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CELLULAR AND MOLECULAR GASTROENTEROLOGY AND HEPATOLOGY

ORIGINAL RESEARCH

Regulation of the Pancreatic Exocrine Differentiation Program and Morphogenesis by Onecut 1/Hnf6

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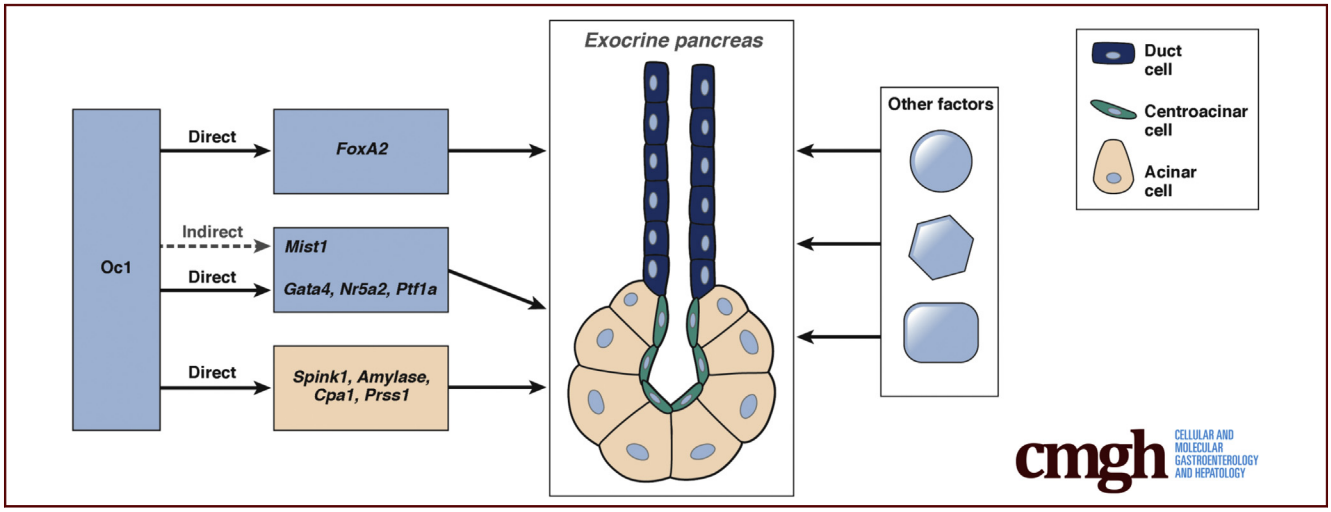

SUMMARY

Using RNA-Seq we analyzed gene expression changes associated with *Oc1/Hnf6* loss in mouse pancreas. We performed chromatin immunoprecipitation sequencing to identify direct transcriptional targets of Oc1/Hnf6 in pancreatic exocrine tissue. Our results solidify a role for Oc1/Hnf6 in establishing pancreas identity and suggest that duct/acinar identity is dependent on differential levels of Oc1/Hnf6 expression.

BACKGROUND & AIMS: The Onecut 1 transcription factor (Oc1, a.k.a. HNF6) promotes differentiation of endocrine and duct cells of the pancreas; however, it has no known role in acinar cell differentiation. We sought to better understand the role of Oc1 in exocrine pancreas development and to identify its direct transcriptional targets.

METHODS: Pancreata from $Oc1^{\Delta panc}$ (*Oc1*$^{fl/fl}$;*Pdx1-Cre*) mouse embryos and neonates were analyzed morphologically. High-throughput RNA-sequencing was performed on control and *Oc1*-deficient pancreas; chromatin immunoprecipitation sequencing was performed on wild-type embryonic mouse pancreata to identify direct Oc1 transcriptional targets. Immunofluorescence labeling was used to confirm the RNA-sequencing /chromatin immunoprecipitation sequencing results and to further investigate the effects of *Oc1* loss on acinar cells.

RESULTS: Loss of *Oc1* from the developing pancreatic epithelium resulted in disrupted duct and acinar cell development. RNA-sequencing revealed decreased expression of acinar cell regulatory factors (*Nr5a2*, *Ptf1a*, *Gata4*, *Mist1*) and functional genes (*Amylase*, *Cpa1*, *Prss1*, *Spink1*) at embryonic day (e) 18.5 in $Oc1^{\Delta panc}$ samples. Approximately 1000 of the altered genes were also identified as direct Oc1 targets by chromatin immunoprecipitation sequencing, including most of the previously noted genes. By immunolabeling, we confirmed that Amylase, Mist1, and GATA4 protein levels are significantly decreased by P2, and Spink1 protein levels were significantly reduced and mislocalized. The pancreatic duct regulatory factors *Hnf1β* and *FoxA2* were also identified as direct Oc1 targets.

CONCLUSIONS: These findings confirm that Oc1 is an important regulator of both duct and acinar cell development in the embryonic pancreas. Novel transcriptional targets of Oc1 have now been identified and provide clarity into the mechanisms of Oc1 transcriptional regulation in the developing exocrine pancreas. Oc1 can now be included in the gene-regulatory network of acinar cell regulatory genes. Oc1 regulates other acinar cell regulatory factors and acinar cell functional genes directly, and it can also regulate some acinar cell regulatory factors (eg, *Mist1*) indirectly. Oc1 therefore plays an important role in acinar cell development. *(Cell Mol Gastroenterol Hepatol 2019;7:841–856; https://doi.org/10.1016/j.jcmgh.2019.02.004)*

Keywords: Pancreas Development; Exocrine; Transcriptome.

Background and Aims

The exocrine pancreas serves a vital function in digestion through production and transport of digestive enzymes. The pancreatic acinar cells produce and secrete digestive enzymes into the lumen of the pancreatic ducts, which in turn transport them to the rostral duodenum. The exocrine pancreas is also the source of serious diseases, such as pancreatitis, intrapapillary mucinous neoplasia, and pancreatic ductal adenocarcinoma (PDAC). The most serious of these, PDAC, afflicts more than 50,000 individuals in the United States every year with only approximately 8% of diagnosed individuals surviving past 5 years.[1] In spite of its name and histologic appearance, PDAC is believed to originate from the pancreatic acinar cells.[2] PDAC development and progression are marked by re-activation of pathways associated with exocrine pancreas development including Wnt, Notch, and Hedgehog (HH) signaling as well as decreased expression of transcription factors that regulate acinar cell identity.[3] For that reason, a more complete understanding of exocrine pancreas development and maintenance of acinar differentiation will provide better avenues to therapeutic approaches.

All cells of the pancreas originate from a pool of multipotent pancreatic progenitor cells (MPCs).[4] Specification and differentiation of pancreatic cell types is orchestrated by a cascade of transcription factors. Two of the most upstream of these are the forkhead box family members Foxa1 and Foxa2. Together they redundantly regulate expression of the essential pancreatic transcription factor, *Pdx1* (pancreatic and duodenal homeobox 1). In the absence of Foxa1 and Foxa2, *Pdx1* expression is lost and severe pancreatic hypoplasia results.[5] Many pancreas transcription factors are initially broadly expressed and then become increasingly restricted to particular cell fates, whereas others are activated specifically in lineage-restricted cells. For example, Pdx1 is initially expressed in all MPCs but as development progresses, it becomes highly upregulated in the β-cell lineage. It is still present at low levels in mature acinar cells and becomes downregulated in ducts.[6] The transcription factors *Ptf1a* (*Pancreas transcription factor, 1a*) and *Nr5a2* (*Nuclear receptor subfamily 5, group A, member 2*) are expressed in MPCs but become restricted to mature acinar cells,[7-14] whereas *Mist1* (*muscle, intestine, stomach transcription factor 1*) is only expressed once cells have become committed to an acinar cell fate.[15-17] Similar to *Ptf1a* and *Nr5a2*, *Hnf1β* (*Hepatocyte nuclear factor 1 β*) and *Sox9* (*SRY-related HMG-box 9*) are transcription factors expressed in MPCs; however, they become restricted to mature duct cells.[18-25] These factors all have key roles in regulating the development, function, and identity of the cell type in which they are expressed. *Ptf1a* inactivation in development results in near complete pancreatic agenesis, and inactivation in adults results in loss of acinar cell identity.[7-10] *Nr5a2* inactivation in development results in a severely hypoplastic pancreas with a disproportionate loss of acinar cells. Loss of *Sox9* during pancreas development results in pancreas hypoplasia, whereas inactivation in adults sensitizes duct cells to dysplasia.[2,20,23,24] *Hnf1β*-null mice similarly develop a severely hypoplastic pancreatic bud, and inactivation later in development results in duct dysmorphogenesis and loss of ductal primary cilia.[18,19,21,22] These studies all demonstrate the importance of lineage-restricted transcription factors in regulation of exocrine pancreas development.

