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## Research Article

# Distinct Murine Pancreatic Transcriptomic Signatures during Chronic Pancreatitis Recovery

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We have previously demonstrated that the pancreas can recover from chronic pancreatitis (CP) lesions in the cerulein-induced mouse model. To explore how pancreatic recovery is achieved at the molecular level, we used RNA-sequencing (seq) and profiled transcriptomes during CP transition to recovery. CP was induced by intraperitoneally injecting cerulein in C57BL/6 mice. Time-matched controls (CON) were given normal saline. Pancreata were harvested from mice 4 days after the final injections (designated as CP and CON) or 4 weeks after the final injections (designated as CP recovery (CPR) and control recovery (CONR)). Pancreatic RNAs were extracted for RNA-seq and quantitative (q) PCR validation. Using RNA-seq, we identified a total of 3,600 differentially expressed genes (DEGs) in CP versus CON and 166 DEGs in CPR versus CONR. There are 132 DEGs overlapped between CP and CPR and 34 DEGs unique to CPR. A number of selected pancreatic fibrosis-relevant DEGs were validated by qPCR. The top 20 gene sets enriched from DEGs shared between CP and CPR are relevant to extracellular matrix and cancer biology, whereas the top 10 gene sets enriched from DEGs specific to CPR are pertinent to DNA methylation and specific signaling pathways. In conclusion, we identified a distinct set of DEGs in association with extracellular matrix and cancer cell activities to contrast CP and CPR. Once during ongoing CP recovery, DEGs relevant to DNA methylation and specific signaling pathways were induced to express. The DEGs shared between CP and CPR and the DEGs specific to CPR may serve as the unique transcriptomic signatures and biomarkers for determining CP recovery and monitoring potential therapeutic responses at the molecular level to reflect pancreatic histological resolution.

## 1. Introduction

Chronic pancreatitis (CP) is a devastating disease characterized by inflammation, fibrosis, and consequent loss of exocrine and endocrine pancreatic function. The most common etiology is alcohol consumption, known to increase the risk of CP in a dose-dependent manner and shown to be related to over 50% of CP cases [1–5]. CP imposes large bur-

dens on patients and healthcare systems, as the economic burden of CP in the US is estimated to be over \$150 million annually [6]. Further, patients with CP are at a higher risk of developing type 3c diabetes and pancreatic cancer [7, 8]. Although initially recognized as a clinical disease entity in 1946 [9], little to no therapeutic advancement has been made over the past six decades, rendering the treatment primarily supportive.

Fundamental to the lack of therapeutic advancement, the pathophysiologic mechanisms underlying pancreatic injury and recovery remain largely undetermined. Animal models continue to serve as a backbone of pancreatic research, as obtaining human pancreatic tissue throughout the disease course is not practicable. Our group has previously demonstrated the pancreas to be capable of recovery from CP lesions in one of the most widely studied animal models, the cerulein-induced mouse model [10]. How pancreatic recovery is achieved, specifically at the molecular level, remains of great interest, as identifying altered specific genes and pathways would increase understanding of the mechanisms and provide a direction for possible therapeutic advancement.

RNA-sequencing (RNA-seq) has been the main approach for studying genome-wide gene expression change and corresponding cellular or phenotypic outcome [11, 12]. Advanced analysis of the transcriptomes derived through RNA-seq provides both the global view and quantitative change of genes in disease versus normal organs. The differentially expressed genes (DEGs) through RNA-seq data analysis reveal exclusive cellular processes during disease progression to further our knowledge of the diseases, with the potential of identifying targetable pathways for therapeutic development [13–15].

We hypothesized that during CP progression and its transition to recovery, pancreatic transcriptomes will undergo an explicit transition pattern to reflect CP recovery, and the key transcriptomic signatures can be used as potential biomarkers for predicting and monitoring such transition and recovery to guide clinical decision-making. In this study, we used RNA-seq to examine the transcriptional changes occurring within the pancreas during the distinct phases of CP injury and the recovery and identified representative sets of up- and downregulated DEGs, which may contribute to CP recovery. These findings provide novel insights into identifying the involved signaling pathways in CP recovery, with potential for developing future interventional therapies leading to improved clinical treatments for CP.

## 2. Materials and Methods

**2.1. Materials.** Cerulein, the decapeptide analog of the potent pancreatic secretagogue cholecystokinin, was purchased from Bachem Americas, Inc. (Torrance, CA).

**2.2. Animals and In Vivo CP and the Recovery Model.** All animal experiments were performed in compliance with the approved IACUC protocol from the Animal Welfare Committee of the University of Texas Health Science Center at Houston. Adult male and female C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a 23°C ambient temperature, 12:12 light-dark cycle facility. Mice were fed standard laboratory chow and given water *ad libitum* and randomly assigned to control or experimental groups.

CP was induced by intraperitoneal injections of cerulein (50 µg/kg, 5 hourly injections/day, 3 days/week, for 4 weeks). Age-matched control mice were given normal saline injections. Pancreata were harvested 4 days after the final injections (CP and control (CON) groups,  $n = 12$  mice (half male and

half female)/group, total 24 mice for this experiment) and 4 weeks after the final injections (CP recovery (CPR) and control recovery (CONR) groups,  $n = 8$  mice (half male and half female)/group, total 16 mice for this experiment). The pancreas samples were fixed in 10% formalin for morphological studies as we previously described [10], frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

**2.3. RNA Extraction.** Total RNAs were extracted from pancreatic tissue samples using RNeasy Plus Universal Mini Kit following the manufacturer's instruction (QIAGEN Inc., Germantown, MD).

**2.4. Library Construction and RNA-Sequencing.** Total RNAs from the female mouse pancreas were designated as CON, CP, CONR, and CPR groups ( $n = 4$  mice/group) and used for library preparation and sequencing, which was performed by Novogene Corporation Inc. (Sacramento, CA). Briefly, quality control of total RNA sample was checked, with the RNA integrity number (RIN)  $\geq 6.0$ , OD260/280 = 1.8 – 2.2, and OD260/230  $\geq 1.8$  in all RNA samples. 1 µg of RNA was used for cDNA library construction at Novogene using an NEBNext® Ultra 2 RNA Library Prep Kit for Illumina® (cat NEB #E7775, New England Biolabs, Ipswich, MA) according to the manufacturer's protocol. Briefly, mRNA was enriched using oligo(dT) beads followed by two rounds of purification and fragmented randomly by adding fragmentation buffer. The first-strand cDNA was synthesized using random hexamer primer, after which a custom second-strand synthesis buffer (Illumina), dNTPs, RNase H, and DNA polymerase I were added to generate the second strand (ds cDNA). After a series of terminal repair, polyadenylation, and sequencing adaptor ligation, the double-stranded cDNA library was completed following size selection and PCR enrichment. The resulting 250-350 bp insert libraries were quantified using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Waltham, MA) and qPCR. Size distribution was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Qualified libraries were sequenced on an Illumina NovaSeq 6000 Platform (Illumina, San Diego, CA, USA) using a paired-end 150 run (2 × 150 bases). Approximately 40 million reads were generated from each library.

