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Original article

Amino acid transporters expression in acinar cells is changed during acute pancreatitis

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ABSTRACT

Pancreatic acinar cells accumulate amino acids against a marked concentration gradient to synthesize digestive enzymes. Thus, the function of acinar cells depends on amino acid uptake mediated by active transport. Despite the importance of this process, pancreatic amino acid transporter expression and cellular localization is still unclear. We screened mouse pancreas for the expression of genes encoding amino acid transporters. We showed that the most highly expressed transporters, namely sodium dependent SNAT3 (Slc38a3) and SNAT5 (Slc38a5) and sodium independent neutral amino acids transporters LAT1 (Slc7a5) and LAT2 (Slc7a8), are expressed in the basolateral membrane of acinar cells. SNAT3 and SNAT5, LAT1 and LAT2 are expressed in acinar cells. Additional evidence that these transporters are expressed in mature acinar cells was gained using acinar cell culture and acute pancreatitis models. In the acute phase of pancreatic injury, when acinar cell loss occurs, and in an acinar cell culture model, which mimics changes occurring during pancreatitis, SNAT3 and SNAT5 are strongly down-regulated. LAT1 and LAT2 were down-regulated only in the *in vitro* model. At protein level, SNAT3 and SNAT5 expression was also reduced during pancreatitis. Expression of other amino acid transporters was also modified in both models of pancreatitis. The subset of transporters with differential expression patterns during acute pancreatitis might be involved in the injury/regeneration phases. Further expression, localization and functional studies will follow to better understand changes occurring during acute pancreatitis. These findings provide insight into pancreatic amino acid transport in healthy pancreas and during acute pancreatitis injury.

1. Introduction

The human pancreas produces approximately 1–1.5 l of fluid daily containing 5–15 g of protein, predominantly digestive enzymes. These are synthesized and secreted upon hormonal stimulation by pancreatic acinar cells and released into the ductal system. Amino acid uptake from blood is required to satisfy the high demand of acinar cells to support protein synthesis. Consequently, a multitude of amino acid transport systems may be expressed in the acinar cells. In the last decades, amino acid transporters have been molecularly characterized, and classified based on sequence homology into SoLute Carrier families (SLC) [1,2]. Many of these transporters are expressed in intestinal and renal epithelial cells localized in the luminal or basolateral membranes [3–5]. Since antiporters and sodium-dependent symporters are expected to be responsible for the accumulation of amino acids in these cells, we hypothesized that these transporters are also expressed and localized in the basolateral membrane of the exocrine pancreas acinar cells.

In the secretory epithelia of the exocrine pancreas, amino acids are transported across the basolateral membrane from the plasma

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against a concentration gradient into acinar cells [6,7]. Functional experiments in perfused pancreas have shown that neutral amino acids are transported into acinar cells by sodium-dependent and -independent mechanisms that are inhibited by competition with several amino acids. L-alanine, glycine, L-methionine, L-leucine, L-phenylalanine, L-tyrosine and L-arginine are transported at a higher rate compared to L-tryptophan and L-aspartate [7]. The transport of L-alanine into acinar cells is sodium-dependent and evokes depolarization of the cells, without concomitant zymogen secretion [8]. This transport is in competition with glycine, but not L-arginine [9]. L-alanine induced depolarization leads to potassium but not calcium efflux, suggesting a sodium-coupled transport with a functional interaction with the Na^+,K^+-ATPase and potassium channels [8]. Another feature of transport through the basolateral membrane is the ability to be increased or trans-stimulated by other amino acids. L-serine and the amino acid analog alpha (methyl amino)-isobutyrate (MeAiB) when accumulated in the exocrine pancreas can be exchanged with several other neutral amino acids, suggesting the presence of antiporters on the basolateral membrane [10]. These and several other studies suggest that various transporters mediate the accumulation of specific amino acids in acinar cells, however, their molecular identity remains still unclear.

In humans, acute injury to the exocrine pancreas, or acute pancreatitis, changes the amino acid requirements [11,12]. Pancreatitis is a self-digestive degenerative process with massive loss of differentiated exocrine acinar tissue, followed by a regenerative process. It has been suggested that amino acid supplementation is useful for the treatment of patients with severe pancreatitis [11–13]. However, changes in the requirements of the tissue during injury, regeneration and repair are unclear. Expression levels of amino acid transporters and their localization on the cell membrane may influence the available amino acid pool. A well-established experimental mouse model (based on injections of the cholecystokinin analogue caerulein) induces tissue damage and recapitulates the dedifferentiation, proliferation and eventual regeneration of acinar cells [14,15]. The loss of differentiated acinar tissue in this model is followed by a total recovery about one week after injury [14].

The aim of this study was to identify and quantify amino acid transporters expressed in the basolateral membrane of acinar cells of the healthy mouse exocrine pancreas and in acute caerulein pancreatitis model to gain a perspective on the amino acid transport changes induced during injury and regeneration.

2. Material and methods

2.1. RNA extraction and reverse transcription

Tissue was collected and snap-frozen in liquid nitrogen until further use. Animal handling was in accordance with the Swiss Animal Welfare laws and approved by the Zurich Cantonal Veterinary office. Total RNA was extracted from the pancreas using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). For RNA extraction, tissues were thawed in RLT buffer (Qiagen) containing 10 μL/ml β-mercaptoethanol (Sigma–Aldrich, Buchs, Switzerland) and homogenized on ice. RNA was treated on column with DNase for 15 min at 25 °C to reduce genomic DNA contamination. RNA quantity was analyzed by ND-1000 NanoDrop® UV-spectrophotometer (NanoDrop Technologies Wilmington, DE, USA) and RNA quality assessed by microchip analysis Agilent 2100 Bioanalyzer® (NanoChip®, Agilent Technologies, Santa Clara, CA, USA). Total human RNA was purchased from Clontech-Takara (Mountain View, CA, USA). Total RNA (20 ng/μL reaction) was used for reverse transcription with the TaqMan Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer recommendations.

2.2. Real-time quantitative PCR

Real-time PCR was performed using 10 ng of cDNA as a template. Reactions were carried out using the Taq-Man Universal PCR master mix (Applied Biosystems) according to the recommendations of Applied Biosystems. Primers and probes used were either previously described [16–24] or as described in Table 1.

Primers were chosen to result in amplicons of 70–100 bp that span intron–exon boundaries or that comprise large introns that exceed the amplification capacity of the enzyme, to avoid the effects of potentially contaminating genomic DNA. The specificity of the primers was first tested on cDNA derived from several tissues and resulted in a single product of the expected size (data not shown). Probes were labeled with reporter dye FAM at the 5′ end and the quencher dye TAMRA at the 3′ end (Microsynth, Balgach, Switzerland) or no dye (Roche, Basel, Switzerland). Reactions were run in 96-well optical reaction plates using a Prism 7700 cycler (Applied Biosystems). Thermal cycles were set to an initial cycle at 50 °C (2 min), then a single cycle at 95 °C (10 min) followed by 45 cycles alternating between 95 °C (15 s) and 60 °C (1 min) with auto ramp time.