The *Onecut1* (*Oc1*, formally known as *Hepatocyte nuclear factor 6* [*Hnf6*]) transcription factor is also expressed in MPCs but, unlike the previously mentioned factors, in adults it is expressed in both acinar and duct cells where it is expressed at a low and high level, respectively.[26,27] Oc1 plays important roles in activating the endocrine specification program during pancreas development[27,28] and during differentiation of the pancreatic ducts.[26] *Oc1* inactivation throughout the pancreatic epithelium in early pancreas development results in a hypoplastic pancreas, ductal cysts, duct hyperplasia, a multilayered duct epithelium, and loss of primary cilia.[26,27,29] Additionally, *Oc1* inactivation during development results in postnatal acinar cell defects resembling pancreatitis including fibrosis, acinar-to-ductal metaplasia (ADM), and inflammation,[27,29] suggesting a role for Oc1 in regulation of both duct and acinar cell development. These findings are further supported by human PDAC studies that correlate progression of precancerous lesions (pancreatic intraepithelial neoplasms) with loss of OC1 protein and gene expression.[30,31]

Very little is known about how Oc1 regulates exocrine pancreas differentiation. Of the known direct Oc1 targets in the pancreas (*Pdx1*, *Ngn3*, *MafA*, and *Hnf4α*), most are endocrine-specific. Only *Pdx1* is expressed in the exocrine lineage (where it is expressed at a low level in subpopulations of acinar cells).[10,28,32-39] Oc1 directly binds to and regulates the *Hnf1β* promoter in liver cholangiocytes,[40,41] but it is unclear if this direct regulation also exists in the pancreatic ducts. The goal of the current study was to identify additional Oc1 targets to better understand how Oc1 regulates exocrine pancreas development. We found that loss of *Oc1* from the developing pancreatic epithelium results in severe exocrine dysplasia and altered gene expression consistent with impaired acinar cell differentiation. We also identified novel direct Oc1 transcriptional targets in late-gestation pancreata using chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-Seq).

Abbreviations used in this paper: **ADM, acinar-to-ductal metaplasia; ChIP-Seq, chromatin immunoprecipitation followed by high-throughput sequencing; CK19, Cytokeratin 19; Cym, Chymosin; HH, Hedgehog; Hnf1β, hepatocyte nuclear factor 1β; Hnf6, hepatocyte nuclear factor 6; Ihh, Indian hedgehog; Inhba, Inhibin, Beta A; Mist1, muscle, intestine, stomach transcription factor 1; MPC, multipotent pancreatic progenitor cell; Nr5a2, nuclear receptor subfamily 5, group A, member 2; Oc1, Onecut1; PDAC, pancreatic ductal adenocarcinoma; Pdx1, pancreatic and duodenal homeobox 1; Ptch2, Patched 2; Ptf1a, Pancreas transcription factor, 1a; RNA-Seq, RNA-sequencing; Smo, Smoothened; Sox9, SRY-related HMG-box 9; Spink 1, serine protease inhibitor Kazal type 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.**

Most current article

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Results

Exocrine Pancreas Dysplasia in Embryonic and Neonatal Oc1$^{\Delta panc}$ Mice

We previously reported pancreatic hypoplasia in Oc1$^{\Delta panc}$ mice at e14.5 and exocrine dysplasia (ADM, fibrosis, periductal hemorrhaging) at 3 weeks of age.[27] These phenotypes demonstrated that Oc1 has a role in regulating exocrine pancreas development; however, we wanted to determine when exocrine dysplasia first developed. We thus examined pancreata from Oc1$^{\Delta panc}$ mice just before birth at e18.5 and immediately after birth at postnatal day (P)2. It was evident that exocrine pancreas development was disrupted in Oc1$^{\Delta panc}$ pancreata at e18.5 as the pancreatic ducts were dilated, there was reduced acinar eosinophilia (Figure 1*A* and *A'*), and reduced acinar cell area (Figure 1*E*, Table 1) with a trend toward increased duct cell area (Figure 1*G*). These findings are consistent with global *Oc1*$^{-/-}$ animals and our previous Oc1$^{\Delta panc}$ animals,[26,27] and were even more pronounced at P2 (Figure 1*B*, *B'*, *F*, and *H*, Table 1). Hematoxylin and eosin staining appeared to show increased fibrosis in both e18.5 and P2 Oc1$^{\Delta panc}$ pancreata, which we analyzed further with sirius red staining for collagen (Figure 1*C*, *C'*, *D*, and *D'*). There was no statistically significant increase in pancreatic collagen at e18.5 (not shown), but there was a significant increase in pancreatic collagen at P2 (Figure 1*I*). Finally, cells expressing markers of both duct (Cytokeratin 19 [CK19]) and acini (amylase) could be detected in P2 Oc1$^{\Delta panc}$ pancreata (Figure 1*J* and *K*) consistent with ADM. Together, these data suggest an impairment in exocrine pancreas development and acinar cell identity.

The relative acinar cell area appeared to progressively decrease from e18.5 to P2, so we predicted that this reduction was caused by either increased acinar cell death or reduced acinar cell proliferation. Acinar cell proliferation was significantly increased at e18.5 (Figure 2*A1*, *A2*, and *C*) but not at P2 (Figure 2*A3*, *A4*, and *C*). Using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labeling to detect apoptotic cells, we found that there was a significant increase in acinar cell death at both e18.5 and P2 in Oc1$^{\Delta panc}$ pancreata (Figure 2*B* and *D*). These findings demonstrate altered acinar cell population dynamics in Oc1$^{\Delta panc}$ samples.

Loss of Oc1 From the Pancreatic Epithelium Results in Significant Gene Expression Changes During Development

To better understand the role of Oc1 in regulating exocrine pancreas development, we used RNA-sequencing (RNA-Seq) to determine how loss of *Oc1* affects global gene expression. Total RNA was extracted from e18.5 exocrine-enriched samples. There was some variability in gene expression between biologic replicates (Figure 3*A*); however, many gene expression changes were consistent in Oc1$^{\Delta panc}$ samples. Of the 1842 affected genes, 1004 showed decreased expression and 838 had increased expression (Figure 3*B*). Among the 25 most significantly affected genes, all had decreased expression except *Cym* (*chymosin*), a peptidase produced by gastric chief cells (Figure 3*C*). Many of the most significantly reduced genes were enzymes, such as amylase, trypsins, trypsin-like peptidases, or peptidase inhibitors that are normally expressed by pancreatic acinar cells (eg, *Amylase*, *Prss1*, *Prss3*, *Try4*, *Try10*, and *Spink1*) (Figure 3*C*). Accordingly, expression of acinar-lineage transcription factors, such as *Mist1*, *Gata4*, *Nr5a2*, and *Ptf1a*, was also decreased. Interestingly, *Onecut2* (*Oc2*) was 1 of the most significantly upregulated transcription factors. However, increased expression of this closely related factor clearly does not compensate for the loss of *Oc1*. Analysis of Biological Processes and Cellular Component categories revealed changes in genes associated with metabolic and developmental processes, membranes and vesicles, and nuclear factors (Figure 3*D*). The finding that acinar cells were disproportionally affected was further supported by gene ontology analysis using WebGestalt, which identified "Enzyme Inhibitor Activity" and "Endopeptidase Activity" as 2 of the 5 most significantly altered groups (Supplementary Tables 1–3). These findings imply that Oc1 regulates expression of acinar cell genes that include other coregulating transcription factors and functional genes.

Another interesting finding was that "Wnt-Activated Receptor Activity," "Wnt-Protein Binding," and "Frizzled Binding" were identified as significantly altered pathways (Supplementary Tables 1–3). Examination of the components of these pathways demonstrated that the differentially expressed genes in Oc1$^{\Delta panc}$ samples were all upregulated. Wnt signaling is known to be an important mediator of exocrine pancreas development, but also acinar cell proliferation. It is possible that the enhanced acinar cell proliferation we observed in Oc1$^{\Delta panc}$ pancreata is caused by activation of the Wnt pathway. Another pathway with noticeable changes was HH signaling. *Ihh* (*Indian hedgehog*, ligand), *Ptch2* (*Patched2* HH receptor), *Smo* (*Smoothened*, patched target), *Gli1*, and *Gli2* (transcription factor effectors of HH signaling) were all upregulated. Of note, HH signaling must be specifically repressed in the foregut endoderm to allow for pancreas specification to take place and is inactive in normal exocrine pancreas homeostasis. It is thus interesting that expression of genes associated with other foregut endoderm-derived organs (*Cym*, *Irx3/4*, *Vill*, *Lgr5*) was significantly increased in Oc1$^{\Delta panc}$ samples. Collectively, these gene expression changes reveal that inactivation of *Oc1* in the developing pancreatic epithelium has a significant impact on the developing exocrine pancreas and potentially alters the identity of pancreatic cells.