**2.5. Processing of the RNA-seq Data and Analysis of Differentially Expressed Genes (DEGs) and Gene Set Enrichment Analysis (GSEA).** We processed the RNA-seq data using the BETSY system [16]. We began by checking quality with FastQC and trimming adapter and low-quality sequence using Trimmomatic [17]. We then aligned the processed short reads to the hg19 reference genome using the STAR aligner [18] and estimated gene expression measured by transcripts per million (TPM) using RSEM [19] and the counts using HTSeq-count [20]. We verified the quality of the data and alignment using Picard [21]. The read counts were then used for calling differential expression by DESeq2 [22], and the differentially expressed genes (DEGs) were determined by the  $\text{Log}_2(\text{fold change}) > 1$  and the false discovery rate (FDR)  $< 0.05$ . For gene set enrichment analysis (GSEA), we identified enriched pathways on the

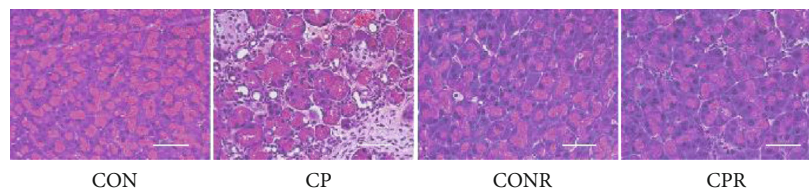


FIGURE 1: Cerulein-induced CP and the recovery mouse model. Adult C57BL/6 mice, both female and male, received cerulein injections (50  $\mu\text{g}/\text{kg}$ , ip, 5 hourly injections/day, 3 days/week) for 4 weeks for CP injury, followed by cessation of cerulein injections for 4 weeks for recovery period (CPR). Time-matched control mice received normal saline injections (CON and CONR). Pancreatic tissue sections were prepared for H&E staining and scored for CP injury as described [10]. Representative images of female mice of CON, CP, CONR, and CPR are shown. Scale bar = 50  $\mu\text{m}$ .

significant gene sets by applying the GATHER algorithm [23] implemented in BETSY using pathway annotations from the MSigDB [24].

**2.6. Quantitative Real-Time PCR (qPCR).** Total RNA samples from both male and female mice were reversely transcribed to cDNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA). The qPCR was performed using the TaqMan gene expression master mix and specific gene probe sets as previously described [25]. The probe sets of mouse *transforming growth factor- $\beta$ 1* (or  $\beta 1$ ) (Mm01178820\_m1), *collagen (Col) 1a2* (Mm00801666\_g1), *fibronectin (Fn1)* (Mm01256744\_m1), *inhibitor of differentiation (Id) 3* (Mm00492575\_m1), *growth differentiation factor (Gdf) 10* (Mm01220860\_m1), *fibroblast growth factor (Fgf) 21* (Mm00840165\_g1), *Hamp2* (Mm07306654\_gH), and *18s* (Hs99999901\_s1) (Life Technology Co., Grand Island, NY) were used in the study. The  $\Delta\text{cycle threshold (Ct)}$  value was acquired by  $(\text{Ct}(\text{gene of interest}) - \text{Ct}(\text{housekeeping gene, 18s rRNA}))$ . The  $2^{-\Delta\text{Ct}}$  value was presented, and the group means of the  $2^{-\Delta\text{Ct}}$  value were used to compare among four groups by ANOVA and to compare between male and female of respective groups by *t*-test, using GraphPad 8.0 (GraphPad Software, La Jolla, California).  $p < 0.05$  was considered statistically significant.

### 3. Results

**3.1. CP Injury and Recovery Phases Were Established from a Representative Cerulein-Induced CP Murine Model.** Chronic pancreatitis was induced in adult male and female mice by cerulein (50  $\mu\text{g}/\text{kg}$ , intraperitoneal (ip), 5 hourly injections/day, 3 days/week), and the pancreata were harvested 4 days (designated as injury group CP) or 4 weeks (designated as recovery group CPR) after the final injections as previously described and quantified on several major functional analyses [10]. The representative images in Figure 1 demonstrate the morphological changes respective to these groups. Overall, compared to the time-matched controls (CON, CONR), the injury groups displayed CP-induced acinar injury and fibrosis. The recovery groups showed a relatively normal acinar structure with over 50% reduced fibrosis compared with the injury groups. No significant differences between males and females were detected by these functional analyses.

**3.2. Distinct DEGs Were Identified in CP and the Recovery Pancreas.** To explore pancreatic recovery after CP induction

at the molecular level, RNA-seq was performed to profile pancreatic transcriptomes during CP injury and recovery. Principal component analysis (PCA) was performed on the acquired RNA-seq data for the gross expression patterns among the groups. As shown in Figure 2(a), the CP group displayed as outliers, while the CPR, CON, and CONR groups were clustered together. Figure 2(b) shows a heatmap of top 500 genes with the highest variability in gene expression for unbiased clustering of samples. Within this heatmap, DEGs in the CP group had higher expression level than other groups, with CON and CONR groups at the lowest and CPR group in the middle. The CP and CPR groups could distinguish themselves from the CON and CONR groups. Compared with the time-matched controls, we identified 3,600 DEGs in CP and 166 in CPR, among which 132 overlapped between CP and CPR, with 3,468 DEGs exclusive to CP and 34 DEGs exclusive to CPR. These results indicate that 4% of DEGs persisted from CP injury to recovery and 34 DEGs newly emerged during CP recovery. A Venn diagram was generated to represent the distribution of the DEGs (Figure 2(c)).

In the 3,468 DEGs exclusive to CP, 3,250 DEGs were upregulated and 218 DEGs were downregulated in the CP group vs. the CON group (Supplementary 1). In the 132 shared DEGs between CP and CPR, 96 DEGs were upregulated and 24 DEGs were downregulated in both CP and CPR; 1 DEG was downregulated in CP while upregulated in CPR; 11 DEGs were upregulated in CP while downregulated in CPR (Table 1). In the 34 DEGs exclusive to CPR, 31 DEGs were upregulated and 3 DEGs were downregulated (Table 2). These results demonstrate the differentially regulated gene expression in the pancreas with CP injury and at the recovery post-CP induction.