All reactions were run as duplicates. The abundance of the target mRNAs was calculated as relative expression ratios to 18S ($R = 2^{-[Ct(\text{Housekeeping gene}) - Ct(\text{test})]}$), where Ct is the cycle number observed at the threshold.

2.3. Immunofluorescence

Both paraffin- and cryosections were used. Healthy pancreas was fixed in 3% paraformaldehyde (PFA) for 4 h. Paraffin embedding was performed with the Microm STP 120 (MICROM International, Walldorf, Germany) by immersion in progressive gradients of EtOH, Xylol and finally paraffin. Deparaffination was achieved by reversing the order of immersion. After fixation specimen for cryosections, were immersed in cryoprotectant (PBS and 30% sucrose) overnight at 4 °C, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek Europe) and snap-frozen in liquid propane. Tissues were

Table 1
Primers and probes used for real time PCR.

Gene	Primers	Probe	Acc. no.
Slc7a13	S: ACCAAGAGCCCAATCTACACAGA A: GGTGATGCCCAACGCTATG	CATATAAGGTGTTTTTACCGTTCA	NM_028746.3
Slc7a12	Applied Biosystems	Assay ID 00499866_m1	NM_080852
Slc16a14	Applied Biosystems	Assay ID 01272722_m1	NM_027421
Slc36a1	S: GTGTTTCTGGCGGACAACTT A: AGTTGGTGGTGGTCCCAT T	Universal probeLibrary Roche#34	NM_153139.4
Slc36a2	S: TCATTTGAAAGCATCGGTGT A: AGACAGAATGGTTGGGAAGC	Universal probeLibrary Roche#34	NM_153170.3
18S	Applied Biosystems	Assay ID 4310893E	Eukaryotic

stored at −80 °C. Serial sections, paraffin- and cryosections (5 and 9 μm thickness respectively) were used for immunofluorescence.

The best conditions for each antibody were established for both paraffin- and cryosections using different antigen retrieval techniques (no retrieval, incubation in 0.1% SDS for 5 min at room temperature and boiling in 10 mM Na^+-Citrate for 10 min). Sections were incubated with primary antibodies (diluted in PBS − 2% BSA, 0.04% Triton X-100) overnight at 4 °C and subsequently with the appropriate secondary antibodies (Table 2). Amylase and insulin were used to identify acinar and beta cells respectively. The lectins Peanut agglutinin (PNA) and Dolichos biflorus (DBA) were used to identify acinar apical membrane and ducts respectively. Sections were mounted using DAKO Glycergel mounting medium (DakoCytomation, Denmark). Sections were viewed on a Nikon Eclipse TE300 epifluorescence microscope (Nikon Instruments Inc, Melville, NY) equipped with a DS-5M Standard charge-coupled device camera (Nikon Instruments Inc) and acquired with NIS-Elements (Nikon Instruments Inc). Images were merged using Photoshop 9.

2.4. Sample preparation and Western Blot analysis

Pancreas of mice injected with saline (control) or caerulein (as described in the following section) were used to the prepare crude membrane used on Western blots. Briefly, as previously described by Dave and Bourgeois et al. [17,25], the pancreas was removed and cleansed in ice-cold PBS. The tissue was homogenized in ice-cold K-Hepes buffer (200 mM, mannitol, 80 mM K-Hepes, 41 mM KOH, pH 7.5) using MagNa Lyser Green Beads (Roche, Switzerland). After centrifugation at 1000× *g* for 20 min at 4 °C, the supernatant was subsequently centrifuged at 100 000× *g* for 1 h at 4 °C to obtain total crude membrane. 25–50 μg of crude membrane protein were solubilized in Laemmli sample buffer, and SDS-Page was performed on 8% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA). After blocking, the blots were incubated with the primary antibodies (for dilutions see Table 2), and donkey anti-rabbit-HRP (1: 5000; Promega, Madison, WI, USA). Antibody binding was detected with the Luminata HRP chemiluminescent substrate (Millipore). Chemiluminescence was detected with the luminescent image analyser LAS-4000 (Fujifilm). Mouse monoclonal anti-actin (42 kDa, Sigma) signal was used to normalize the samples. Quantification of the bands was performed using the free software imageJ (http://rsbweb.nih.gov/ij/index.html).

2.5. Pancreatitis mouse models

Acute pancreatitis was induced *in vivo* in C57Bl/6 mice (either starved for 12 h or fed ad libitum) by treatment with supra-physiological repetitive doses of caerulein (Sigma, Louis, MO, USA) for 12 h (12 injections of 50 μg/kg every hour). The animals were sacrificed 12 h after the last injection.

To model pancreatitis *in vitro*, acinar cells were isolated and cultured as previously described [26]. In brief, male CD1 mice (10–14 weeks) were utilized and dissected pancreata were digested in a collagenase solution. The digested tissue was filtered (500 and 100 mesh gauze) to separate the acini prior to culture in suspension with RPMI-1640 medium containing 10% FBS for 5 days.

2.6. Expression arrays and data analysis

Total RNA was extracted from the acinar cell cultures using RNaqueous kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions and RNA quality was determined using the Agilent 2100 bioanalyzer. mRNA expression analysis was performed using the MoGene-1_0-st-v1 platform from Affymetrix and raw expression data was extracted using the Expression Console software. Differential expression analysis was performed using the web-based suite, Pomelo II. A limma paired *t*-test was used to determine the significance of differentially regulated genes. A false discovery rate (FDR) of less than 0.05 was used to assign significantly differentially expressed genes.

2.7. Statistics

Pooled data are shown as means ± SEM (n) where n represents the number of independent observations. For each gene analyzed (35 genes in total, 2 were not detected in at least 1 group) an ANOVA (Analysis of Variance) was performed followed by Bonferroni post-test comparing control fed with pancreatitis fed animals and fed versus starved pancreatitis using appropriate software (Prism v. 4.0, GraphPad, San Diego, Calif., USA). For the 70 pairwise Bonferroni comparison, false discovery rate (FDR) correction was performed [27,28]. The FDR was set at 0.1, what allows us to infer with 95% confidence that the means between the groups compared by Bonferroni are different. After FDR correction, only p values $\leq$0.02 were considered significant.

Table 2
Antibodies detecting amino acid transporters and cell markers used for the localization experiments and the dilutions used for Western Blot.