Oc1 Inactivation in the Developing Pancreatic Epithelium Has Persistent Impacts on Postnatal Gene Expression

Analysis of RNA-Seq from exocrine-enriched samples at P2 also revealed significantly altered expression of 280 genes in Oc1$^{\Delta panc}$ samples despite some variability between biologic replicates (Figure 4*A*). Expression of 172 genes was downregulated, whereas 108 genes showed increased expression (Figure 4*B*). Approximately half of the genes

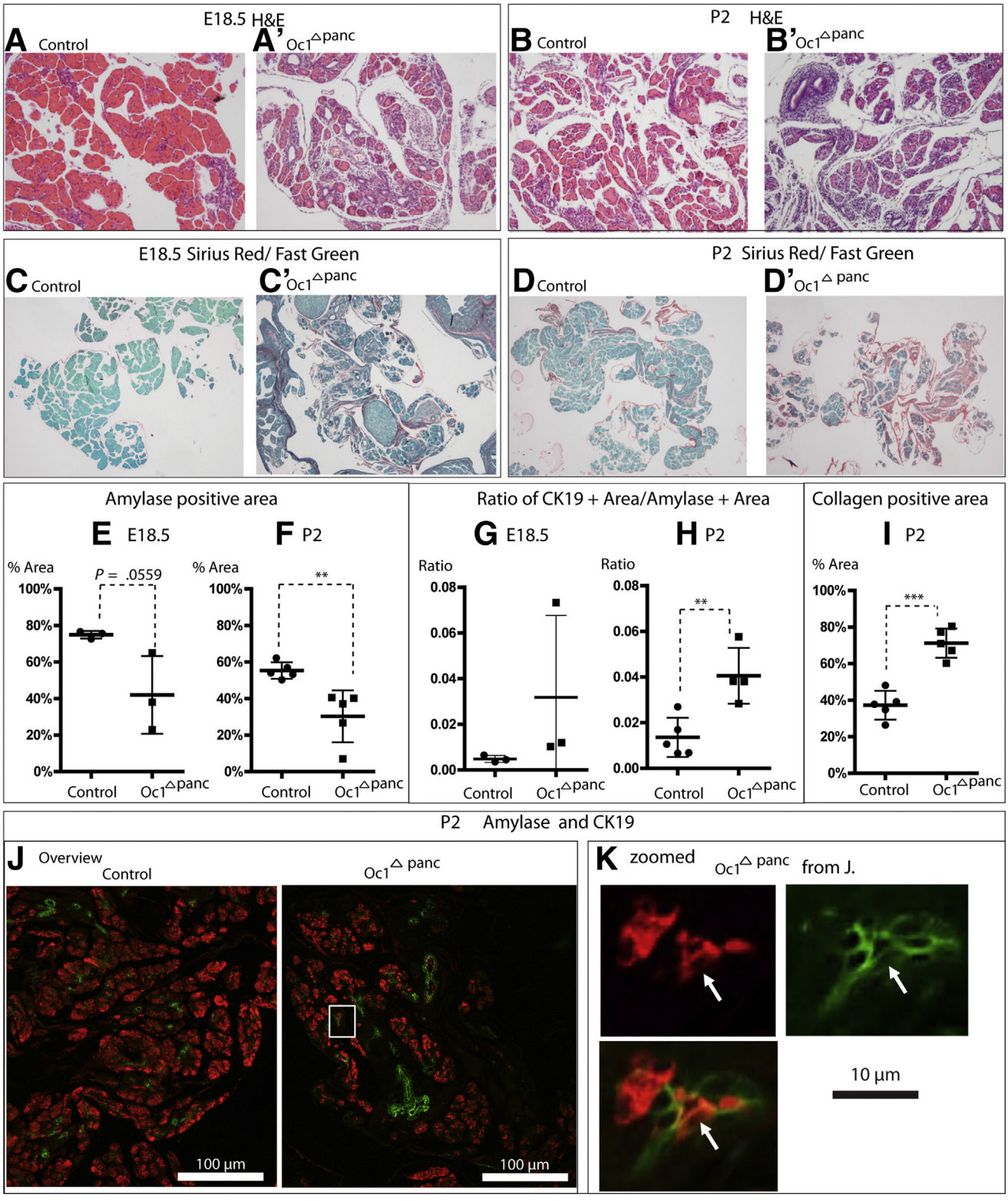

Figure 1. Exocrine dysplasia in Oc1$^{\Delta panc}$ pancreata. Representative hematoxylin and eosin images from e18.5 (*A* and *A'*) and P2 (*B* and *B'*) Control (*A* and *B*) and Oc1$^{\Delta panc}$ pancreata (*A'* and *B'*). Representative images of sirius red/fast green staining from e18.5 (*C* and *C'*) and P2 (*D* and *D'*) Control (*C* and *D*) and Oc1$^{\Delta panc}$ pancreata (*C'* and *D'*). e18.5 (n = 3 mice); P2 (n = 5 mice). Images captured at ×20. Amylase-positive area at e18.5 (*E*, n = 3 mice) and P2 (*F*, n = 5 mice); ratio of CK19+:Amylase+ area at e18.5 (*G*, n = 3 mice) and P2 (*H*, n = 5 mice). (*I*) Collagen-positive area at P2 (n = 5 mice). (*J*) Representative immunofluorescence images of amylase (*red*) and CK19 (*green*) in Control and Oc1$^{\Delta panc}$ pancreata at P2. Scale bar represents 100 µm. (*K*) Split channel of boxed area in J Oc1$^{\Delta panc}$. $^{**}P \leq .01$, $^{***}P \leq .001$ by 2-tailed Student *t* test. H&E, hematoxylin and eosin.

Table 1. Quantification of Acinar Area

Stage	% Acinar area/pancreas area		*P* value
	Control	$Oc1^{\Delta panc}$	
E18.5 (n = 3)	55.3% ± 6.1%	29.0% ± 13.2%	.0345
P2 (n = 5)	40.9% ± 6.4%	18.1% ± 7.1%	.0007

Data are presented as the mean ± standard deviation. *P* value derived from 2-tailed Student *t* tests.

altered in P2 $Oc1^{\Delta panc}$ samples are shared with e18.5 $Oc1^{\Delta panc}$ samples (Figure 4*C*). Several acinar cell functional genes were altered at both ages (eg, *Amylase*, *Cela1*, *Cpa1/2*, *Prss2*, and *Spink1*) suggesting that Oc1 directly regulates acinar cell function at both stages. Examination of the 25 most significantly altered genes (Figure 4*D*) supports previously published findings that Oc1 is required for endocrine cell differentiation.[27,28]

WebGestalt analysis identified pathways associated with peptidase activity as 4 of the 5 most significantly affected pathways in the Biological Function category at P2 (Figure 4*E*, Supplementary Tables 4–6). These gene expression changes support the observed exocrine dysplasia in $Oc1^{\Delta panc}$ pancreata because dysplastic acinar cells have impaired peptidase expression. Similar to e18.5, components of the HH pathway were upregulated (*Ihh*, *Gli1*, *Ptch1*, and *Ptch2*) at P2 but components of the Wnt signaling pathway were largely unaffected. Interestingly, *Inhba* (Inhibin, Beta A), which has been associated with acinar cell dysplasia in pancreatic cancers,[42] was 1 of the most significantly upregulated genes (5.788-fold). Its activity could contribute to the increased extracellular matrix deposition observed in Figure 1. *Oc2* was also upregulated at P2 (albeit to a lesser extent than at e18.5). In all, the RNA-Seq results from P2 $Oc1^{\Delta panc}$ samples confirm a continued decrease in expression of multiple acinar cell functional genes.

Identification of Direct Oc1 Targets in e18.5 Pancreata

To better understand how Oc1 directly regulates development of the exocrine pancreas we performed ChIP-Seq on whole pancreata from wildtype e18.5 mice. At this age, Oc1 is excluded from all hormone$^+$ cells thereby allowing us to select for targets in the pancreatic ducts and acinar cells. There were approximately 7400 peaks identified, which were associated with 4962 genes. These peaks were enriched in the 5' untranslated regions and proximal promoters (<500 bp from transcription start sites) of the associated genes (Figure 5*A*). Of the 4962 genes associated with Oc1 binding peaks, 499 had altered gene expression in the e18.5 $Oc1^{\Delta panc}$ RNA-Seq indicating that Oc1 directly regulates transcription of these genes (Figure 5*B*). Motif analysis of our ChIP-Seq data also identified peaks of transcription factors that are known to associate with Oc1 (Figure 5*C*), suggesting that in acinar cells, Oc1 regulates gene expression cooperatively with other transcription factors.

Among the most noticeable direct Oc1 targets were *Ptf1a*, *Nr5a2*, and *Gata4* (Figure 5*D–F*), transcription factors that play vital roles in the specification, differentiation, and function of pancreatic acinar cells. All 3 of these factors had reduced expression in the $Oc1^{\Delta panc}$ RNA-Seq samples implying that Oc1 positively regulates their expression. A significant Oc1 binding peak was also identified in Area III of the *Pdx1* promoter (Figure 5*G*), the area that is known to promote nonendocrine expression of *Pdx1*.[43] Taken together, these data suggest that in acinar cells Oc1 functions, at least in part, through regulating other transcription factors. Oc1 also directly bound to regions associated with acinar cell functional genes, such as *Amylase*, *Prss1/2*, *Prss2*, *Pnlip*, *Pnliprp1/2*, and *Spink1* (not shown). Expression of each of these functional genes was decreased in $Oc1^{\Delta panc}$ pancreata. Thus, Oc1 directly regulates genes important for acinar cell identity and function and other acinar cell transcription factors.

We and others have demonstrated that loss of Oc1 from the developing pancreas results in ductal cysts, duct hyperplasia, and tortuous ducts.[26,27] Thus, we predicted that the ChIP-Seq analysis would identify direct Oc1 targets that could be attributed to duct cells. The transcription factors *Hnf1β* and *FoxA2*, both of which are expressed in MPCs at early stages and later in the pancreatic ducts, were identified as direct Oc1 targets (Figure 5*H* and *I*). Other duct-specific genes, such as *Prox1* or *Sox9*, were not identified as targets in our analysis.

Although Oc1 functions as a transcriptional activator for many genes important in exocrine pancreas development, it also seems to function as a transcriptional repressor for genes associated with other endoderm-derived lineages. Both gastric and intestinal-associated genes were identified as direct Oc1 targets in the ChIP-Seq experiment and had increased expression the $Oc1^{\Delta panc}$ RNA-Seq. Most notable among these genes were the transcription factor *Irx3*, the G-protein coupled receptor *Lgr5*, and the cytoskeleton-related factor *Vill*.