**3.3. Identified RNA-seq Signature Genes Were Validated in Both Female and Male Mice.** To validate the RNA-seq data, we utilized RNA samples from both female and male mice for validation and explored sex-dependent gene expression patterns. Figure 3(a) demonstrates the RNA-seq data of the selected pancreatic fibrosis-relevant genes. Extracellular matrix molecules *Coll1a2* [26] and *Fn1* [27], *Tgf- $\beta$ 1* [28] and one of the downstream molecules *Id3* [29], and *Gdf10* [30] displayed a similar expression pattern, highly upregulated in the CP group and returned to the control levels, except *Gdf10* which was still elevated in the CPR group. However, the RNA-seq data of *Fgf21*, a gene protective in

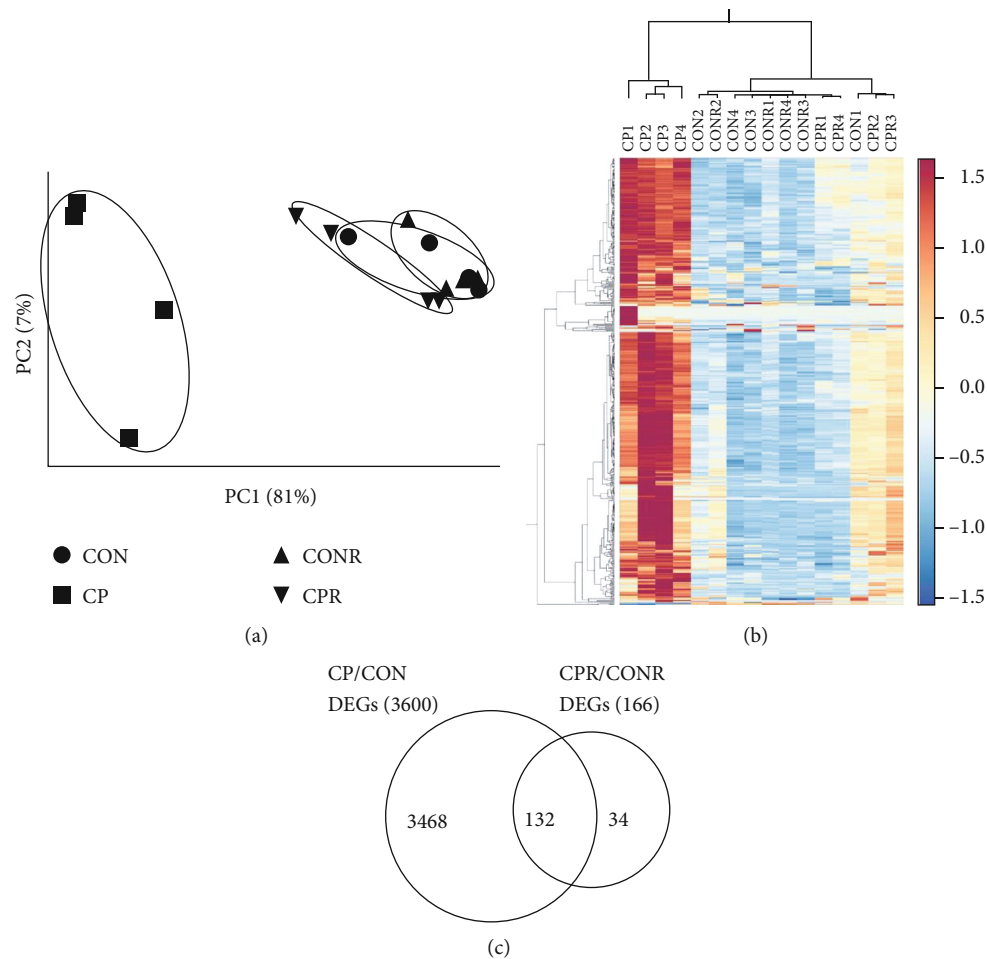


FIGURE 2: RNA-seq data analysis for the CON, CP, CONR, and CPR groups. Total RNAs were prepared from the mouse pancreata as mentioned in Figure 1, and RNA-seq was performed. (a) Principal component analysis (PCA) illustrates the variance of all genes expressed within each sample and among the groups. (b) The heatmap demonstrates the expression level of the DEGs among samples. Each column is a sample, and each row is a gene. The value is the z-score of normalized gene expression counts. For any given gene, the red color represents gene expression that is greater than the overall mean, and the blue color represents gene expression that is less than the overall mean. Hierarchical clustering of genes and samples is represented by the dendrograms on the left and across the top of the heatmap. For the CP and CPR groups, each could cluster as a unique group. (c) The Venn diagram depicts the distribution of the DEGs. The statistical criteria for a gene to be considered differentially expressed were a fold change of  $\text{Log}_2 > 1$  and false discovery rate (FDR)  $< 0.05$ .

pancreatic fibrosis [31], and *Hamp2*, a gene critical in tissue iron homeostasis [32], exhibited a different expression pattern, dramatically reduced in the CP group and partially recovered in the CPR group. These RNA-seq data were validated by qPCR (Figure 3(b)). For a better understanding of the change in gene expression pattern, we presented the qPCR data as the  $2^{-(\Delta\Delta C_t)}$  value for each gene and individual mouse in the groups (Supplementary 2) with group means in Figure 3(b). We compared CON vs. CP for gene expression in CP injury, CONR vs. CPR for gene expression in CP recovery, and CP vs. CPR for differences between CP recovery and CP injury. We also compared gene expression between female and male mice of respective groups to identify sex-dependent gene expression.

Similar gene expression patterns and levels were observed in female and male mice on *Col1a2*, *Fn1*, *Tgf-beta1*, *Id3*, and *Gdf10*, which were dramatically increased in CP ( $p < 0.05$ )

and declined to control levels or close to control levels in CPR, and consistent with RNA-seq data. Similar gene expression patterns were also observed in female and male mice on *Fgf21* and *Hamp2*, which were dramatically reduced in the CP group ( $p < 0.05$ ) and partially recovered in the CPR group. Furthermore, different expression levels of *Gdf10* and *Fgf21* were identified between female and male mice in the CP and CPR groups and *Hamp2* in the CON, CP, and CONR groups.

In light of clinical implication of our findings derived from mouse models to human diseases, we have searched relevant published articles and databases. We found respective human homologs of 6 out of 7 genes validated in qPCR assays, including ECM genes *Col1a2* and *Fn1*, TGF-beta superfamily and signaling pathway genes *Tgf-beta1*, *Id3*, and *Gdf10*, and *Fgf21* (<https://www.ncbi.nlm.nih.gov/geo/profiles>). Mouse *Hamp2* is unique in mice, and mouse

TABLE 1: DEGs shared between CP and CPR.