Antigen	Primary antibody	Antigen retrieval	Section, antibody dilution	Secondary antibody (molecular probes)	Dilutions used for Western Blot analysis
LAT2[a]	CPIFKPTPVKDPDSEEQP Eurogentec (Seraing, Belgium)	None/SDS	cryosection, 1:1000	Alexa Fluor 594 donkey-anti-rabbit IgG (1:500)	1:2000
LAT1	Cosmo Bio (KE026)	None/SDS	cryosection, 1:100		1:500
SNAT3[a]	MEIPRQTEMVELVPNGKC Pineda (Berlin, Germany)	None/SDS	cryosection, 1:200		1:500
SNAT5[a]	MEMQEPKMNGTLSAGC Pineda (Berlin, Germany)	None/SDS	cryosection, 1:5000		1:5000
TAT1[a]	CGSSGIFKKDASII Genosphere (Paris, France)	Na^+-citrate	Paraffin, 1:200	Alexa Fluor 488 donkey-anti-rabbit IgG (1:500)	
Insulin	Abcam (ab7842)	Na^+-citrate	Paraffin, 1:1000	Alexa Fluor 568 goat anti-ginea pig IgG (1:500)	
Amylase	Lifespan (LS-C11236)	None/SDS	cryosection, 1:1000	Donkey-anti-sheep (DyLight® 488) Abcam (1:500)	
Dapi (Nucleus)	Merk (124653)		1:5000		
PNA-FITC	Vector		1:500		
DBA-FITC	Vector		1:100		

[a] Tailor-made antibodies.

3. Results

3.1. The mouse pancreas expresses several amino acid transporters

Currently 44 genes from 11 solute carrier families are known to encode amino acids and peptide transporters [1,2]. We analyzed the mRNA expression of 37 genes encoding amino acid, oligopeptide, orphan transporters and accessory proteins from eight Slc families in total mouse pancreas by real-time qPCR (Fig. 1A–D). From the Slc7 family, which is divided into cationic amino acid transporters (CAT) and heterodimeric amino acid transporters (HAT) [29], we analyzed the mRNA expression of one CAT (*slc7a1*) and 9 HAT members in addition to the Slc3 family glycoprotein accessory proteins genes (Fig. 1A). The genes with the highest mRNA expression were *slc7a8* and *slc3a2* that encode the transporter LAT2-4F2. LAT2-4F2 is a sodium-independent antiporter for large neutral amino acids. Also transcripts for *slc7a5*, *7a6*, *7a7* and *7a11* respectively encoding the transporters LAT1, y^+LAT2, y^+LAT1 and