Confirmation of Direct Oc1 Targets in e18.5 Pancreata

We next performed immunofluorescence imaging to validate the Oc1 targets identified by ChIP-Seq and RNA-Seq. For example, expression of *Mist1*, an acinar-lineage transcription factor, was found to be decreased by RNA-Seq analysis, but there were no Oc1 binding motifs associated with the *Mist1* gene (suggesting indirect regulation). To confirm the RNA-Seq data, we examined Mist1 protein expression by immunolabeling. At e18.5 most acinar cells in $Oc1^{\Delta panc}$ pancreata have lower Mist1 levels than cells in control pancreata (Figure 6*A1* and *A2*). However, the average number of Mist1-positive cells was not altered (Figure 6*C1*). In contrast, by P2, both the level of Mist1 protein and the number of Mist1-positive cells were decreased in $Oc1^{\Delta panc}$ (Figure 6*A3*, *A4*, and *C2*).

GATA4 is another acinar-lineage transcription factor. RNA-Seq analysis found that *Gata4* expression decreased in $Oc1^{\Delta panc}$ pancreata. Chip-Seq analysis further identified the

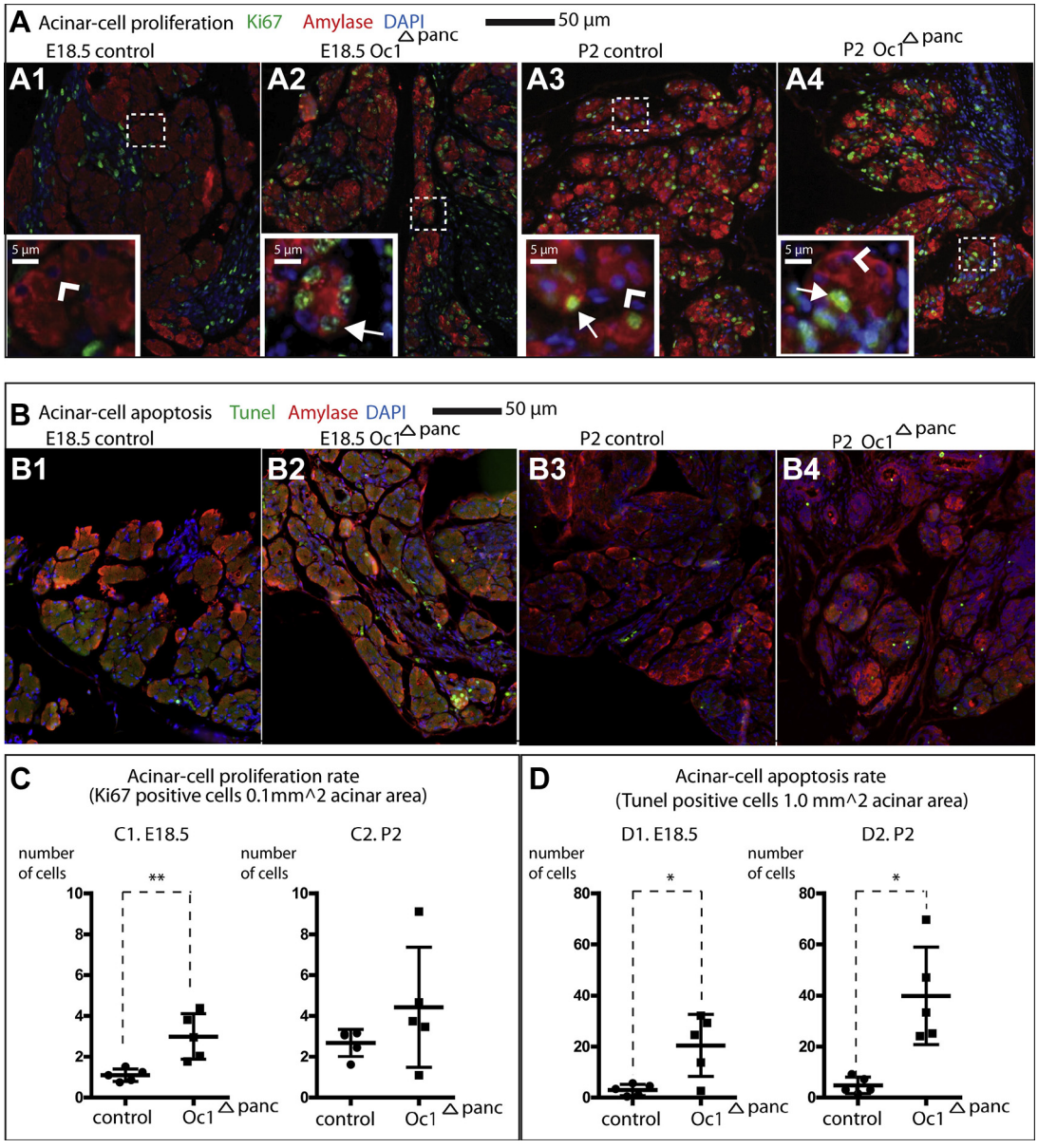

Figure 2. Altered acinar cell proliferation and death in Oc1$^{\Delta panc}$ pancreata. (*A*) Representative immunofluorescence images of amylase (*red*), Ki67 (*green*), and DAPI (*blue*) from e18.5 (*A1* and *A2*) and P2 (*A3* and *A4*) Control (*A1* and *A3*) and Oc1$^{\Delta panc}$ (*A2* and *A4*) pancreata. Images in the *white dotted line box* are shown at increased magnification at lower left. *Arrows* indicate Ki67-positive cells, *arrowheads* indicate Ki67-negative cells. (*B*) Representative immunofluorescence images of amylase (*red*), TUNEL (*green*), and DAPI (*blue*) from e18.5 (*B1* and *B2*) and P2 (*B3* and *B4*) Control (*B1* and *B3*) and Oc1$^{\Delta panc}$ (*B2* and *B4*) pancreata. (*C*) Quantification of acinar cell proliferation at e18.5 (*C1*) and P2 (*C2*). (*D*) Quantification of acinar cell apoptosis at e18.5 (*D1*) and P2 (*D2*). n = 5 mice for each group. $^{**}P \leq .01$, $^{*}P \leq .05$ by 2-tailed Student *t* test.

Gata4 gene as a direct target of Oc1. By immunolabeling, we found that at e18.5 there was increased heterogeneity of GATA4 expression in acinar cells of Oc1$^{\Delta panc}$ pancreata. A subpopulation of cells had lower GATA4 expression in Oc1$^{\Delta panc}$ (compare Figure 6*B1* with *B2*). However, overall, using quantitative immunofluorescence we did not find a significant difference in the average GATA4 intensity between Oc1$^{\Delta panc}$ and control acinar cells (Figure 6*D1*). By P2, GATA4 intensity became significantly reduced in Oc1$^{\Delta panc}$ (Figure 6*B3*, *B4*, and *D2*). Putting our RNA-Seq, ChIP-Seq, and immunofluorescence data together, we propose that Oc1 directly and positively regulates GATA4 expression. We also examined the expression of Ptf1a, another acinar lineage transcription factor, by immunofluorescence. Both our ChIP-Seq and RNA-Seq data suggest that Oc1 positively and directly regulates *Ptf1a*, but unfortunately, we cannot draw any firm conclusions at this time because of the variation in immunolabeling between samples (data not shown).

In addition to transcription factors, we also explored the expression level of the acinar cell functional protein Spink1, which was identified as a direct transcriptional target of Oc1. Spink1 is important to prevent the

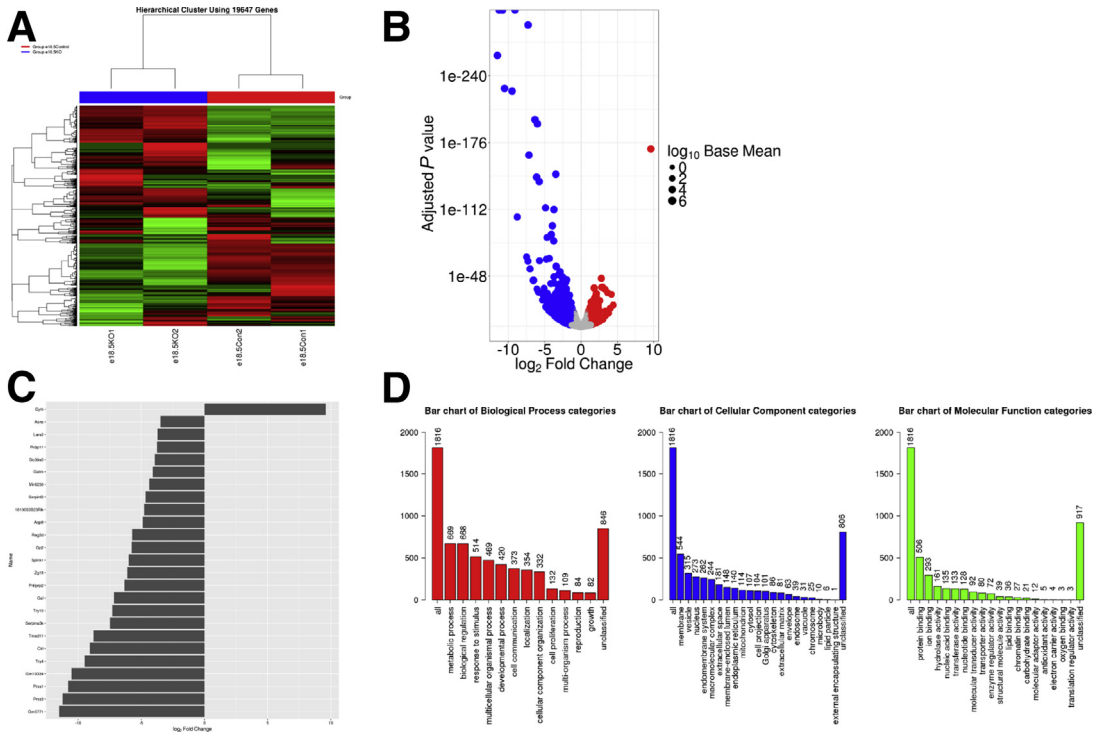

Figure 3. Gene expression changes in e18.5 exocrine-enriched Oc1$^{\Delta panc}$ samples. (*A*) Heatmap of gene expression from RNA-Seq for each of 2 biologic replicates for Control (*red bar at top*) and Oc1$^{\Delta panc}$ (*blue bar at top*). *Green* indicates high expression; *red* indicates low expression. (*B*) Volcano plot of genes with differential expression in Oc1$^{\Delta panc}$ samples. Significant gene expression changes were determined by an FDR of 0.05 and $\log_2$ fold change of 1. *Blue dots* represent significantly reduced genes; *red dots* represent significantly increased genes with $\log_2$ fold change on the x-axis and adjusted *P* value on y-axis. (*C*) Plot of 25 most significantly altered genes. $\log_2$ fold change is plotted on the x-axis and gene names are plotted on the y-axis. (*D*) Bar graphs representing gene ontology analysis of biologic process, cellular component, and molecular function. See Supplementary Tables 1–3 for further detail.