Group*	No. of DEGs	DEGs**
UP in CP and CPR	96	<i>Cilp, Pgc, Fetub, Kcnj15, Sfrp1, Myoc, Obp2b, Gm830, Ptn, Gdf10, Pdgfrl, Hmcn2, Lbp, Cpxm2, Inmt, Plod2, Hmcn1, Ephb2, Cygb, Fam107b, Nkd2, Slit2, Itgbl1, Eph3, Olfml3, Igsf9, Zfp521, Ms4a6d, Prune2, Sparcl1, C7, Gypc, Ngfr, Casp1, Tmem45a, Wnt5a, Klhl29, Mfap4, Reg3a, Mgp, Stum, Reg3g, Zim1, Ccr2, Pcsk5, Gm10334, Vgll3, Gfpt2, Galnt15, Ifi211, Prox1, Plekho2, Mdk, Slc39a10, Dst, Eln, Njam1, Pld1, Fbln1, Mrc1, Flt3l, Prss1, Fam102b, Ifi207, Cacna1c, Sult5a1, C1qtnf7, Ear2, Ogn, Reln, Nox4, Gli1, Lyl1, Retnla, Gask1b, Zfhx4, Ahnak, Elmo1, Cdkn1c, Bgn, Sulfb, Pla2g7, Renbp, Fes, Numbl, Ms4a7, Pcdh18, Kcnb1, Prrg3, Tenm4, Lgals9, Stab1, Ckb, Col14a1, Olfml2b, Fcrls</i>
DN in CP and CPR	24	<i>1810007D17Rik, Nxph2, Amy2-ps1, Smtnl2, Iyd, Cldn9, Dclk3, Gal, Cela3a, Cdhr2, Scn4b, Camk2b, Cyp3a13, Tulp2, Itih4, Smim24, Fgf21, Nat8f1, Padi2, Slc38a5, Hamp2, Sypl2, Vtn, Ctrl</i>
DN in CP, UP in CPR	1	<i>Reg2</i>
UP in CP, DN in CPR	11	<i>Fxyd1, Upp2, Slc6a4, Abhd11os, Susd2, Dpf1, Usp2, Sdr9c7, Pkd1l2, Apip, Try10</i>
Total	132	

\*UP: upregulated; DN: downregulated. \*\*DEGs were listed in an order of Log<sub>2</sub>(fold change) value high to low.

TABLE 2: DEGs specific to CPR.

Group*	No. of DEGs	DEGs**
UP in CPR	31	<i>Agr2, Thbs4, Lyz1, D630003M21Rik, Plekhh2, Slc6a2, Prr33, Cldn2, Tmod2, Tnfrsf18, Col8a2, Hap1, Mboat1, Eif5a2, Dnm1, 3110039I08Rik, Sema6a, Golm1, Coro2a, Rnasel, Wdr78, Ptgir, Pde4b, Speg, Dnm3, Elovl6, Nrp2, Slc37a2, Pde3a, Piezo2, Dbp</i>
DN in CPR	3	<i>Nsg1, Car4, Clec11a</i>
Total	34	

\*UP: upregulated; DN: downregulated. \*\*DEGs were listed in an order of Log<sub>2</sub>(fold change) value high to low.

*Hamp1* is the human homolog *Hamp*. In this study, *Hamp1* level was not significantly altered in CP and CPR respective to controls based on RNA-seq data, thus not validated in qPCR. The expression of these seven genes is variable in normal human pancreas. ECM genes, TGF-beta signaling pathway genes, and *Fgf21* remain low expression compared to those in other organs (<https://www.ncbi.nlm.nih.gov/geo/profiles>), while *Gdf10* expression level is the 2<sup>nd</sup> highest to that of the lungs among over 12 organs tested ([https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS422:41245\\_at](https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS422:41245_at)), and *Hamp* expression level is the 2<sup>nd</sup> highest to that of the liver among over 36 organs tested ([https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1096:220491\\_at](https://www.ncbi.nlm.nih.gov/geo/tools/profileGraph.cgi?ID=GDS1096:220491_at)).

Furthermore, in human CP pancreas, expression levels of ECM and TGF-beta signaling pathway genes are elevated [33, 34]. Taken together, the mouse pancreatic transcriptome in this study largely recapitulates human pancreatic transcriptome. However, the existing differences between mouse and human should be taken into consideration when interpreting data derived from animal studies as well as proceeding to translational studies for human diseases.

**3.4. Differential Extracellular Matrix (ECM) Remodelling and Cancer Metabolism Gene Sets Were Enriched Using GSEA.** To explore the relevant signaling pathways from the DEGs with a focus on pancreatic recovery after CP induction,

GSEA was performed for the 132 DEGs that overlapped between CP and CPR, as well as the 34 DEGs exclusive to CPR. The total gene sets generated from the 132 DEGs shared by CP and CPR groups were demonstrated in the plot as shown in Figure 4(a), with the top 20 gene sets shown in Figure 4(b) and the matched DEGs summarized in Table 3. Of note, four gene sets, NABA MATRISOME, NABA\_CORE\_MATRISOME, NABA\_MATRISOME\_ASSOCIATED, and NABA\_ECM\_GLYCOPROTEINS, are relevant to extracellular matrix (ECM) remodelling [35], and nine gene sets, CHICAS\_RB1\_TARGETS\_CONFLUENT [36], SMID\_BREAST\_CANCER\_NORMAL\_LIKE\_UP [37], DELYS\_THYROID\_CANCER\_DN [38], SCHAEFFER\_PROSTATE\_DEVELOPMENT\_48HR\_DN [39], SCHUETZ\_BREAST\_CANCER\_DUCTAL\_INVASIVE\_UP [40], WEST\_ADRENOCORTICAL\_TUMOR\_DN [41], VECCHI\_GASTRIC\_CANCER\_ADVANCED\_VS\_EARLY\_UP [42], SMID\_BREAST\_CANCER\_LUMINAL\_B\_DN, and SMID\_BREAST\_CANCER\_BASAL\_DN [37], are involved in cancer metabolism. Within the top gene sets, top three regulated DEGs in both CP and CPR are *Cilp* (2.9-fold in CP, 3.2-fold in CPR), *Pgc* (5.3-fold in CP, 2.7-fold in CPR), and *Fetub* (2.9-fold in CP, 2.4-fold in CPR).

Regarding the total gene sets of the 34 DEGs unique to the CPR group, the plot of Figure 4(c) depicts the total gene sets, with the top 10 gene sets further identified in