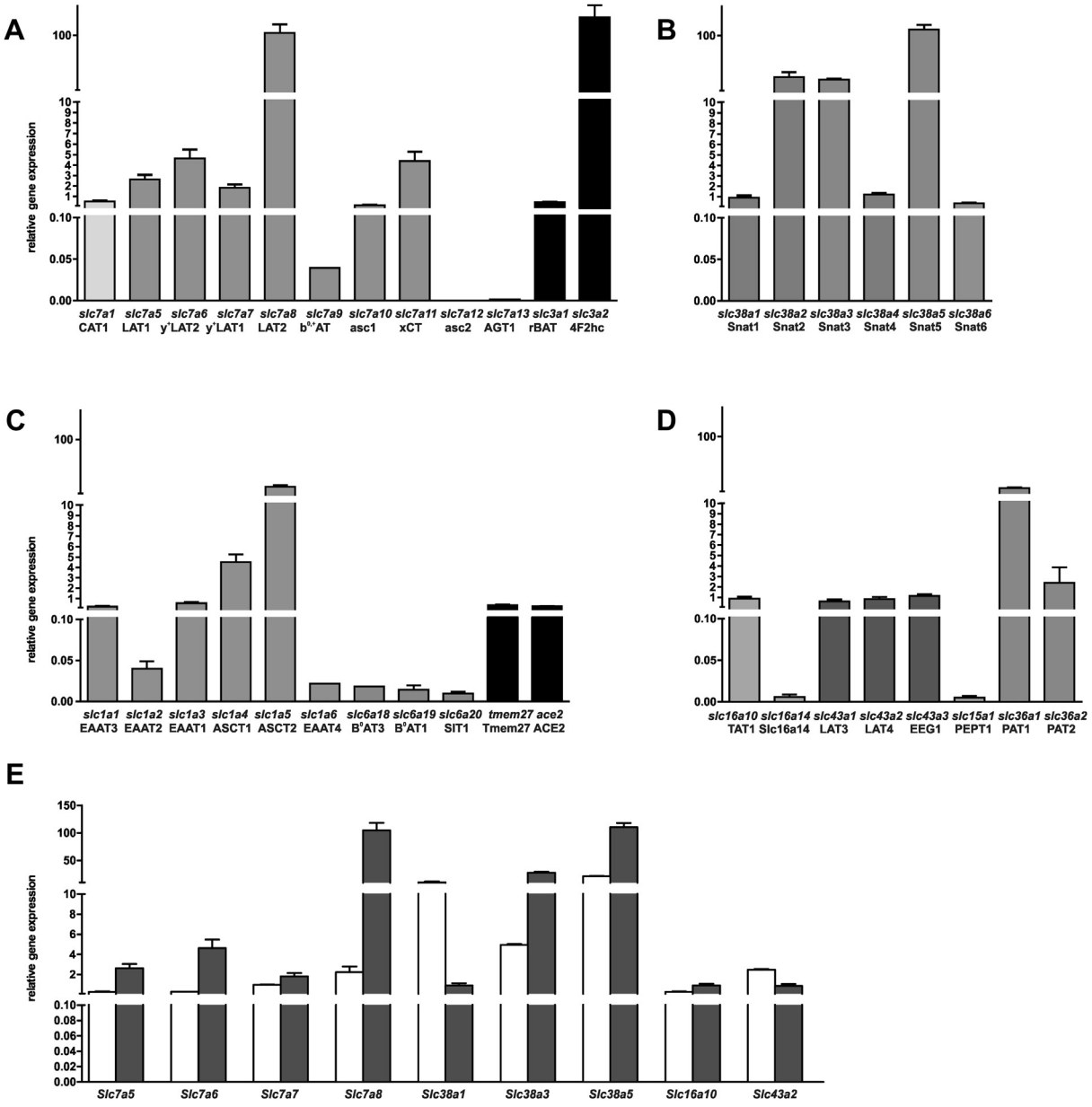

Fig. 1. The mouse pancreas expresses several amino acid transporters. Total pancreas of wild type animals were used to analyze the gene expression of amino acid transporters by qPCR. The expression of genes encoding members of several amino acids families was analyzed. A) Relative expression of genes encoding amino acid transporters from family Slc7: *slc7a1* (CAT1), *slc7a5* (LAT1), *slc7a6* (y^+LAT2), slc7a7 (y^+LAT1), *slc7a8* (LAT2), *slc7a9* ($b^{0,+}$AT), *slc7a10* (asc1), *slc7a11* (xCT), *slc7a12* (asc2), *slc7a13* (AGT1), and *slc3a1* (rBAT) and *slc3a2* (4F2hc). B) Genes encoding the amino acid transporters from the Slc38 family, System A, *slc38a1* (SNAT1), *slc38a2* (SNAT2), *slc38a4* (SNAT4); system N: *slc38a3* (SNAT3), *slc38a5* (SNAT5); *slc38a6* (Orphan SNAT6). C) Genes expressing amino acid transporters from families Slc1 and Slc6: *slc1a1* (EAAT3), *slc1a2* (EAAT2), *slc1a3* (EAAT1), *slc1a4* (ASCT1), *slc1a5* (ASCT2), *slc1a6* (EAAT4), *slc6a18* (B^0AT3), *slc6a19* (B^0AT1), *slc6a20* (SIT1) and the RAS members identified as the Slc6 family accessory proteins *tmem27* (Tmem27/collectrin) and *ace2* (ACE2). D) Genes encoding transporters from Slc16, 43, 15 and 36 families: *slc16a10* (TAT1), orphan *slc16a14*, *slc43a1* (LAT3), *slc43a2* (LAT4), *slc43a3* (EEG1), *slc15a1* (PEPT1), *slc36a1* (PAT1) and *slc36a2* (PAT2). The expression is represented relative to the house-keeping gene ($18S \times 10^6$). Data points represent mean values of 3 animals ± SEM. Asc2 not detected. Black bars, accessory proteins. Gene expression is expressed relative to the housekeeping gene ($18S \times 10^6$). E) Expression of SLC38A5 is high in humans and mouse. The expression of several genes from the SLC families 7, 38, 16 and 43 were assayed in human pancreatic RNA and compared with the expression in mouse. Gene expression from both species is expressed relative to the same housekeeping gene ($18S \times 10^6$). The white columns represent the human genes, while the gray columns depict the mouse homolog.

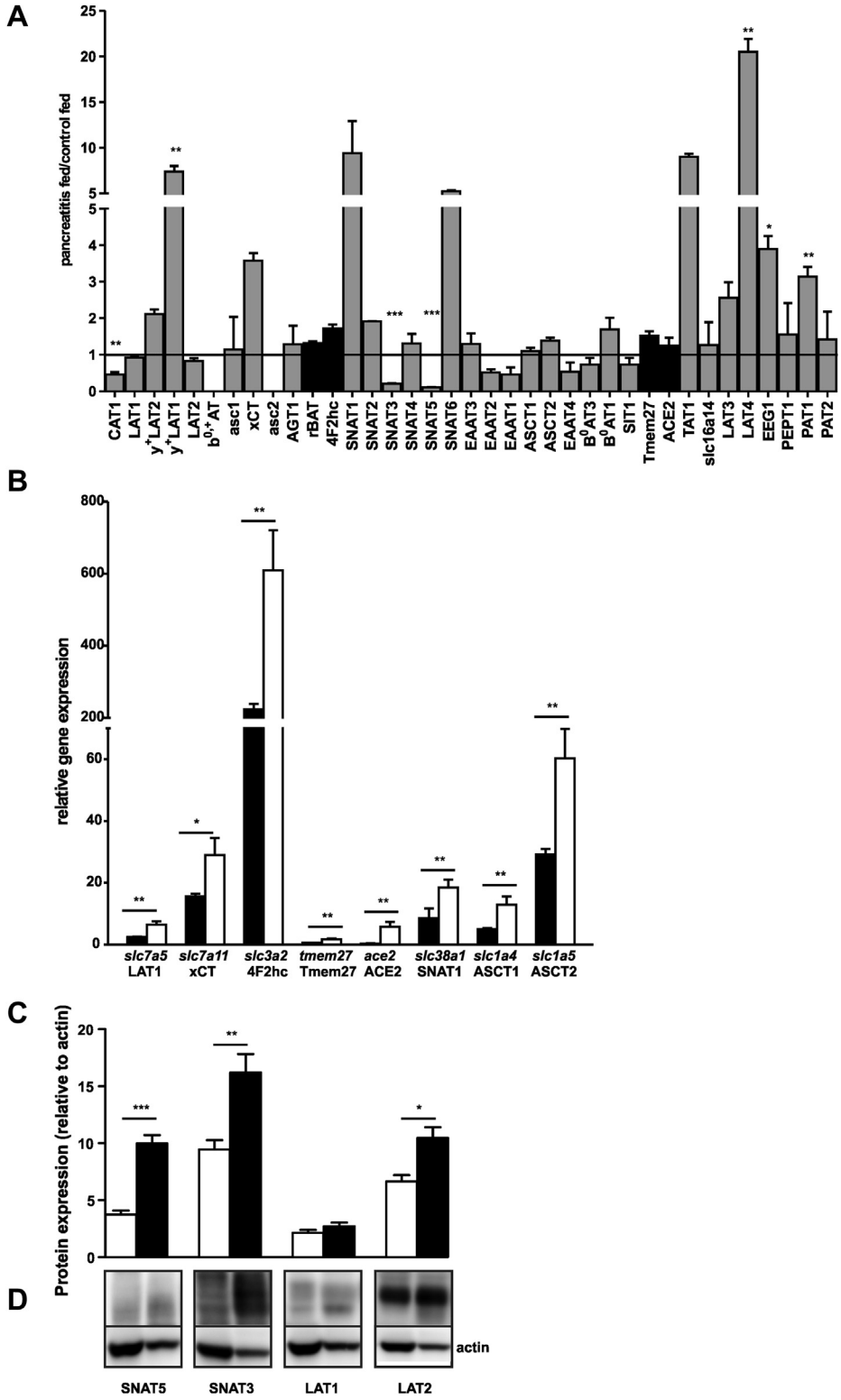

Fig. 2. The expression of a subset of amino acid transporters is changed in pancreatitis. A) Modified amino acid transporter genes expression in the acute phase of pancreatitis. The relative expression of the genes in animals with induced pancreatitis was normalized to control animals. Slc7a9 ($b^{0,+}AT$) and slc7a12 (asc2) were not detected. Black bars, accessory proteins. $^{*}p < 0.02$ after FDR B) In starved animals the genes encoding LAT1, xCT, 4F2, Tmem27, ACE2, SNAT1, ASCT1 and ASCT2 (*slc7a5*, *slc7a11*, *slc3a2*, *tmem27*, *ace2*, *slc38a1*, *slc1a4* and *slc1a5* respectively) were up-regulated when compared with fed animals. Data points represent mean values of 3 animals ± SEM, $^{*}p < 0.02$ after FDR. The expression is represented relative to the house-keeping gene ($18S \times 10^6$) for starved (white bars) and fed (black bars) animals. C and D) Expression of SNAT3 and SNAT5 at protein level are also reduced in the acute phase of pancreatitis. LAT1, LAT2, SNAT3 and SNAT5 expression was assayed by immunoblotting. 50 or 25 μg of crude membrane protein from 3 control or pancreatitis induced animal were used for the analysis. The bands were quantified and data is expressed relative to actin in C. A representative control/pancreatitis blot is depicted in D. The approximate size of the bands is 65 kDa for SNAT3 and SNAT5, 36 kDa LAT1, 55 kDa LAT2 and 42 kDa actin. Data points represent mean values of 3 animals ± SEM, $^{***}p < 0.0001$, $^{**}p < 0.001$ and $^{*}p < 0.05$. White bars represent pancreatitis and black bars control animals.