premature activation of trypsin to prevent autodigestion of the pancreas. At e18.5, Spink1 protein intensity was significantly reduced in Oc1$^{\Delta panc}$ (compare Figure 7*A* and *B*, quantified in *E1*). We also found that in most acinar cells in control pancreata, Spink1 was uniformly distributed (Figure 7*A2* and *A3*), but in Oc1$^{\Delta panc}$ pancreata, there are high-intensity patches of Spink1 protein localized at the cortex of some cells (Figure 7*B2* and *B3*), indicating defects in intracellular localization of Spink1 in Oc1$^{\Delta panc}$ mutants. At P2, the disparity in Spink1 localization between control and Oc1$^{\Delta panc}$ pancreata becomes more pronounced. In most acinar cells in control pancreata, Spink1 is evenly distributed throughout the cytoplasm (Figure 7*C* and *F1*). However, in a subpopulation of cells in the mutants, Spink1 accumulates at the cell cortex (closely apposed to E-cadherin labeling) with almost no protein in the central cytoplasm (Figure 7*D3*, *D4*, and *F2*). Cells with cortical Spink1 often show additional defects in epithelial organization as indicated by E-cadherin immunolabeling, and are potentially undergoing ADM, as we have previously observed with loss of *Oc1*.[27] There remains a subpopulation of acinar cells with relatively normal cytoplasmic Spink1 distribution in the Oc1$^{\Delta panc}$ pancreata (Figure 7*D1*).

Conclusions

The current study provides the first report of direct transcriptional targets of the critical transcription factor OC1 (formerly known as Hnf6) in the developing exocrine pancreas. Together our data suggest that in the exocrine pancreas cells, Oc1 functions via at least 3 mechanisms: (1) direct and indirect regulation of acinar lineage transcription factors, (2) direct regulation of acinar cell functional genes, and (3) direct regulation of duct lineage genes. Our data also suggest that Oc1 cooperates with other endoderm transcription factors that bind near Oc1 target sequences to regulate exocrine gene expression. Our results also solidify a role for Oc1 in establishing pancreas identity, because expression of posterior foregut genes normally restricted from the pancreas anlagen was elevated in the absence of Oc1.

Loss of *Oc1* from the developing pancreatic epithelium results in impaired development of the pancreatic ducts and acinar cells.[26,27] These findings, paired with the findings that OC1 expression is lost from acinar cells in human PDAC,[30,31] suggest that Oc1 has a role in regulation of acinar cell identity. Here we show that inactivation of *Oc1* during pancreas development results in embryonic acinar cell dysplasia that becomes progressively more severe after birth. These findings are consistent with other models of

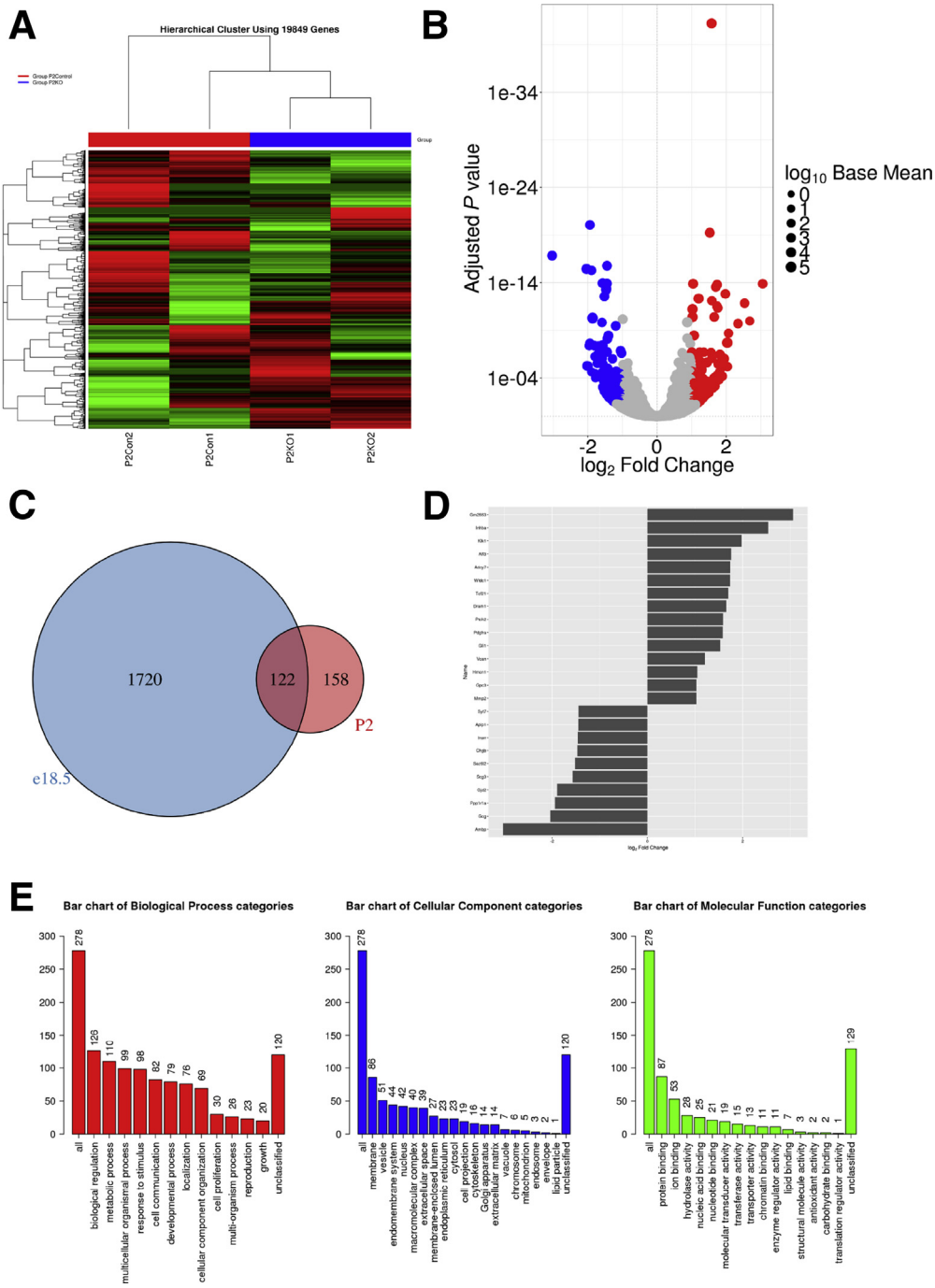

Figure 4. Gene expression changes in P2 exocrine-enriched Oc1$^{\Delta panc}$ samples. (*A*) Heatmap of gene expression from RNA-Seq for each of 2 biologic replicates for Control (*red bar at top*) and Oc1$^{\Delta panc}$ (*blue bar at top*). *Green* indicates high expression; *red* indicates low expression. (*B*) Volcano plot of genes with differential expression in Oc1$^{\Delta panc}$ samples. Significant gene expression changes were determined by an FDR of 0.05 and $\log_2$ fold change of 1. *Blue dots* represent significantly reduced genes; *red dots* represent significantly increased genes with $\log_2$ fold change on the x-axis and adjusted *P* value on y-axis. (*C*) Venn diagram illustrating overlapping gene expression changes at e18.5 and P2. (*D*) Plot of 25 most significantly altered genes. Log_2 fold change is plotted on the x-axis and gene names are plotted on the y-axis. (*E*) Bar graphs representing gene ontology analysis of biologic process, cellular component, and molecular function. See Supplementary Tables 4–6 for further detail.

impaired acinar cell differentiation, such as inactivation of *Nr5a2*, which results in acinar hypoplasia and disrupted acinus morphology.[13] The *Oc1*$^{\Delta panc}$ phenotype is distinct from knockout models of acinar cell transcription factors, such as *Mist1* or *Gata6*, which are dispensable for acinar cell differentiation, but vital for postnatal acinar cell identity.[16,44-48] Indeed, both *Mist1* and *Gata6* embryonic knockout models have little to no phenotype before birth. Of note, GATA4 and GATA6 have partially redundant roles in pancreas organogenesis and inactivation of both factors during embryogenesis has severe consequences for both endocrine and exocrine pancreas development.[49-51] Thus, morphology of the *Oc1*$^{\Delta panc}$ pancreas is more similar to that of knock-out models for factors regulating acinar cell differentiation (*Nr5a2*) than acinar cell identity and function (*Mist1*, *Gata6*) suggesting that *Oc1* contributes to regulation of acinar cell differentiation.