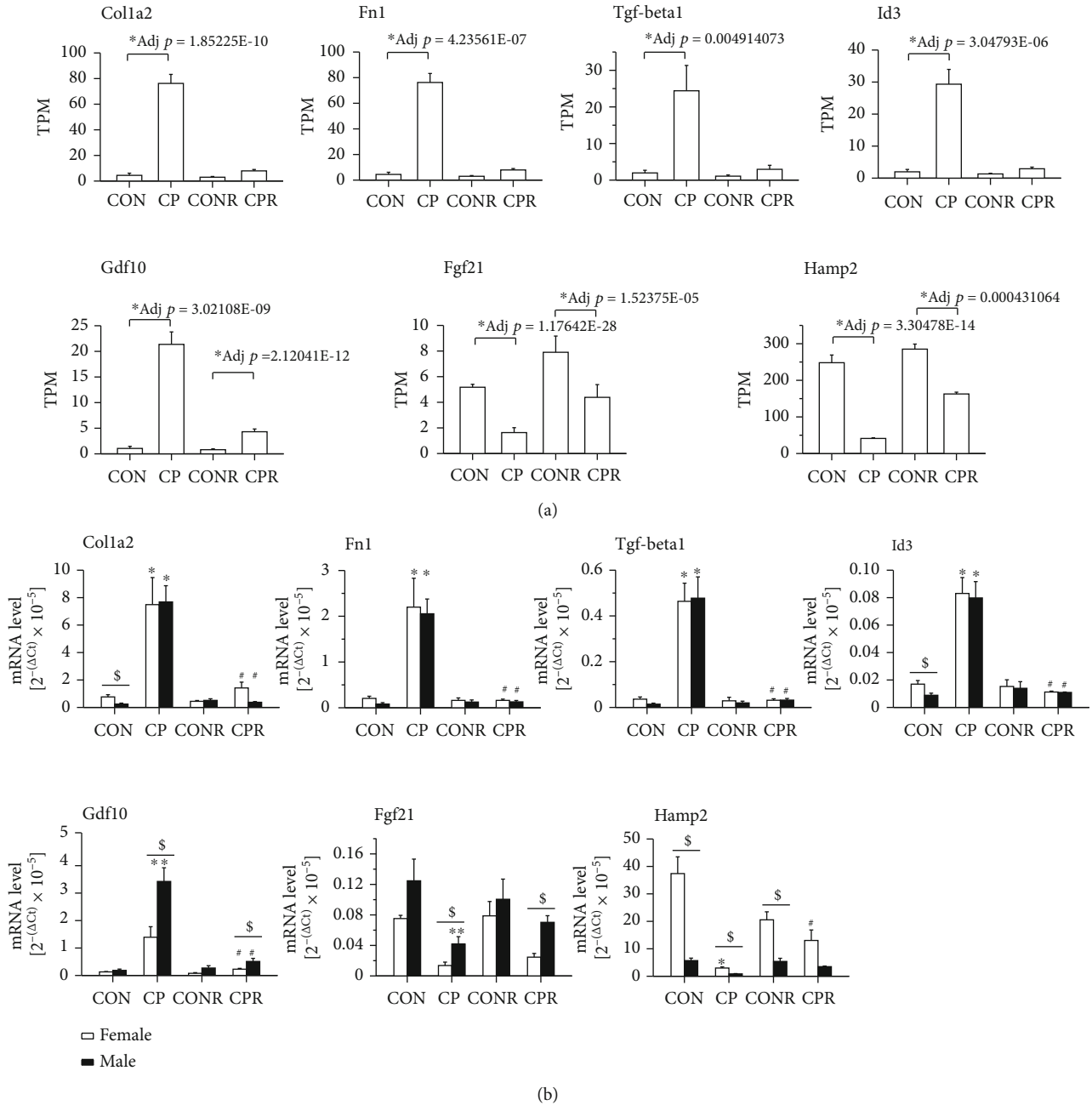


FIGURE 3: Validation of the selected DEGs from the RNA-seq data by qPCR. (a) The expression levels of the selected DEGs from RNA-seq data. TPM: transcripts per million. RNA-seq data are presented as mean of TPM  $\pm$  SEM.  $n = 4$  mice/group. \* $p < 0.05$  compared between CON and CP, between CONR and CPR. (b) The mRNA level of specific genes by qPCR. The qPCR data are presented as [group means of  $2^{-(\Delta Ct)} \pm SEM$ ]  $\times 10^{-5}$ .  $n = 3 - 6$  mice/group. \* $p < 0.05$  compared between CON and CP or CONR and CPR. # $p < 0.05$  compared between CP and CPR. \$ $p < 0.05$  compared between female and male of respective groups.

Figure 4(d) and the matched DEGs summarized in Table 4. Among the top 10 gene sets, 2 sets, FIGUEROA\_AML\_METHYLATION\_CLUSTER\_2\_UP [50] and MEISSNER\_NPC\_HCP\_WITH\_H3\_UNMETHYLATED [51], are relevant to DNA methylation, while 4 sets, DURCHDEWALD\_SKIN\_CARCINOGENESIS\_UP [52], YOSIMURA\_MAPK8\_TARGETS\_UP [53], FEVR\_CTNNB1\_TARGETS\_UP [54], and ONKEN\_UVEAL\_MELANOMA\_DN [55], are related to cell signaling pathways. Within the top gene sets,

top two upregulated DEGs in CPR are *Agr2* (2.9-fold) and *Thbs4* (1.7-fold).

#### 4. Discussion

Fibrosis of the pancreas has long been considered an end point of chronic disease. In recent years, studies of chronic pancreatic lesions in experimental animal CP models have revealed regression after withdrawal of insults, as reported



