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ORIGINAL RESEARCH

# Targeted Inhibition of Pancreatic Acinar Cell Calcineurin Is a Novel Strategy to Prevent Post-ERCP Pancreatitis

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**SUMMARY**

This work establishes that pancreatic acinar cell calcineurin is a critical mediator of post–endoscopic retrograde cholangiopancreatography pancreatitis, using a mouse model. Importantly, the work led us to discover that calcineurin inhibitors, infused along with the radiocontrast, largely can prevent the procedural complication.

**BACKGROUND & AIMS:** There is a pressing need to develop effective preventative therapies for post–endoscopic retrograde cholangiopancreatography pancreatitis (PEP). We showed that early PEP events are induced through the calcium-activated phosphatase calcineurin and that global calcineurin deletion abolishes PEP in mice. A crucial question is whether acinar cell calcineurin controls the initiation of PEP in vivo.

**METHODS:** We used a mouse model of PEP and examined the effects of in vivo acinar cell-specific calcineurin deletion by either generating a conditional knockout line or infusing a novel adeno-associated virus–pancreatic elastase improved Cre (I–iCre) into the pancreatic duct of a calcineurin floxed line.

**RESULTS:** We found that PEP is dependent on acinar cell calcineurin in vivo, and this led us to determine that calcineurin inhibitors, infused within the radiocontrast, largely can prevent PEP.

**CONCLUSIONS:** These results provide the impetus for launching clinical trials to test the efficacy of intraductal calcineurin inhibitors to prevent PEP. *(Cell Mol Gastroenterol Hepatol 2017;3:119–128; http://dx.doi.org/10.1016/j.jcmgh.2016.08.006)*

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Endoscopic retrograde cholangiopancreatography (ERCP) is a common gastrointestinal procedure that confers a risk of acute pancreatitis ranging between 1% and 15%.[1] The efficacy of widely accepted strategies to prevent post-ERCP pancreatitis (PEP) such as pretreatment with rectal indomethacin[2] recently have been challenged.[3,4] The search for PEP prevention requires uncovering central mechanisms that initiate PEP. By using an ex vivo surrogate model of PEP, derived by isolating primary mouse and human pancreatic acinar cells, we recently showed that common radiocontrast agents used during ERCP induce acinar cell inflammatory signaling and injury through the activation of the calcium-activated phosphatase calcineurin (Cn).[5] In an in vivo model of PEP in mice, we found that global *Cn* knockout mice (deficient in *CnAβ*) or systemic inhibition of Cn with frequent dosing of the Cn inhibitors FK506 or cyclosporine A (CsA) prevented PEP. Because Cn is expressed ubiquitously, a crucial unanswered question is whether acinar cell Cn blockade by itself is sufficient to prevent PEP in vivo.

## Materials and Methods

### Reagents and Animals

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise. Mice carrying *loxP*-flanked (floxed) alleles of *CnB1* (*CnB1*$^{f/f}$; backcrossed to a C57BL/6 strain) were a kind gift from Dr Gerald Crabtree.[6] The Cre recombinase estrogen receptor T2 (*Ela-CreERT2*) mutant line was a kind gift from Dr Craig Logsdon, and it contains a transgenic insertion of a full-length acinar cell-specific mouse pancreatic elastase I (Ela) promoter that drives a tamoxifen-inducible *CreERT2*.[7] This line also was backcrossed to a C57BL/6 strain. Lox-Stop-Lox (LSL)-tdTomato Red reporter (Tm) mice were obtained from the Jackson Lab (Farmington, CT).[8] Both male and female genetically engineered mice were used for the in vivo studies. Eight- to 10-week-old wild-type male and female

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***Abbreviations used in this paper:*** **AAV, adeno-associated virus; Cn, calcineurin; CreERT2, Cre recombinase estrogen receptor T2; CsA, cyclosporine A; Ela, pancreatic elastase I; ERCP, endoscopic retrograde cholangiopancreatography; IL, interleukin; LSL, Lox-Stop-Lox; LVNS, low volume normal saline; MPO, myeloperoxidase; PEG, polyethylene glycol; PEP, post-ERCP pancreatitis; Tm, tdTomato Red reporter.**

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Swiss Webster mice weighing 25 g were used to assess the efficacy of intraductal administration of FK506 and CsA. All mice were housed at 22ºC with a 12-hour light-dark cycle and maintained on standard laboratory chow with free access to food and water. All animal experiments were performed using a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

### Generation of Conditional Pancreatic Acinar Cell-Specific CnB1 Knockouts

*CnB1*$^{f/f}$ mice were crossed with *Ela-CreERT2* mice to generate homozygous *Ela-CreERT2/CnB1*$^{f/f}$ strains. To delete *CnB1* in pancreatic acinar cells (*CnB1*$^{\Delta/\Delta}$), *CreERT2/CnB1*$^{f/f}$ mice received a cumulative dose of 5–6 mg tamoxifen given intraperitoneally either daily or every other day for a total duration of 5–6 days. PEP was induced 1 week after the last tamoxifen injection. *CnB1*$^{f/f}$ lines lacking the *Ela-CreERT2* insertion served as controls, and they also received tamoxifen.