The reduction in acinar cell area in Oc1 mutants likely results from a combination of reduced specification and differentiation from MPCs as well as increased acinar cell

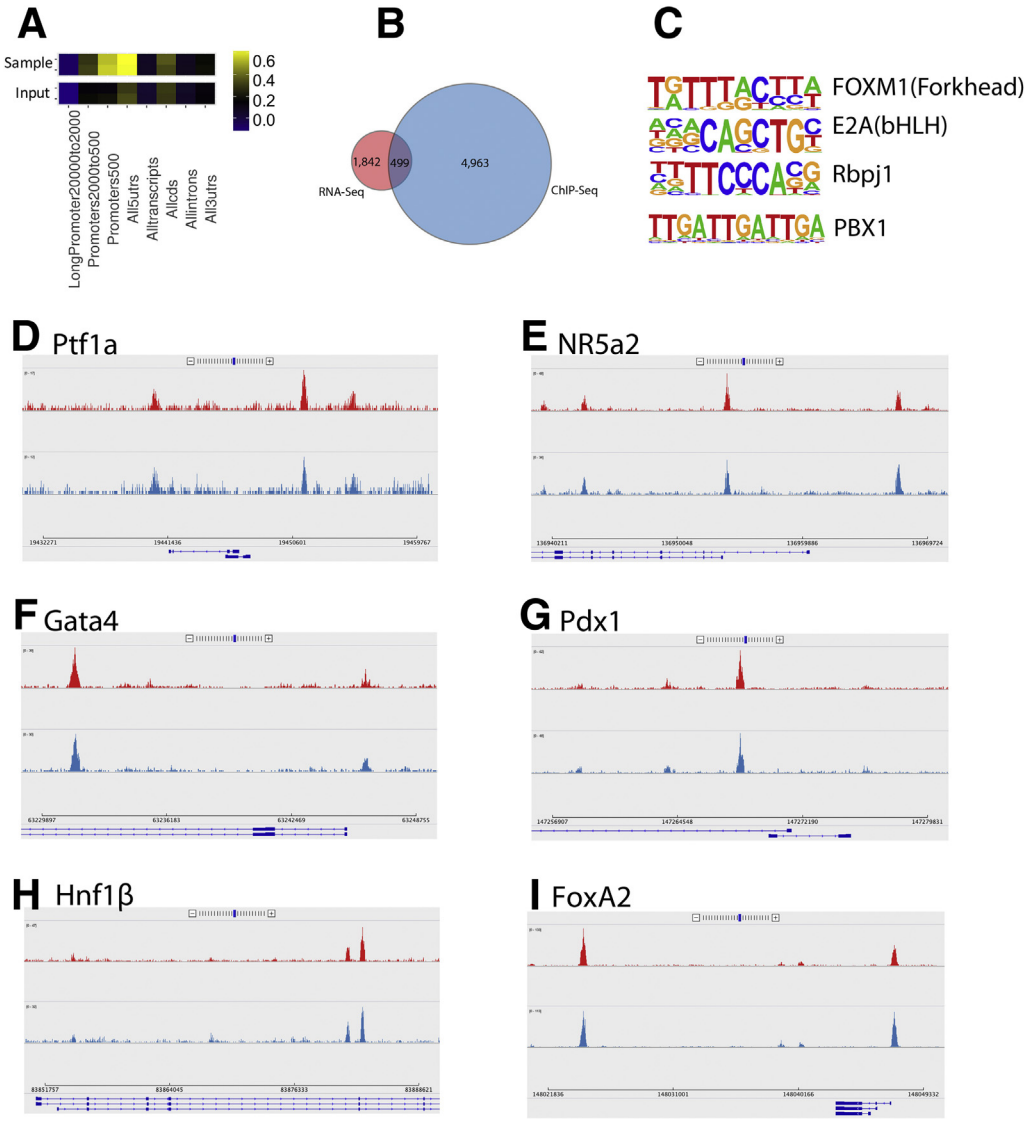

Figure 5. Identification of direct Oc1 targets in e18.5 pancreata. (*A*) Genomic regions with enrichment of reads in Oc1 ChIP-Seq samples. *Yellow* indicates higher incidence of reads. (*B*) Venn diagram illustrating overlap of genes with altered expression in e18.5 Oc1$^{\Delta panc}$ RNA-Seq samples (*red*) with genes associated with Oc1 binding peaks in e18.5 ChIP-Seq samples (*blue*). (*C*) Motif analysis of other transcription factor binding sites identified near Oc1 binding cites in Oc1 targets. (*D–I*) Sashimi plots of Oc1 binding peaks. Each track (*red or light blue peak build-up*) shows alignments from an individual biologic replicate. Gene coding regions are represented as *darker blue* annotations at the bottom of each plot. (*D*) *Ptf1a*. (*E*) *Nr5a2*. (*F*) *Gata4*. (*G*) *Pdx1*. (*H*) *Hnf1β*. (*I*) *FoxA2*.

apoptosis. Our finding that acinar cell death was increased in *Oc1*$^{\Delta panc}$ pancreata was not surprising given the severely disrupted acinar compartment; however, the finding that acinar cell proliferation was increased at e18.5 was unexpected. Increased proliferation to compensate for reduced specification or differentiation is not unprecedented in the pancreas. We have previously demonstrated that such a process can occur in β cells[52] and others have shown that that epithelial cells of primitive ducts can proliferate in order to produce more endocrine progenitors.[53] Additionally, acinar cells show increased proliferation following injury in adult pancreata,[54,55] so it is possible that such a mechanism is also functioning in *Oc1*$^{\Delta panc}$ acinar cells at e18.5 to compensate for the reduced acinar cell area. Postnatal acinar cell proliferation is partially regulated by Wnt signaling.[54-56] Our RNA-Seq results indicated that e18.5 *Oc1*$^{\Delta panc}$ pancreata had increased expression of multiple components of the Wnt pathway, so it seems likely that Wnt-activation is closely connected with the observed increase in proliferation. Of note, *Wnt7b* (which contributes to regulation of pancreas morphogenesis[57]) was identified as a direct target of Oc1 in our ChIP-Seq analysis and also showed increased mRNA expression, likely caused by direct loss of *Oc1* activity.

Our RNA-Seq results revealed decreased expression of many important acinar cell genes including regulatory