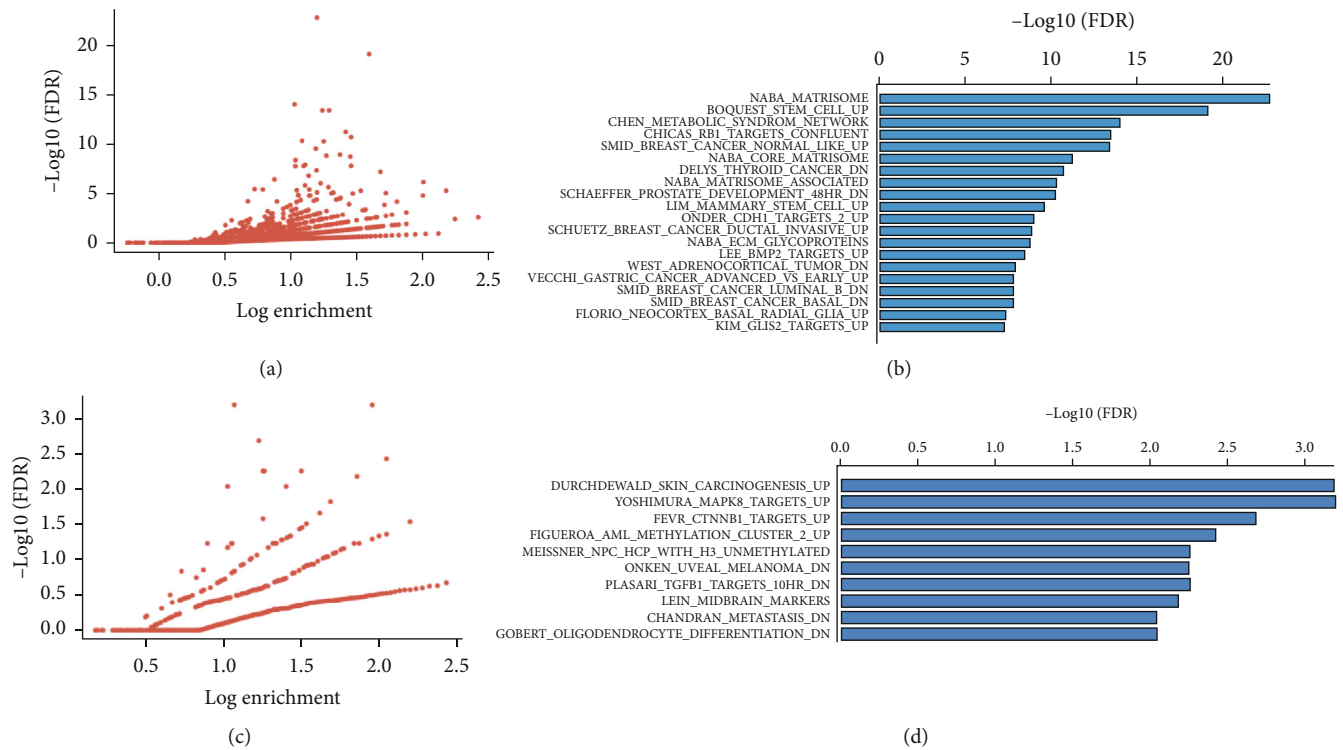


FIGURE 4: Gene set enrichment analysis of the DEGs identified from CON/CP and CONR/CPR. Distribution of GSEA enrichment FDR versus enrichment  $p$  value, both with log scale for the (a) 132 common genes between CP and CPR and the (c) 34 unique genes in CPR. (b) The top 20 gene sets enriched in the 132 common genes and (d) the top 10 gene sets enriched in the 34 unique genes, respectively.

by others [60] and our group [10]. These findings provide novel insights and the potential for the therapeutic strategies in improving CP-induced pancreatic fibrosis and function. Based on our prior findings that pancreatic histological lesions in cerulein-induced CP mice recovered after cessation of cerulein [10], in this study, we applied RNA-seq to explore CP recovery at the whole transcriptome level, with the goal of identifying potential therapeutic targets. We identified 132 shared DEGs between the CP and CPR groups, 34 unique DEGs in the CPR group, and generated the respective gene sets based on the relevant functions of these DEGs.

The top 20 gene sets generated from the 132 shared DEGs between CP and CPR are most relevant to ECM remodelling and cancer metabolism (Table 3). The ECM is a fundamental and essential component in the pancreatic stroma that provides architectural and biochemical support to cells. The core structural components of ECM include fibronectins, collagens, laminins, and proteoglycans [61]. Moreover, the ECM serves as a reservoir for growth factors, cytokines, and ECM-remodelling enzymes that collaborate with structural proteins to enhance cell signaling. In addition to its role in normal physiology, alterations of ECM have been associated with various pathologies such as fibrotic diseases, tumor development, and metastasis [62]. ECM is a key regulator of tissue repair and fibrosis development after injury [63]. In the tumor microenvironment, ECM plays an essential role in the development of distant metastasis and specific tumorigenic signaling pathways through modification of cellular adhesion proteins, promotion of angiogenic cytokines, and

immune system evasion [64]. The genes involved are differentially expressed in different cancer types and, thus, facilitate metastasis to different organ systems [65].

Furthermore, within the top 20 gene sets generated from the 132 shared DEGs between CP and CPR, we focused on several DEGs that are relevant to ECM remodelling and appear frequently in the top 20 gene sets. Cartilage intermediate layer protein (CILP) is an ECM protein that possesses an antifibrotic activity in ECM remodelling [66]. CILP-1 was reported to inhibit TGF- $\beta$ 1-induced Smad3 phosphorylation and the subsequent fibrogenic events during cardiac fibrosis remodelling. It suppressed Smad3 phosphorylation by increasing Akt phosphorylation and therefore promoting the interaction between Akt and Smad3 [66]. In this study, *Cilp* expression was increased 2.9-fold in CP and 3.2-fold in CPR and was involved in 7 out of the top 20 gene sets (Table 3). *Gdf10* has been reported to act as a tumor suppressor in mammary epithelial cells that limits proliferation and suppresses epithelial-mesenchymal transition in breast cancer via upregulation of Smad7 [67]. Here, we found that *Gdf10* expression increased in CP and declined to normal level at CP recovery (Figure 3) and was involved in 4 out of the top 20 gene sets (Table 3). These data suggest that *Cilp* and *Gdf10* play important roles in ECM remodelling during the pancreatic repairing process. *Fgf21* has been shown to protect the pancreas from inflammation and proteotoxic stress, which was downregulated by oncogenic KRAS in acinar cells to promote pancreatic tumorigenesis in mice [68]. In our study, *Fgf21* was downregulated in CP and recovered

TABLE 3: Top 20 gene sets enriched in 115 DEGs shared between CP and CPR\*.