xCT, showed a relatively high expression. LAT1 has similar substrate selectivity as LAT2, whereas y^+LAT1 and 2 are antiporters for intracellular cationic amino acids against extracellular neutral amino acids and sodium. xCT functions as a sodium-independent antiporter for the extracellular dipeptide cystine and intracellular L-glutamate. All these transporters have the accessory protein 4F2 (Slc3a2) in common and, in other epithelia, were shown to localize to the basolateral membrane. The transporter $b^{0,+}$AT-rBAT (*slc7a9-slc3a1*), an apical transporter in kidney and intestinal epithelia, exhibited relatively low mRNA expression. The CAT member *slc7a1* (CAT1) showed moderate mRNA expression compared to the other Slc7 family members.

The second family analyzed included the Slc38 genes, known as sodium-coupled neutral amino acid transporters family (Fig. 1B) [30]. These transporters are divided into two subgroups, previously called system A, for Na^+-dependent L-alanine transporters and system N, for Na^+ and pH dependent L-asparagine transporters. Slc38a1, 2 and 4 correspond to system A, whereas Slc38a3 and 5 correspond to system N transporters. Slc38a6 is an orphan transporter. Both systems transport L-glutamine with different apparent affinities as well other neutral amino acids. Transcripts for both system N members (*slc38a3* and *5*), as well as the system A transporter *slc38a2* were shown to have the highest gene expression, whereas *slc38a1*, *4* and *6* exhibited lower expression.

Fig. 1C depicts the expression of Slc1 and Slc6 families. These genes encode Na^+, H^+-glutamate co-transporters (members 1–3) and the two neutral Na^+-dependent antiporters (members 4 and 5) [31]. These transporters are highly expressed in the central nervous system but also in the periphery. *Slc1a1* encodes the transporter EAAT3/EAAC1 and is highly expressed in the apical membrane of epithelial kidney and small intestine cells. The four members responsible for glutamate transport, *slc1a1*, *2*, *3* and *6* showed lower expression compared to the neutral amino acid antiporters (*slc1a4* and *5*). ASCT1 (*slc1a4*) was shown to be expressed in the basolateral membrane of pancreatic acinar cells and other epithelial cells [32]. ASCT2 (*slc1a5*) is also expressed in epithelial cells, but its localization is not yet clear. ASCT1 and ASCT2 are Na^+-dependent transporters for L-alanine, L-serine, L-cysteine and L-threonine [33]. Members of the Slc6 family, the neurotransmitter and amino acid Na^+- and Cl^--dependent transporters, were also assessed (Fig. 1C). The major neutral amino acid transporter B^0AT1 in kidney and intestine (*slc6a19*), as well *slc6a18* and *slc6a20*, encoding the neutral (B^0AT3) and imino (SIT1) transporters respectively, showed very low expression in the pancreas. Additionally, the genes encoding the accessory proteins for several of the Slc6 family members, ACE2 and Tmem27/Collectrin [16,34], are expressed. However, these proteins have functions in the pancreas beyond acting as accessory proteins, e.g. they appear to be involved in the docking of insulin vesicles [35], in the renin-angiotensin system (RAS) and Angiotensin II local system activity [36].

The expression of members of the facilitated diffusion transport families Slc16 and Slc43, encoding aromatic and branched chain amino acids (BCAA) transporters was also analyzed (Fig. 1D). There are evidences that these transporters are expressed in the basolateral membrane of epithelial cells [20,37]. Relatively low expression of the genes encoding the aromatic amino acid transporter TAT1 (*slc16a10*), and the neutral amino acid facilitative diffusion transporters LAT3 (*slc43a1*) and LAT4 (*slc43a2*), and the orphan transporter EEG1 (*slc43a3*) was observed. Fig. 1D also illustrates the expression of the genes encoding the H^+-dependent L-proline transporters (*slc36a1* and *2*). They are co-transporters with similar substrate selectivity but different substrate affinity [38,39]. The gene Slc36a1, encoding the low affinity L-proline transporter PAT1 has a higher expression than the high affinity transporter PAT2 (*Slc36a2*). These transporters were shown to be highly expressed in the small intestine and kidney and are localized to the apical membrane of the epithelial cells. *Slc15a1* encoding the peptide transporter PEPT1 is hardly detectable in total pancreas, suggesting that in acinar cells, the transport of free amino acids is the main absorptive pathway for precursors for protein synthesis.

We compared the expression of selected genes in human and mouse tissue (Fig. 1E) and observed the variation between the species. This variation can also be seen in gene expression databases (for example http://biogps.org). Since the commercial sample used was from a single donor, the results should not however be quantitatively analyzed. Interestingly, the gene Slc38a5 was highly expressed in both our mouse and human our samples. According to the work of Nishimura and Saito [40,41], also performed using commercial human RNA sources, SLC38A5 is expressed highest in pancreas out of the 23 organs compared in their study. A further careful analysis of expression should be carried out with multiple human samples at RNA and protein levels to allow a better understanding of the human organ in health and disease.

3.2. The expression of a subset of amino acid transporters is changed in pancreatitis

The expression of the transporters was analyzed in total pancreas from the caerulein-induced pancreatitis mouse model by real time qPCR and Western Blot 12 h after the last injection (Fig. 2A, C and D). The mRNA expression of *slc7a7*, (encoding y^+LAT1), *slc43a2*, *slc43a3* (LAT4 and EEG1), and *slc36a1* (PAT1) was more than two-fold up-regulated relative to the control group and reached significance after correction. *Slc38a3* and *5* encoding SNAT3 and SNAT5 respectively were down-regulated while *slc7a5* and *Slc7a8* (LAT1 and LAT2) did not change. At the protein level, SNAT3 and SNAT5 expression were also reduced, while LAT1 was unchanged as observed at the RNA level and LAT2 tended toward a decreased expression at the protein level, a phenomenon that was not observed at RNA level (Fig. 2C and D). Since the expression of cytoskeleton components, including actin, is known to be changed during acute pancreatitis [42], a normalization to protein load was also performed (data not shown). The results did not differ from those previously obtained by normalizing protein expression to β-actin. It is also worth mentioning that amino acid transporters may have different glycosylated forms [43], which are likely responsible for the blurriness of the bands.