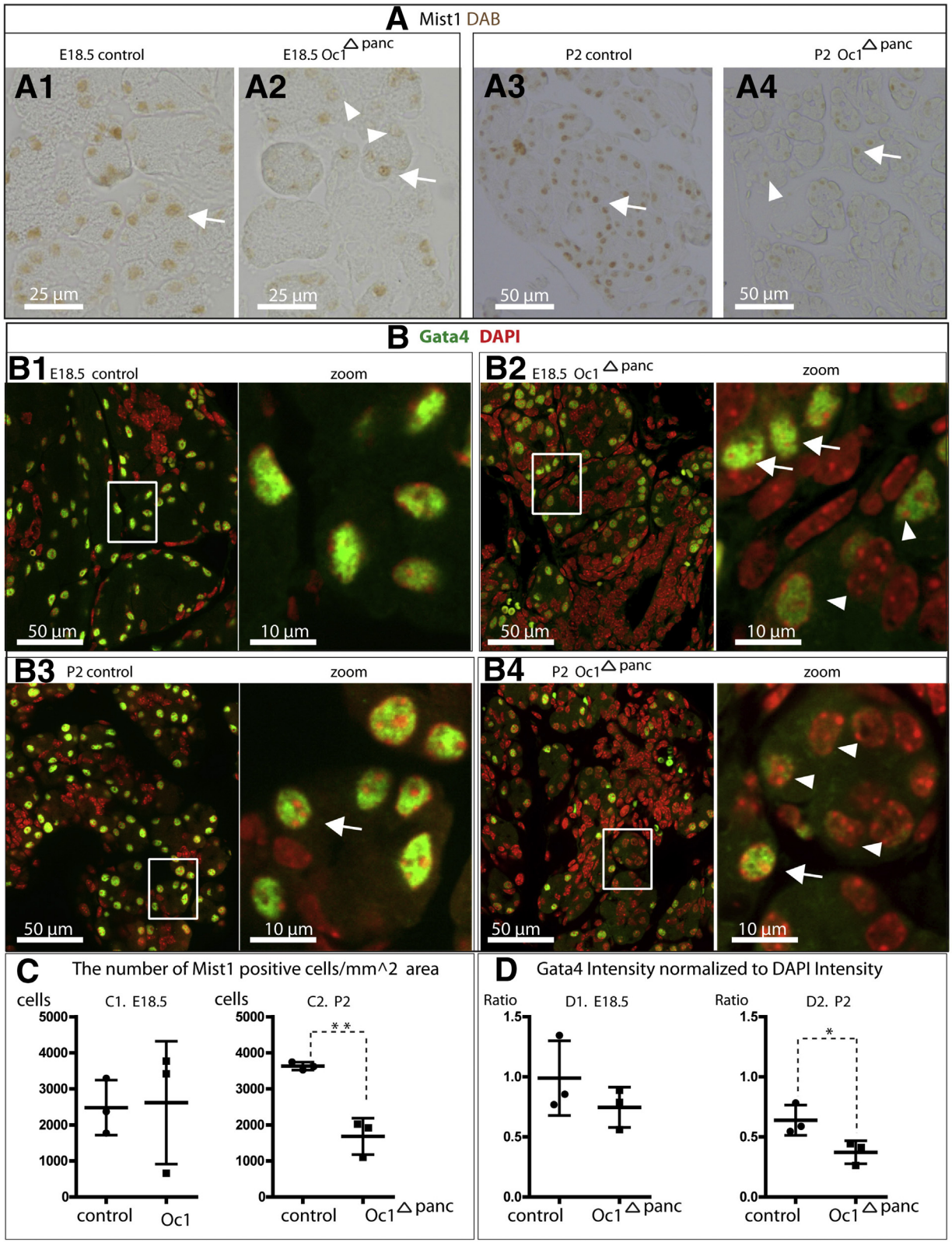

Figure 6. (*A*) Representative immunohistochemistry images of Mist1 (*brown*) from e18.5 (*A1* and *A2*) and P2 (*A3* and *A4*) Control (*A1* and *A3*) and Oc1$^{\Delta panc}$ pancreata (*A2* and *A4*). *Arrows* show cells with high Mist1 expression; *arrowheads* show cells with low Mist1 expression. (*B*) Representative immunofluorescence images of Gata4 (*green*) and DAPI (*red*) from e18.5 (*B1* and *B2*) and P2 (*B3* and *B4*) Control (*B1* and *B3*) and Oc1$^{\Delta panc}$ pancreata (*B2* and *B4*). (*C*) Quantification of the number of Mist1-positive cells at e18.5 (*C1*) and P2 (*C2*). (*D*) Quantification of Gata4 intensity at e18.5 (*D1*) and P2 (*D2*). n = 3 mice for each group. *$P \leq .05$ by 2-tailed Student *t* test.

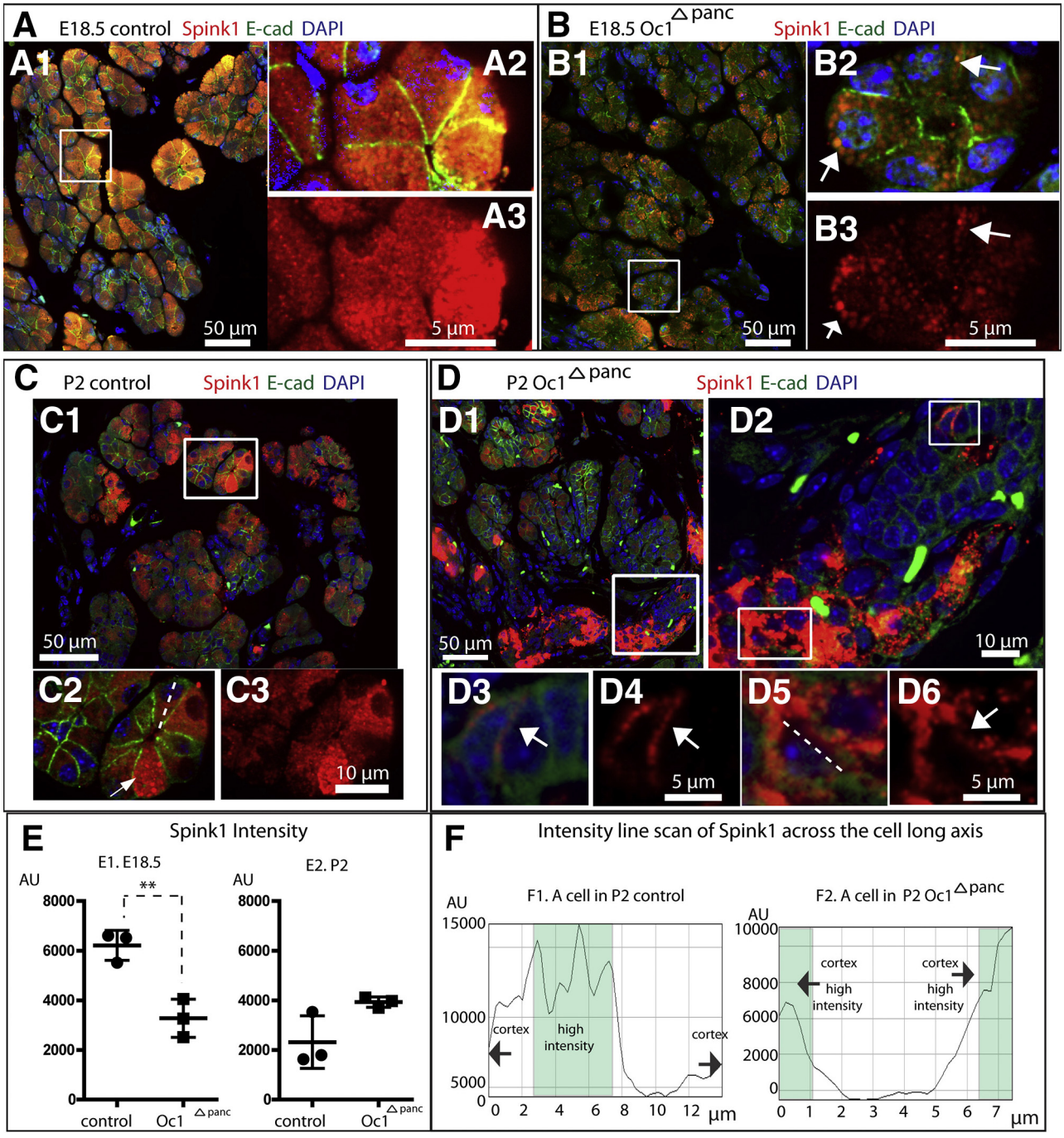

Figure 7. Representative immunofluorescence images of Spink1 (*red*), E-cadherin (*green*), and DAPI (*blue*) from e18.5 (*A* and *B*) and P2 (*C* and *D*) Control (*A* and *C*) and Oc1$^{\Delta panc}$ pancreata (*B* and *D*). *Boxed region* in *A1* is shown at higher magnification in *A2* and *A3*. *Boxed region* in *B1* is shown at higher magnification in *B2* and *B3*. *Boxed region* in *C1* is shown at higher magnification in *C2* and *C3*. *White dotted line* in *C2* indicates the long axis of the cell used for scan in *F1*. *Boxed region* in *D1* is shown at higher magnification in *D2*. Two *boxed regions* in *D2* are further zoomed in *D3* and *D4* or *D5* and *D6*. *White dotted line* in *D5* indicates the long axis of the cell used for scan in *F2*. (*B*) *Arrows* indicate high intensity Spink1 patches. (*D*) *Arrows* indicate Spink1 protein accumulated at the cell cortex. (*E*) Quantification of Spink1 intensity at e18.5 (*E1*) and P2 (*E2*). n = 3 mice for each group. $^{**}P \leq .001$ by 2-tailed Student *t* test. (*F*) Intensity line scan across the long axis of the cell indicated by the *white lines* in *C2* (*F1*, Control) and *D5* (*F2*, Oc1$^{\Delta panc}$).

transcription factors (*Ptf1a*, *Nr5a2*, *Mist1*) and functional genes (*Prss1/2*, *Spink1*, *Amylase*). These findings suggest an impairment in acinar cell identity and are consistent with the observed morphologic changes. Additionally, we detected increased expression of genes associated with other endoderm-derived organs, such as *Cym*, *Vill*, *Irx3/4*, and *Lgr5* as well as increased expression of components of the HH signaling pathway. Expression of these genes could

indicate a misallocation of pancreatic cells to alternative endoderm fates. Although histologic analyses did not reveal any obvious morphologic changes consistent with other endodermal derivatives, it is possible that cells in *Oc1*$^{\Delta panc}$ pancreata express a complement of genes associated with overlapping cell fate phenotypes. This possibility suggests that *Oc1* functions in the pancreatic epithelium in part to repress specification of nonpancreatic foregut endoderm fates.

Notably, the observed gene expression changes were still significant in spite of the variability between samples. The sample variability may be caused by the following: (1) inclusion of some endocrine cells in control samples, (2) the stress of dissociating the tissue and the subsequent selection of exocrine-enriched samples, and (3) "survival bias" whereby mutant acinar cells showing the strongest phenotype are less likely to survive the tissue dissociation procedure. The presence of activated digestive enzymes likely contributed to the greater RNA-Seq variability observed at P2 compared with e18.5. Yet, consistent and significant gene expression changes were still detected in our biologic replicates providing support for the veracity of these findings.

We were surprised that few of the genes affected in Oc1$^{\Delta panc}$ samples were associated with pancreatic ducts, and it is unlikely that duct cells were not significantly affected by loss of *Oc1*. Duct cells only constitute a small percentage of pancreatic cells and thus the changes in expression could have been masked by the changes in acinar cell gene expression. In spite of the limited ability to detect these targets, *FoxA2* and *Hnf1β* were identified as direct Oc1 targets. These findings are consistent with Oc1 directly regulating these 2 genes in hepatocytes and cholangiocytes.[35,58-61] Sorting of labeled pancreatic duct cells would likely yield additional gene expression changes and Oc1 targets associated with the ductal phenotype.