Gene set	No. of DEGs (%)	-Log <sub>10</sub> (FDR)	DEGs	Ref.
NABA MATRISOME	30 (26.1)	22.806	<i>Bgn, C1qtnf7, Cela3a, Cilp, Col14a1, Fbln1, Fgf21, Flt3lg, Gdf10, Hmcn1, Hmcn2, Itih4, Lgals9b, Mdk, Mfap4, Mgp, Ogn, Pcsk5, Plod2, Prss1, Ptn, Reg1b, Reg3g, Reln, Sfrp1, Slit2, Sparcl1, Sulf2, Vtn, Wnt5a</i>	[35]
BOQUEST STEM CELL UP	18 (15.7)	19.127	<i>C7, Cilp, Col14a1, Fbln1, Fxyd1, Gdf10, Gfpt2, Itgbl1, Mfap4, Mgp, Myoc, Ogn, Olfml2b, Olfml3, Pdgfrl, Slit2, Tmem45a, Wnt5a</i>	[43]
CHEN_METABOLIC_SYNDROM_NETWORK	24 (20.9)	14.042	<i>Bgn, Casp1, Col14a1, Cygb, Fes, Fetub, Gask1b, Gdf10, Ifi16, Itgbl1, Lyl1, Mrc1, Ms4a6a, Ms4a7, Nfam1, Numbl, Olfml3, Padi2, Pdgfrl, Pla2g7, Renbp, Stab1, Vtn, Znf521</i>	[44]
CHICAS_RB1_TARGETS_CONFLUENT	18 (15.7)	13.430	<i>Ahnak, Bgn, C7, Ephb2, Fbln1, Galnt15, Gask1b, Ifi16, Itgbl1, Mdk, Mfap4, Olfml3, Pdgfrl, Ptn, Reln, Sfrp1, Tenm4, Vgll3</i>	[36]
SMID_BREAST_CANCER_NORMAL_LIKE_UP	17 (14.8)	13.430	<i>C7, Casp1, Ccr2, Col14a1, Elmo1, Flt3lg, Fxyd1, Ifi16, Lyl1, Mfap4, Mrc1, Ms4a6a, Pcsk5, Ptn, Reln, Sfrp1, Sparcl1</i>	[37]
NABA_CORE_MATRISOME	13 (11.3)	11.257	<i>Bgn, Cilp, Col14a1, Fbln1, Hmcn1, Hmcn2, Mfap4, Mgp, Ogn, Reln, Slit2, Sparcl1, Vtn</i>	[35]
DELYS_THYROID_CANCER_DN	12 (10.4)	10.732	<i>Elmo1, Epha3, Fbln1, Fxyd1, Gdf10, Mgp, Myoc, Ogn, Pla2g7, Ptn, Reln, Sfrp1</i>	[38]
NABA_MATRISOME_ASSOCIATED	17 (14.8)	10.353	<i>C1qtnf7, Cela3a, Fgf21, Flt3lg, Gdf10, Itih4, Lgals9b, Mdk, Pcsk5, Plod2, Prss1, Ptn, Reg1b, Reg3g, Sfrp1, Sulf2, Wnt5a</i>	[35]
SCHAEFFER_PROSTATE_DEVELOPMENT_48HR_DN	14 (12.2)	10.300	<i>C1qtnf7, Cpxm2, Ephb2, Fam107b, Fbln1, Gypc, Lbp, Mfap4, Ms4a7, Pcdh18, Prox1, Slit2, Sparcl1, Znf521</i>	[39]
LIM_MAMMARY_STEM_CELL_UP	14 (12.2)	9.564	<i>Col14a1, Cygb, Fbln1, Fxyd1, Gypc, Khlh29, Ngfr, Pcdh18, Pla2g7, Reln, Scn4b, Slc38a5, Slit2, Vgll3</i>	[45]
ONDER_CDH1_TARGETS_2_UP	11 (9.6)	8.959	<i>Bgn, Ephb2, Fbln1, Gypc, Itgbl1, Olfml3, Pdgfrl, Plod2, Prune2, Wnt5a, Zfx4</i>	[46]
SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_UP	12 (10.4)	8.846	<i>Bgn, Casp1, Cilp, Col14a1, Gfpt2, Ifi16, Itgbl1, Ms4a6a, Nox4, Olfml2b, Olfml3, Sparcl1</i>	[40]
NABA_ECM_GLYCOPROTEINS	10 (8.7)	8.758	<i>Cilp, Fbln1, Hmcn1, Hmcn2, Mfap4, Mgp, Reln, Slit2, Sparcl1, Vtn</i>	[35]
LEE_BMP2_TARGETS_UP	15 (13.0)	8.396	<i>Casp1, Cilp, Ckb, Cpxm2, Fam102b, Fxyd1, Galnt15, Mfap4, Mgp, Ngfr, Nkd2, Pld1, Renbp, Vtn, Znf521</i>	[47]
WEST_ADRENOCORTICAL_TUMOR_DN	13 (11.3)	7.912	<i>Ahnak, Bgn, C7, Fbln1, Fxyd1, Mgp, Ngfr, Ogn, Olfml3, Pld1, Plekho2, Stab1, Vtn</i>	[41]
VECCHI_GASTRIC_CANCER_ADVANCED_VS_EARLY_UP	9 (7.8)	7.818	<i>Bgn, Gfpt2, Itgbl1, Mgp, Nox4, Pla2g7, Plod2, Slit2, Tmem45a</i>	[42]
SMID_BREAST_CANCER_LUMINAL_B_DN	13 (11.3)	7.802	<i>C7, Ccr2, Col14a1, Fbln1, Gal, Ifi16, Lbp, Mfap4, Mrc1, Padi2, Ptn, Sfrp1, Tmem45a</i>	[37]
SMID_BREAST_CANCER_BASAL_DN	14 (12.2)	7.788	<i>Ahnak, Camk2b, Cilp, Col14a1, Epha3, Fbln1, Gask1b, Itgbl1, Mfap4, Ogn, Olfml3, Pcsk5, Slc6a4, Sparcl1</i>	[37]
FLORIO_NEOCORTEX_BASAL_RADIAL_GLIA_UP	11 (9.6)	7.362	<i>C1qtnf7, C7, Ccr2, Cela3a, Epha3, Itgbl1, Iyd, Kcnj15, Pcdh18, Slc6a4, Wnt5a</i>	[48]
KIM_GLIS2_TARGETS_UP	7 (6.1)	7.219	<i>Ccr2, Col14a1, Mfap4, Mgp, Ogn, Pdgfrl, Sparcl1</i>	[49]

\*Only 115 out of the 132 DEGs were found in those gene sets for the enrichment analysis.

TABLE 4: Top 10 gene sets derived from the 32 unique DEGs in CPR\*.

Gene set	No. of DEGs (%)	-Log10(FDR)	DEGs	Ref.
DURCHDEWALD_SKIN_CARCINOGENESIS_UP	4 (12.5)	3.194	<i>Dbp, Elov16, Plekhh2, Tnfrsf18</i>	[52]
YOSIMURA_MAPK8_TARGETS_UP	8 (25.0)	3.194	<i>Clec11a, Dbp, Hap1, Nsg1, Pde3a, Pde4b, Slc6a2, Thbs4</i>	[53]
FEVR_CTNNB1_TARGETS_UP	6 (18.8)	2.689	<i>Agr2, Ca4, Coro2a, Dbp, Elov16, Nrp2</i>	[54]
FIGUEROA_AML_METHYLATION_CLUSTER_2_UP	3 (9.4)	2.431	<i>Dnm3, Nrp2, Thbs4</i>	[50]
MEISSNER_NPC_HCP_WITH_H3_UNMETHYLATED	5 (15.6)	2.259	<i>Ca4, Col8a2, Slc6a2, Speg, Thbs4</i>	[51]
ONKEN_UVEAL_MELANOMA_DN	5 (15.6)	2.259	<i>Dbp, Nrp2, Nsg1, Pde4b, Sema6a</i>	[55]
PLASARI_TGFB1_TARGETS_10HR_DN	4 (12.5)	2.259	<i>Dbp, Elov16, Plekhh2, Rnase1</i>	[56]
LEIN_MIDBRAIN_MARKERS	3 (9.4)	2.184	<i>Dnm3, Eif5a2, Hap1</i>	[57]
CHANDRAN_METASTASIS_DN	4 (12.5)	2.041	<i>Col8a2, Golm1, Pde4b, Thbs4</i>	[58]
GOBERT_OLIGODENDROCYTE_DIFFERENTIATION_DN	6 (18.8)	2.041	<i>Dbp, Dnm3, Mboat1, Pde4b, Rnase1, Speg</i>	[59]

\*Only 32 out of the 34 DEGs were found in those gene sets for the enrichment analysis.

partially in CPR (Figure 3) and was involved in 2 of the top 20 gene sets (Table 3), supporting a protective role of Fgf21 in pancreatitis.