It has been previously shown that the acute injury induced by caerulein is more severe in fed animals than in starved animals due to a reduction in the release of cholecystokinin [44]. To determine the possible relevance in our models, we compared animals that were starved against those fed before induction of acute pancreatitis. For most of the transporters, no difference between the two feeding regimes was observed. The transcripts *slc7a5* (LAT1), *slc7a11* (xCT), *slc3a2* (4F2), *tmem27* (Tmem27/collectrin), *ace2* (ACE2), *slc38a*1 (SNAT1) and *slc1a4* and *5* (ASCT1 and 2) were significantly changed in starved compared to fed animals (Fig. 2B). Other genes in fed animals were equivalently regulated in starved animals during pancreatitis.

The above analyses were carried out using whole tissue, including exocrine, endocrine, stromal and inflammatory cells. To identify those genes specifically expressed in acinar cells and which undergo functionally relevant changes in pancreatitis, we analyzed cDNA expression arrays of a cell culture model of purified acinar cells that phenocopies pancreatitis. As recently shown, acini in this cell culture model have reduced expression of mature acinar cell markers (e.g. Mist1), decrease in functional (chymotrypsin, amylase, lipase), and increase in embryonic ductal markers (Hes1, Krt19) [26]. Table 3 compares acinar cells after culture (i.e. the pancreatitis–like state) to freshly purified acini (i.e. the normal

Table 3
Expression of genes encoding amino acid transporters in a pancreatitis-mimicking culture model of acinar cells. Acinar cells were isolated and cultured, the gene expression is represented as the fold change between after culture/before culture. The genes significantly down-regulated (gray) are suggested as being typical of mature acinar cells. The genes significantly up-regulated (black) are typical of dedifferentiated acinar cells.

Protein name	gene	accession number	acinar cell culture
EAAT3	**slc1a1**	NM_009199	0.75
EAAT2	**slc1a2**	NM_011393	
	slc1a2	NM_001077514.3	0.76
EAAT1	**slc1a3**	NM_148938	0.51
ASCT1	**slc1a4**	NM_018861	0.90
ASCT2	**slc1a5**	NM_009201	0.57
EAAT4	**slc1a6**	NM_009200	0.93
CAT1	**slc7a1**	BC051230	
	slc7a1	NM_007513	0.94
LAT1	**slc7a5**	NM_011404	0.48
y^+LAT2	**slc7a6**	NM_178798	1.40
	slc7a6 (opposite strand)	NM_001007567	1.85
y^+LAT1	**slc7a7**	NM_011405	1.06
LAT2	**slc7a8**	NM_016972	0.18
$b^{0,+}$	**slc7a9**	NM_021291	0.88
asc1	**slc7a10**	NM_017394	0.88
xCT	**slc7a11**	NM_011990	3.93
asc2	**slc7a12**	NM_080852	0.99
AGT1	**slc7a13**	NM_028746	1.02
SNAT1	**slc38a1**	NM_134086	5.61
SNAT2	**slc38a2**	NM_175121	1.02
	slc38a2	AK047403	
SNAT3	**slc38a3**	NM_023805	0.46
SNAT4	**slc38a4**	NM_027052	0.92
SNAT5	**slc38a5**	NM_172479	0.03
SNAT6	**slc38a6**	NM_001037717	1.56
PAT1	**slc36a1**	AK017918	
	slc36a1	NM_153139.4	0.89
PAT2	**slc36a2**	NM_153170	0.90
TAT1	**slc16a10**	AK036743	
	slc16a10	166999493	0.78
Slc16a14	**slc16a14**	AK049846	
	slc16a14	41281605	0.93
LAT3	**slc43a1**	BC053747	
	slc43a1	NM_001081349.2	1.05
LAT4	**slc43a2**	NM_173388.2	1.09
EEG1	**slc43a3**	NM_021398	0.72
Chymotrypsin	**Ctrc**	NM_001033875	0.03
Amylase	**Amy1**	NM_007446	0.11
Lipase	**Pnlip**	NM_026925	0.24
Mist1	**Bhlha15/Mist1**	NM_010800	0.23
Cytokeratin-19	**Krt19**	NM_008471	4.89
Hes1	**Hes1**	NM_008235	1.98

state) and depicts these expression changes. The down-regulated genes typically represent genes characteristic of differentiated acinar cells (labeled gray). As for the transporters analyzed in Fig. 1, these results support the acinar expression of *slc7a5*, *slc7a8*, *Slc38a3*, *Slc38a5* (LAT1, LAT2, SNAT3 and SNAT5 respectively). The results also suggest that *slc1a3* (EAAT1) and *slc1a5* (ASCT2) are expressed in differentiated acinar cells. On the other hand, *slc7a6*, (y^+LAT2), *slc7a11* (xCT), and *slc38a1* and *6* (SNAT1 and SNAT6), are increased in acinar cells once dedifferentiated in pancreatitis (genes labeled black). The genes *slc16a10*, *slc43a1*, *slc43a2*, *slc43a3*, and *slc36a1* were not changed in dedifferentiated acinar cells.

3.3. Antiporters and Na^+-dependent transporters are localized to the basolateral membrane of acinar cells

LAT1, LAT2, SNAT3 and SNAT5 are expressed in the exocrine pancreas on acinar cells labeled with amylase (Fig. 3B, E, H, K respectively). They do not co-localize with the duct marker DBA