The identification of direct Oc1 targets in e18.5 pancreas provides novel information about the mechanism of how Oc1 regulates gene expression in the developing pancreas. These results demonstrate that Oc1 functions to promote expression of select acinar cell regulatory factors, such as *Ptf1a*, *Nr5a2*, and *Pdx1*. Notably, each of these factors is necessary for specification and differentiation of acinar cells during pancreas development. Oc1 did not bind to any known regulatory sequence of the *Mist1* gene even though its expression was reduced in *Oc1*$^{\Delta panc}$ samples, suggesting an indirect mechanism of regulation. Additionally, our studies revealed that Oc1 directly binds Area III of the *Pdx1* promoter, suggesting a nonendocrine role for regulation of *Pdx1* by Oc1. Pdx1 is indeed expressed in acinar cells, albeit at low levels, so we were surprised to find that *Pdx1* gene expression was not significantly reduced in our RNA-Seq analysis. It is possible that Oc1 does not actively regulate *Pdx1* gene expression at this time in spite of its binding to Area III. Interestingly, *Gata4* was identified as a direct Oc1 target but not *Gata6*. As noted above, these 2 factors have partially redundant roles in pancreas development, so it is possible that Oc1 contributes to the more nuanced regulation of these 2 factors. Oc1 also directly regulates acinar cell functional genes, such as *Prss1/2*, *Amylase*, and *Spink1*. These findings demonstrate that Oc1 has a previously unknown role in actively regulating acinar cell function through promoting expression of select digestive enzymes.

The accumulation of Spink1 at the cell cortex may be a consequence of the defects of cell polarity in Oc1$^{\Delta panc}$. Under normal conditions, Spink1 is packaged together with trypsin into the zymogen granules and then secreted from apical surface of acinar cells in a tightly regulated manner.[62,63] Our previous work has demonstrated that *Oc1* deletion leads to defects of cell polarity.[27] It is possible that when acinar cell polarity was disrupted, Spink1 failed to be secreted properly. However, the intracellular transportation machinery is still transporting Spink1 toward the cortex, leading to the accumulation of Spink1 underneath the cortex. Overall, our immunofluorescence analysis shows stronger defects in protein expression and localization at P2 than e18.5. The stronger phenotype at P2 likely reflects the cumulative defects of losing *Oc1* at earlier stages.

Both ChIP-Seq and RNA-Seq provide a starting point for understanding gene function. However, because of biologic complexity, candidate pathways/genes identified by such analysis need to be further validated at the protein expression level. Our immunofluorescence analysis found that Gata4, Mist1 expression start to decrease at E18.5, and the defects deteriorate at P2, suggesting Oc1 regulates acinar cell function through these transcription factors. Among the functional genes, we are particularly interested in *Spink1* (also called Spink3 in mouse). Our ChIP-Seq shows *Spink1* is a direct target of Oc1. Spink1 is important to prevent the self-digestion of the pancreas by the premature activation of trypsin. It was shown that Spink1 prevents serine protease-dependent cell apoptosis.[64] Losing Spink1 in mice directly leads to autophagic cell death of acinar cells.[65] Therefore, Oc1 might regulate acinar cell survival through Spink1.

Together, the current findings suggest that Oc1 has an important role in regulating differentiation and identity of acinar cells. However, it remains possible that some of the acinar dysplasia phenotypes observed in *Oc1*$^{\Delta panc}$ samples involve cell-nonautonomous effects of *Oc1* inactivation in the ducts. Defects in the pancreatic ducts have been previously demonstrated to have secondary effects on the acinar cells[66,67] and inactivation of *Oc1* in ducts results in a phenotype very similar to the Oc1$^{\Delta panc}$ model described here.[29] This group has also shown that overexpression of Oc1 in acinar cells can cause ADM and loss of acinar cell identity.[68] It is possible that *Oc1* dosage is an important determining factor for acinar cell identity versus duct cell identity. The current work provides a foundation for future work into the mechanisms whereby Oc1 regulates duct and acinar cell development.

Methods

Animals

Oc1 floxed mice are described elsewhere.[27] The *Pdx1-Cre* transgenic mice are described elsewhere.[69] Control mice carried the *Pdx1-Cre* transgene; *Oc1*$^{\Delta panc}$ mice had the genotype *Oc1*$^{fl/fl}$;*Pdx1-Cre*. All mice were on a mixed genetic

background. Mice were maintained on a 12-hour light/dark cycle and provided food and water *ad libitum*. All experiments involving mice were approved by the Institutional Animal Care and Use Committee of Vanderbilt University Medical Center. All authors had access to the study data and reviewed and approved the final manuscript.

Tissue Preparation and Imaging

Digestive organs were fixed for 4 hours in 4% paraformaldehyde at room temperature, dehydrated, cleared in xylenes, and embedded in paraffin. Paraffin-embedded tissues were cut at 5 μm, deparaffinzed in xylene, and rehydrated in water. Fluorescent and bright field images were captured using either an Olympus BX41 microscope, the Aperio ScanScope microscope and slide scanner (Vista, CA), or a Nikon spinning disk confocal driven by Nikon Elements or Nikon 600 with MagnaFire software (Optronics Engineering, Goleta, CA). Hematoxylin and eosin staining was performed as described elsewhere.[70] For sirius red/fast green staining, slides were incubated 1 hour in the staining solution (1 mg/ml Direct Red 80 [Sigma, St. Louis, MO], 1 mg/ml Fast Green [Sigma] in 3% picric acid solution), washed 2 × 5' in acidified water (1% glacial acetic acid solution), dehydrated, and mounted with a xylene-based mounting medium.

Immunofluorescence

For embryonic and postnatal analyses, tissue sections approximately 150 μm apart (2–4 per animal) were analyzed. Primary antibodies were: rat α-CK19 (TROMA III; Developmental Studies Hybridoma Bank, Iowa City, IA; 1:500), goat α-amylase (Santa Cruz Biotechnologies, Dallas, TX; 1:500), rabbit α-Ki67 (Jackson Laboratories, West Grove, PA; 1:500), mouse α-GATA4 (Invitrogen, Carlsbad, CA, 1:500), rabbit α-Spink1 (LSBio, Seattle, WA, 1:100), and rabbit α-Mist1 (a gift from Dr Stephen Konieczny, Purdue University). TUNEL labeling was carried out using the ApoAlert DNA Fragmentation Assay Kit (Clontech, Mountain View, CA) following the manufacturer's protocol. Species-specific secondary antibodies were conjugated to Cy2, Cy3, or Cy5 and diluted 1:500. Labeling with the α-CK19 antibody required incubation with 0.2 mg/mL proteinase K. Labeling with the α-Ki67 antibody required heat-mediated antigen retrieval in a 10-mM sodium citrate buffer.

RNA Acquisition and Sequencing

Pancreata were dissected from e18.5 and P2 mice, dissociated with collagenase, and exocrine-enriched samples (islets excluded) were collected for RNA extraction in 500 μl Trizol reagent (ThermoFisher, Waltham, MA). Total RNA was generated from 2 animals per genotype. RNA was isolated using the RNeasy Micro kit (Qiagen, Germantown, MD) according to manufacturer's instructions. RNA concentration and integrity were assessed using a ND-1000 Spectrophotometer (NanoDrop) and the 2100 Electrophoresis Bioanalyzer (Agilent, Santa Clara, CA) at Vanderbilt Technologies for Advanced Genomics (VANTAGE) Core. Libraries of 100-bp fragments were generated for each sample by the VANTAGE Core. Libraries were pair-end sequenced to a depth of 50 million reads on an Illumina hiSeq2000 (San Diego, CA). Sequences were aligned to the mm10 genome and DESeq2[71] was used to determine differentially expressed genes using an FDR cutoff of 0.05 and $\log_2$-fold change of 1 by the Vanderbilt Technologies[72,73] was used to perform gene ontology analysis.

Chromatin Immunoprecipitation Preparation and Sequencing

For each of 2 biologic replicates, 3–4 e18.5 pancreata were combined and minced with fine scissors. Samples were fixed 10 minutes in 1.11% formaldehyde at room temperature and reactions quenched by adding glycine to a final concentration of 0.125 M. Samples were homogenized by hand with plastic pestles. Chromatin was sheared with a Diagenode Bioruptor for a total of 22.5 minutes to an average length of 300 bp. ChIP was performed with 200 μg of DNA and 10 μg of Oc1 antibody (Rabbit α-Hnf6, Santa Cruz Biotechnology sc-13050) and Protein A/G plus agarose beads (Santa Cruz Biotechnology). DNA was purified from each reaction with the MinElute PCR Purification kit (Qiagen). Libraries were generated by HudsonAlpha Institute for Biotechnology (Huntsville, AL). Libraries were pair-end sequenced at 50 bp on an Illumina HiSeq 2000 platform to a depth of 50 million reads. Sequences were aligned to the mm10 genome and peaks were called using MACS2 software with an FDR of 0.05 by VANGARD.

Data Analysis and Statistics

To quantify acinar cell area, pancreas tissue was stained with hematoxylin and eosin. Colors were separated using a customized algorithm in Python. Image J particle analysis was used to quantify the whole pancreas area and acinar area after color separation. For immunofluorescence labeling shown in Figures 1, 2, 6, and 7, a total of 3–5 mice were analyzed for each group. For each mouse, 2–4 tissue sections approximately 150 μm apart were analyzed. The average value for each mouse was then used to compare between different groups. Image analysis was performed either with ImageScope software from the Aperio suite (Vista, CA) or with Image J. Image contrast, brightness, and gamma were adjusted equally for each image to bring out the details of the images for the purpose of illustration. Data are presented as the mean ± standard deviation. Statistical analyses were performed as 2-tailed Student t tests using GraphPad Prism 6 software.

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