For the top 10 gene sets from the 34 DEGs specific to CPR, the top gene sets are related to DNA methylation and signaling pathways (Table 4). DNA methylation is a biochemical process that represses gene transcription. Physiological DNA methylation is crucial for normal development as well as cellular processes such as genomic imprinting, X chromosome inactivation, and normal aging. Inflammatory stimuli may lead to aberrant DNA methylation and the altered gene expression [69]. Abnormal DNA methylation has been implicated in pathological processes, including the hypo- and hypermethylation to oncogenes and tumor suppressor genes, respectively [70]. The progressive increase of DNA methylation levels has been associated with the progression of chronic inflammatory diseases developing into cancer [71]. In our study, DNA methylation was involved in 2 out of the top 10 gene sets (Table 4), suggesting that DNA methylation may be critical for disease recovery. Though few genes were reported to be methylated during CP, an increase of genes methylated during the development of pancreatic tumor was widely reported [71]. Relevant to the signaling pathways, the top gene set DURCHDEWALD\_SKIN\_CARCINOGENESIS\_UP [52] involves Podoplanin, a small membrane glycoprotein expressed in cancer-associated fibroblasts, which was reported to contribute to the progression of multiple types of tumors including pancreatic ductal adenocarcinoma [72, 73]. The gene set YOSIMURA\_MAPK8\_TARGETS\_UP [53] contains the c-Jun N-terminal kinase (JNK) pathway, one of the major signaling cassettes of the mitogen-activated protein kinase (MAPK) signaling pathway, which controls a large number of cellular processes, including proliferation, embryonic development, and apoptosis [74]. Inhibition of JNK is reported to be protective in AP [75–77] and CP [78]. The gene set FEVR\_CTNNB1\_TAR-

GETS\_UP [54] includes the Wnt/ $\beta$ -catenin signaling pathway, which is critical for the embryonic development of numerous tissues [79] and was reported to be protective in AP [80]. The gene set ONKEN\_UVEAL\_MELANOMA\_DN [55] contains *Id2* and *E-cadherin*. We previously reported that *Id2* was a downstream target of TGF- $\beta$  [81], a major profibrogenic factor in the pancreas [82]. TGF- $\beta$  suppressed *Id2*, and overexpression of *Id2* attenuated TGF- $\beta$ -induced apoptosis [81]. E-cadherin is a Ca<sup>2+</sup>-dependent adhesion protein of the classical cadherin family. Upregulation of E-cadherin expression is a protective response and promotes the repair of cell-cell adhesions of pancreatic acinar cells [83].

Furthermore, within the top 10 gene sets generated from the 34 unique DEGs in CPR, we focused on several DEGs that appear frequently in the top 10 gene sets. Thrombospondin-4 (*Thbs4*) is a top DEG upregulated in CPR with 1.7-fold and present in 3 out of the 10 gene sets. *Thbs4* is a member of the extracellular calcium-binding protein family, which plays a critical role in cellular adhesion and migration [84]. Anterior gradient 2 (*Agr2*) is another top DEG upregulated in CPR at 2.9-fold. *Agr2* has conserved roles in tissue development and regeneration and is positively correlated with human cancers. In the pancreas, *Agr2* plays an important role in cancer cell growth and survival, and the expression and secretion of *Agr2* is increased during pancreatic tumorigenesis [85, 86]. Our data suggest a promoting role of *Thbs4* and *Agr2* in pancreatic recovery and regeneration.

The regeneration gene family is known to be involved in regeneration of various tissues including pancreas  $\beta$  cells. Among this family, regenerating islet-derived 2 (*Reg2*) is significantly induced in response to diabetes, pancreatitis, high-fat diet, and during pancreatic regeneration [87, 88]. Interestingly, in our study, *Reg2* is the only DEG downregulated in CP but upregulated in CPR (Table 1), suggesting that *Reg2* may suppress inflammation and assist in tissue recovery.

The protein Hepcidin-2, which is coded by gene *Hamp2*, is an important regulator of iron entry into cells. Loss of Hepcidin signaling in mice leads to iron overload-induced chronic pancreatitis [89]. Furthermore, bone morphogenic protein (BMP) 2, an antifibrogenic protein in the pancreas [90, 91], and BMP6 have nonredundant roles in Hepcidin regulation of iron [89, 92]. In our study, *Hamp2* was downregulated in CP and CPR (Table 1, Figure 3), suggesting a potential protective role of *Hamp2* in CP.

Overall, there are several aspects of novelty in this study. First, comparing the CP injury and recovery phases against their respective time-matched controls allows us to recapitulate a dynamic change of transcriptomes during the disease and the recovery. Second, the unique DEGs identified in the CP recovery imply that the repairing/recovery phase may also be an active process that involves the newly emerging DEGs during the recovery phase. Third, several DEGs identified in the CP recovery are shared with those identified in a cerulein-induced AP recovery mouse model [93], i.e., upregulation of *Cilp* and *Agr2* and downregulation of *Fgf21* and *Hamp2*. These findings intriguingly suggest that there are commonly shared mechanisms that may contribute to AP and CP recovery. Our findings support the speculation that AP and CP, the two most commonly seen pancreatic inflammation lesions, may exist on a spectrum, shedding light on developing disease-specific therapies for AP and CP patients. We acknowledge that there are limitations in this study. For feasibility reasons, only female mouse pancreatic RNA samples were utilized in RNA-seq assays. However, using female mice for RNA-seq experiment is unique because of its contrast to the dominant male animal studies in biomedicine [94]. Furthermore, the RNA-seq data were validated using qPCR in both female and male mice. No major differences were identified between the female and male mice, except a few variable magnitudes in *Gdf10*, *Fgf21*, and *Hamp2*. Although we reported the functional analysis of the cerulein-induced CP and recovery mouse model previously [10], further investigation is warranted for the identified DEGs in CP and CP recovery for mechanistic exploration and therapeutic development.

## 5. Conclusion

Our RNA-seq profile analysis revealed more than 3,600 DEGs in the cerulein-induced CP and the recovery animal model, demonstrating a characteristic transcriptional modification or signatures for the disease recovery, with 3,600 DEGs in CP vs. 166 in CPR. From a biological standpoint, these data offer an opportunity to better understand the transcriptomic mechanisms through which CP develops and then recovers. Further investigation is warranted for exploring the functional contribution of the identified DEGs critical for the disease recovery and repair, which may lead to identification of molecular targets for therapeutic development against CP.

## Data Availability

The RNA-seq data used to support the findings of this study are included within the supplementary information file Table S1

and can be accessed at [https://www.uth.edu/bioinfo/Cao\\_RNA-seq.xlsx](https://www.uth.edu/bioinfo/Cao_RNA-seq.xlsx) data.

## Disclosure

This study has been presented as a meeting abstract in the American Pancreatic Association annual meeting 2020.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Authors' Contributions

Yinjie Zhang and Baibing Yang contributed equally to this work.

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## Supplementary Materials

*Supplementary 1.* Supplementary Table S1: a complete list of all identified DEGs.

*Supplementary 2.* Supplementary Table S2: mRNA level by qPCR assay presented as  $[2^{-(\Delta Ct)}] \times 10^5$ .

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