### CnB1 $^{\Delta/\Delta}$ Genotyping

Genomic DNA was prepared from freshly isolated mouse pancreas and liver tissue, as described.[9] Briefly, the tissue was minced on ice and homogenized in sodium chloride Tris-EDTA buffer containing proteinase K. The homogenates were incubated at 55ºC for 3 hours with intermittent vortexing. After inactivation of proteinase K, the homogenates were centrifuged at 4ºC, and the supernatants containing genomic DNA were precipitated with isopropanol. The precipitated genomic DNA was pelleted at 4ºC, washed with 70% ethanol, air-dried, and dissolved in 200 $\mu$L of 1 × Tris-EDTA buffer for polymerase chain reaction. A schematic of the location and size of the expected amplicons are provided in Figure 1. Primer sequences are shown in Table 1.

The polymerase chain reaction products were separated on a 2% agarose gel and imaged. They were cut out, purified, and sequenced. All sequences were aligned to the National Center for Biotechnology Information database and manually verified to confirm *CnB1* deletion and that each component (eg, *Ela*, *Cre*, and *ERT2*) was in frame.

### Nuclear Factor of Activated T Cells–Luciferase Activity Assay

Isolated pancreatic acinar cells were infected with nuclear factor of activated T cells–luciferase adenovirus as previously described.[10] Briefly, cells were incubated with adenovirus (titer 2 × $10^9$ infectious units) for 30 minutes, and then were exposed to radiocontrast for approximately 6 hours. After stimulation, cells were collected, washed with phosphate-buffered saline once, lysed with 1× lysis buffer (E397A; Promega, Madison, WI), and centrifuged at 12,000 × g for 5 minutes at 4ºC. Luminescence was measured from the supernatant using the Luciferase Assay System (E1483; Promega) in a Synergy H1 plate reader (BioTek, Winooski, VT), and total protein, determined by the BCA kit (Thermo Scientific, Rockford, IL), was used to normalize the data.

### Adeno-Associated Virus 6 Constructs

Adeno-associated virus (AAV)6 plasmids were generated by cloning a pEla-iCre or pCMV-ZsGreen control vector into a pAAV-multiple cloning site plasmid (VPK-410; Cell Biolabs, San Diego, CA), as previously described.[11,12] Once cloned, the AAV6 plasmid was transfected into HEK293 cells along with 2 helper plasmids: (1) pAAV-RepCap (0912-06; Applied Viromics, Fremont, CA), which is a packaging plasmid that carries the serotype 6 rep and cap genes; and (2) pHelper (0913; Applied Viromics), which is a plasmid that carries the helper genes. Cells were collected 72 hours after transfection and suspended in lysis buffer containing 50 mmol/L Tris, 150 mmol/L NaCl, and 2 mmol/L $MgCl_2$.

### Purification of AAV6 for In Vivo Administration

AAV6 was purified as previously described.[12,13] Briefly, transfected HEK293 cells were freeze-thawed 3 times to release the AAV6 virus. Cell lysates were treated with benzonase (0.05 U) at 37ºC for 30 minutes, followed by 1% sodium deoxycholate at 37ºC for 30 minutes. Lysates were spun at 2500 × g for 10 minutes, and the supernatant was collected. AAV6 was precipitated using a 1:4 mixture of 40% polyethylene glycol (PEG-800) and 2.5 mol/L sodium chloride for 2 hours at 0ºC. The solution was spun at 2500 × g for 30 minutes to collect the PEG pellet. The pellet was resuspended in HEPES buffer (50 mmol/L), treated with an equal volume of 100% chloroform, spun at 2500 × g for 10 minutes, and air-dried for 30 minutes. Two-phase partitioning was performed using 50% ammonium sulfate and 40% PEG-800 and spun at 2500 × g for 15 minutes. The ammonium sulfate phase was collected and dialyzed using a 10-kilodalton molecular weight cut-off Slide-A-Lyser Dialysis Cassette (66810; Thermo Scientific) for 4 hours. Dialysis was repeated a second time for 16 hours. The AAVs were concentrated using a 50-kilodalton centrifugal filter unit (UFC905024; Millipore, Billerica, MA) and stored at -80ºC. The QuickTiter AAV Quantitation Kit (VPK-145; Cell Biolabs) was used to measure viral concentrations.

### Pancreatic Ductal Infusion of AAV6 Into CnB1$^{f/f}$ Mice and Immunofluorescence

The surgical procedure for retrograde pancreatic ductal infusion of the AAV6 was as previously described.[5] Briefly, 100 $\mu$L of purified AAV6 (titer 2 × $10^{12}$ plaque-forming units) was infused into the biliopancreatic duct at a rate of 10 $\mu$L/min for 10 minutes using a P33 peristaltic syringe pump (Harvard Apparatus, Holliston, MA). Surgical anesthesia was achieved by inhaling isoflurane and oxygen. A single injection of the analgesic buprenorphine (0.075 mg/kg) was given immediately after the surgery. Mice recovered on a heating pad for 30 minutes and were housed for 4–6 weeks with free access to food and water before induction of PEP. To verify the efficacy of the AAV6 infusion, LSL-Tm mice were used. One hundred microliters of purified AAV6Ela-iCre (titer 2 × $10^{12}$ plaque-forming units) was infused into the pancreatic duct as described earlier. Five weeks after the surgery, pancreas tissue, along with the abdominal organs en bloc, was imaged using a fluorescence