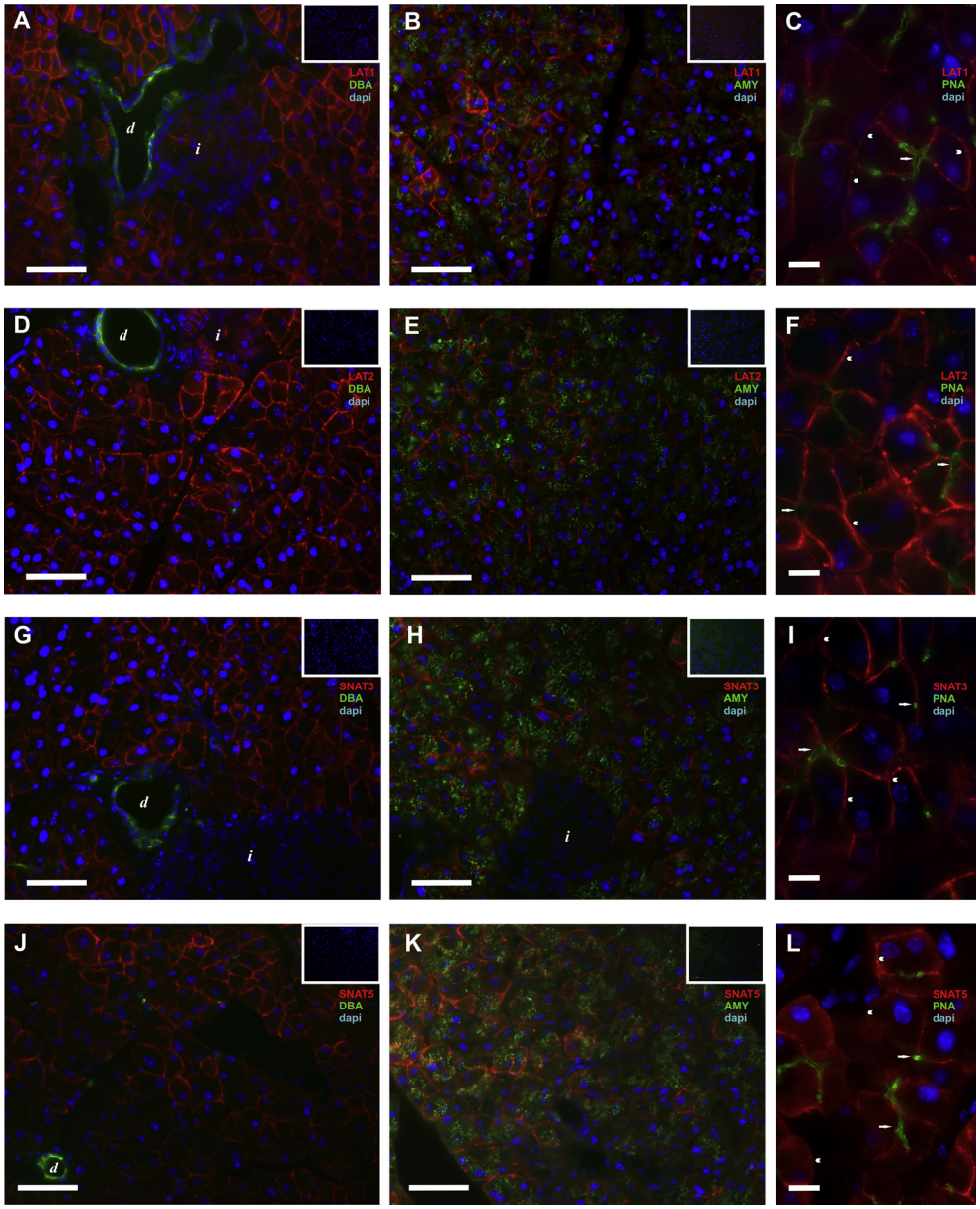

Fig. 3. Antiporters and Na^+-dependent transporters are localized to the basolateral membrane of acinar cells. The amino acid transporters LAT1 (A, B and C), LAT2 (D, E and F), SNAT3 (G, H and I), SNAT5 (J, K and L) were detected by immunofluorescence in mouse pancreas. These transporters do not co-localize with ductal marker DBA (A, D, G and J). They are expressed in acinar cells labeled with amylase (B, E, H and K) and are localized at the basolateral membrane since they do not co-localize with acinar cell marker PNA (C, F, I and L). The insert on the upper right corner of A, B, D, E, G, H, J, and K panels represent the signal in the absence of a primary antibody. Pancreas sections from control animals were co-labeled with antibodies against transporters (red), amylase, PNA or DBA (green) and the nuclei were stained (blue). The pictures in the left and middle panels were performed with 40× oil objective and the bar length corresponds to 50 μm. Right panels were obtained with a 100× oil objective and the bar length represents 10 μm *i*, islets; *d*, duct, *arrows*, apical membrane, *arrowheads*, basolateral membrane.

(Fig. 3A, D, G and J). In Fig. 3C, F, I and L we can also observe that the transporters do not co-localize with the apical acinar marker PNA, suggesting that their localization is on the basolateral membrane of acinar cells. LAT1 and LAT2 are localized to the basolateral membrane of acinar cells, and in addition demonstrated a weak signal in islets (Fig. 3A and D respectively). The transporters SNAT3 and SNAT5 were exclusively localized to the basolateral membrane of the acinar cells (Fig. 3G, H and J, K respectively).

The gene *slc16a10* (TAT1) had a tendency to be increased in pancreatitis (Fig. 2A), but this induction was not reproduced in our acinar culture model (Table 3). Therefore, we further explored TAT1 expression by immunofluorescence and found it localized exclusively in the endocrine islets (Fig. 4A). High levels of TAT1 mRNA were found in islets compared to exocrine tissue, confirmed by targeted real time PCR (Fig. 4B). In islets of *tat1* knockout animals [45], TAT1 was completely ablated, strongly supporting its selective localization to the endocrine compartment (Fig. 4C).

4. Discussion

In this study, we demonstrated that the antiporters LAT1 and LAT2, which accumulate neutral amino acids, and the L-glutamine and L-alanine transporters SNAT3 and 5, are expressed in the basolateral membrane of acinar cells in healthy pancreas. We further showed that amino acid transporter expression changes during pancreatitis.

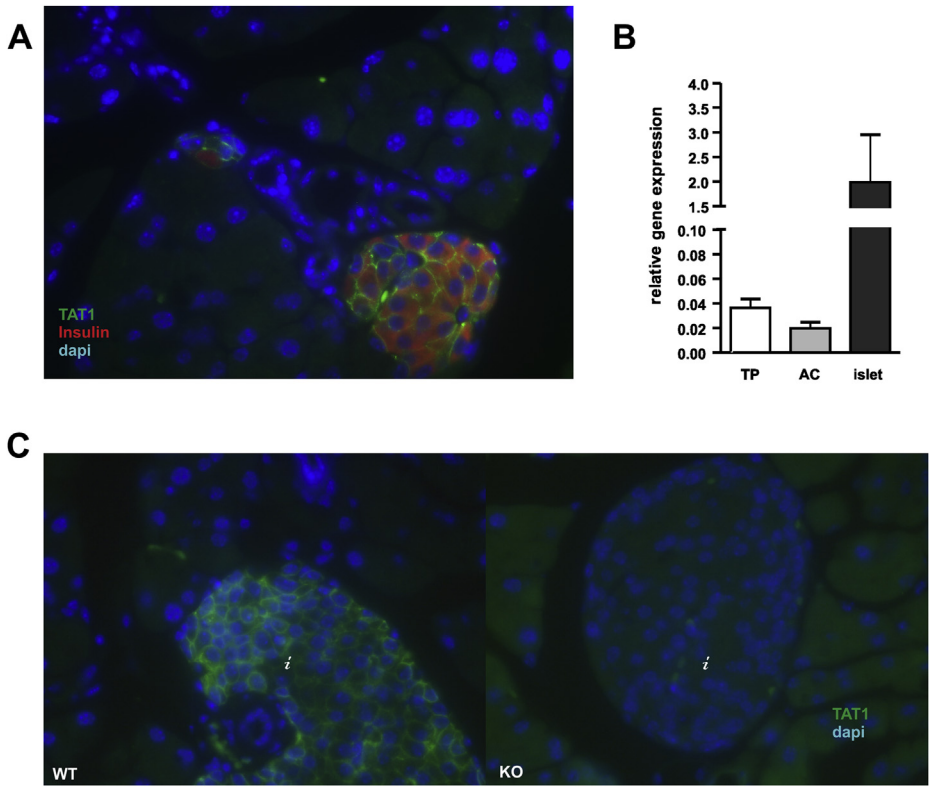

Fig. 4. The amino acid transporter TAT1 (Slc16a10) is expressed in islets. A) TAT1 co-localizes with insulin. Pancreas sections from control animals were co-labeled with antibodies against TAT1 (green), insulin (red) and the nuclei were stained (blue). B) Expression of the gene encoding *slc16a10* (TAT1) is enriched in islets cells. qPCR analysis in isolated cell populations (acinar cells, $n = 3$ (AC) and islets, $n = 4$ (islets)) and compared with total pancreas, $n = 7$ (TP) The expression is represented relative to the house keeping gene. C) The islets transporter TAT1 expression is absent in tat1 knock out animals. Left panel shows wild type (WT), right panel *tat1* knock out (KO). (*i*) Islet. The panels were obtained with 60× oil objective.

