fabpl*, is expressed in hepatocytes, enterocytes, and enteroendocrine cells. Along the anterior-posterior axis of the small intestine, FABP1 is expressed in a gradient with jejunal expression greater than that in the duodenum or ileum [40]. In that regard, spatial restriction of FABP1 closely mimics that of lactase and sucrase–isomaltase in the intestine. Sweetser et al. have mapped distinct cis-acting elements in the 5′ flanking region of the *fabpl* gene that are necessary for specifying appropriate anterior-posterior spatial patterning of transgene expression in mice [40]. It is of interest that promoter activity of the *FABP1* 0.6 kb promoter-reporter construct, pFABP1luc, was repressed by Pdx1 co-transfection in the present study. PDX1 regulated repression of *FABP1* promoter activity in intestinal cells is consistent with the reduced abundance of FABP1 protein in the proximal duodenum where PDX1 is most abundant. A similar correlation exists between reports of PDX1 repression of promoter activity of the lactase [30] and sucrase–isomaltase genes [11], reduced *in vivo LCT* and *SI* expression in the

proximal duodenum, and decreased *LCT* and *SI* transcript levels in Caco-2 cells overexpressing PDX1 (present study). Our findings thus suggest that Pdx1 regulation of candidate target genes including *FABP1*, *LCT*, and *SI* may play a role in specifying the anterior duodenal boundary of spatial expression for enterocytes expressing those genes.

Disclosures

No conflicts of interest are declared by the author(s).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.05.113.

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