LAT1 and LAT2 have an important function in the accumulation of substrates into cells [46]. It has been shown that these transporters have a high affinity for amino acids in the extracellular space in contrary to a very low affinity in the intracellular space, thereby favoring the cytosolic accumulation of amino acids [47]. For this exchange system to work, a transport system with a different mechanism must provide intracellular substrates. The sodium-dependent co-transporters SNAT3 and 5 are good candidates for this function as they transport L-glutamine, L-histidine, L-serine, L-asparagine and L-alanine with low affinity [30]. The amino acids transported by the SNAT3 and 5 are intracellular substrates for LAT1 and LAT2 in exchange for other neutral amino acids not transported by the Na^+-dependent carriers. The functional relationship between the antiporters (or exchangers) and symporters (co-transporters) or uniporters has been previously described in epithelial cells where they play a pivotal role in controlling the transepithelial transport of amino acids [48] and during starvation-induced cellular autophagy [49]. We hypothesize that the functional interaction of sodium dependent co-transporters SNAT3 and SNAT5 with the antiporters LAT1 and LAT2 may supply pancreatic acinar cells with neutral amino acids that are transported by the exchangers but not the co-transporters. Additionally, this symbiosis among the transporters might control cellular volume, autophagy and metabolism during starvation, parenteral nutrition or recovery after injury.

Indeed, during experimental acute pancreatitis, acinar cells change their cellular program in ways that may be reflected in differences in tissue nutrient needs. The accumulation of amino acids in the exocrine pancreas decreases [50,51] and in the early phase of acute pancreatitis in humans serum concentrations of most amino acids are changed. Among them, L-arginine, and L-glutamine are reduced and L-glutamate increased. These amino acids are key players on the production of NO, GSH, ammonia and urea metabolism [52]. L-glutamine has been used in animal experiments and clinical trials as supplementation of enteral/parenteral nutrition and showed to have beneficial effects on acute pancreatitis [12,13,51]. The protective mechanism is unknown, as is the effect of the injury/regeneration on the acinar cells' L-glutamine transporters. The Na^+-dependent glutamine transporters SNAT3 and SNAT5 are highly expressed in mature healthy acinar cells, and a decrease in their RNA and protein levels in whole pancreas isolated from mice with caerulein induced pancreatitis was observed. This reduction of exchanger substrate accumulation may impair the function of LAT1 and LAT2. However, the whole tissue expression mRNA levels of LAT1 and LAT2 were unchanged, while LAT2 protein had a tendency to decrease. We used a cell culture model to analyze the acinar cell specific expression changes during the pancreatitis state. Though we can expect differences comparing the *in vivo* model with the *in vitro* data due to different proliferative states and spatiotemporal changes, the results are concordant for SNAT 3 and 5. These data further suggest that LAT1 and LAT2 may be expressed in another cell population of which expression increases and masks the loss in the acinar cells in the *in vivo* model.

In the caerulein pancreatitis model we also found transporters up-regulated or with tendency to be up-regulated, in particular the transporter y^+LAT1 and 2, and SNAT1. These genes have also been found up-regulated in dedifferentiate primary cultured cells [18]. SNAT1 co-transports L-alanine and L-glutamine with sodium and y^+LAT2 exchanges intracellular L-arginine with extracellular L-glutamine and other neutral amino acids and might play a role in

nutrient accumulation [53]. Thus, it appears that a different subset of transporters may be involved in the accumulation of amino acids in a healthy state compared to the phases of injury, regeneration and proliferation. Further analysis of y^+LAT1 and 2, and SNAT1 localization in pancreas will allow a better understanding of these transporters role during pancreatitis. In the liver, similarly to the pancreas, the regeneration depends on the capacity of differentiated hepatocytes to act as facultative progenitor cells [54]. Interestingly, after partial hepatectomy the levels of SNAT5 and LAT2 were reported to be up-regulated [55], suggestive of injury- and tissue-specific requirements for amino acid transport.

In the healthy animal, starvation was shown to change the expression of amino acid transporters [56,57]. During acute pancreatitis, some of the transporters analyzed had a differential expression in animals starved or fed before injury, suggesting starvation may introduce another variable in gene expression and possible in the metabolism. This observation emphasizes the need to take the feeding state into consideration when assessing experimental results.

In conclusion, we have demonstrated selective expression and localization of LAT1, LAT2, SNAT3 and SNAT5 transporters in the basolateral membrane of the acinar cells. During acute pancreatitis, the expression of SNAT3 and SNAT5 as well of other amino acid transporters are reduced, suggesting these transporters may be important for the accumulation of amino acids in healthy tissue. LAT1 and LAT2 expression was not reduced in the *in vivo* pancreatitis model at RNA level and neither was LAT1 at protein level. In an acinar cell culture model resembling pancreatitis however there was a decrease. These transporters might be over-expressed in other cells during pancreatitis, masking acinar changes *in vivo*. Further analysis of amino acid transporter expression and localization in pancreatic cells after acute injury will be analyzed. A more detailed description of the changes occurring in amino acid transporters expression in acinar and, as well, in ductal cells may help to understand the significance of the metabolic changes undergone by the exocrine pancreas during injury and regeneration.

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Authorship credit

I.R. and S.M.R.C. conception and design of research; I.R., S.M.R.C., C.L., A.V.P., K.H. and T.R. performed experiments; I.R., S.M.R.C., C.L., and A.V.P. analyzed data; I.R., S.M.R.C, and R.G. interpreted results of experiments; I.R., C.L. and S.M.R.C. prepared figures; I.R. and S.M.R.C. drafted manuscript; I.R., S.M.R.C., C.L., T.L., R.G. and F.V. edited and revised manuscript; I.R., S.M.R.C., C.L., A.V.P., K.H., T.R., T.L., R.G. and F.V. approved final version of manuscript.

Disclosures

No conflicts of interest exist.

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