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Acinar-to-ductal metaplasia in pancreatic cancer: regulatory genes as a target for therapy.

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"This master's thesis came about (in part) during the period in which higher education was subjected to a lockdown and protective measures to prevent the spread of the COVID-19 virus. The process of formatting, data collection, the research method and/or other scientific work the thesis involved could therefore not always be carried out in the usual manner. The reader should bear this context in mind when reading this Master's thesis, and also in the event that some conclusions are taken on board".

Acinar-to-ductal metaplasia in pancreatic cancer: regulatory genes as a target for therapy.

Van den Bossche J.[1]

Abstract

Background/aim: Pancreatic ductal adenocarcinoma is one of the deadliest cancers and has a 5-year-survival rate of 5%. It is believed that the process of acinar-to-ductal metaplasia (ADM) can give rise to a big part of these cancers. However, more research is needed on this topic before it can be used as a therapeutic target for (chemo)prevention. In this research, the aim is to identify and characterize genes of interest that play a role in ADM. In addition, the druggability is tested for xCT, which is the main target of this study due to its interesting characteristics in cancer and promising preliminary data.
Methods: For xCT, a full knockout mouse model was used and acinar cells were used for qPCR for a specific gene panel for ADM. For the other four genes of interest, namely GFRA1, GDF15, MXRA5 and CXCL17, we aimed at obtaining an shRNA-mediated knockdown by lentiviral transduction in primary human acinar cells.
Results: Results of this study showed that for the main target xCT, we observed an upregulation at day 1 of ADM in both human and murine acinar cells. In its absence, mouse acinar cells acquire lower expression levels of the ductal marker Keratin-19 (whose upregulation around day four is a hallmark of ADM) at day four. Furthermore, TGFβ, which is described as a primary regulator of ADM, is downregulated over time in xCT knock-out cells.
Conclusion: These findings suggest that xCT has a function in the process of ADM and could be involved in a more expanded pathway initiating ADM.

Key words: Acinar-to-ductal metaplasia, pancreatic ductal adenocarcinoma, xCT.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer and is a very lethal disease, with a 5-year survival rate of 5% [1]. The most effective treatment option is to surgically remove the entire tumor [2]. However, at the time of diagnosis the patient is often already in an advanced stage of the disease, rendering the complete removal of the tumor impossible. Therefore, research on the onset of the cancer is highly necessary.

The adult pancreas is very plastic, meaning that it can react to internal or external stress to maintain organ integrity [3]. One mechanism by which the pancreas responds to stress is acinar-to-ductal metaplasia (ADM), which happens for example during pancreatitis [3-5]. During this process, acinar cells undergo morphologic and transcriptional changes and dedifferentiate into a progenitor-like state with proliferative capacities. These cells can either re-differentiate into acinar cells or fully dedifferentiate into a duct-like cell. In the case of acute pancreatitis, most cells will return after the insult to their initial differentiated state as acinar cells and some will transdifferentiate into duct cells. In the case of chronic pancreatitis, where the damage is prolonged, cells tend to maintain a dedifferentiated progenitor-like state [3-5]. However, during sustained stress and due to accumulation of oncogenic mutations, these progenitor-like cells are thought to be the cell of origin for at least part of the pancreatic ductal adenocarcinoma cases [6,7].

Several genes have already been linked to acinar-to-ductal metaplasia, but more research is needed to fully understand the mechanisms of this process [4,8-11]. In this study, five genes of interest (xCT, MXRA5, GDF15, CXCL17 and GFRA1) have been selected based on their upregulation in human dedifferentiated acinar cells in respect of ductal cells in an RNAseq analysis [5].

The gene xCT or SLC7A11 has been linked to multiple cancers, including pancreatic cancer [12,13]. It is a cystine/glutamate antiporter, whose unique function is the import of cystine, which is required for the production of glutathione, and thus the scavenging of reactive oxygen species (ROS). Literature has already shown that xCT is upregulated during pancreatitis, which is a known ADM-inducing pathology [14]. However, to this day it has not yet been linked to the process of ADM.

Matrix remodeling-associated protein 5 (MXRA5) is a member of the MXRA-family, whose function has not yet been completely described [15]. It has been shown that MXRA5 is upregulated in some cancers and it is even proposed as a biomarker for colorectal cancer [15]. In addition, based on online databases, MXRA5 is associated with a significantly reduced overall survival in PDAC patients [16].

The gene GDF15, or Growth Differentiation Factor 15, was also shown to be implicated in cancers, including pancreatic cancer [17,18]. In addition, literature suggests that it is a possible biomarker for PDAC [18].

CXCL17 is involved in immune surveillance and is upregulated during pancreatic carcinogenesis [19]. It is suggested to have an anti-tumor function, while some studies also state it has a tumor-promoting effect [20]. In online databases, it is seen that high CXCL17 expression is associated with significantly reduced survival in PDAC patients [16].

Finally, GFRA1, or glycosylphosphatidylinositol-linked GDNF (glial cell derived neurotrophic factor) receptor alpha 1, is involved in differentiation and proliferation of neuronal cells [21]. This gene and its ligand GDNF have also been implicated in pancreatic cancer [22].

Thus, the process of ADM is an attractive topic for PDAC research, since a better comprehension and characterization of how the genes are involved in the acinar cell dedifferentiation will be important for pancreas regeneration after pancreatitis and ideally also for better understanding the onset of PDAC. Several studies have already identified some genes that are involved in ADM, but much more information is needed to obtain reliable and specific markers for dedifferentiating acinar cells or to find genes that play a crucial role in the process of ADM. For that reason, five genes with interesting characteristics in cancer and more specifically pancreatic cancer were selected in this study.

Results

Upregulation of genes in dedifferentiated human acinar cells

An *in vitro* model has previously been reported for dedifferentiation of acinar cells, resembling acinar-to-ductal metaplasia [23]. In this model, human acinar cells are cultured in suspension dishes for up to eight days (Fig. 1A). Previous RNA-sequencing data generated by our research group has resulted in a list of genes that were differentially expressed in human acinar cells compared to ductal counterpart. Based on their differential expression in dedifferentiated acinar cells and on literature research, five genes of interest were selected. These shortlisted candidates were xCT (SLC7A11), MXRA5, CXCL17, GDF15 and GFRA1.
Here, across the days of culture, the levels of expression were measured by means of RT-qPCR. It should be noted that there can be variability between patients due to conditions like age, lifestyle or the period between the isolation of the cells and delivery in the lab. The analysis confirmed that all of the genes of interest were modulated in human samples during *in vitro* dedifferentiation (Fig. 1B). In addition, some of the genes showed consistent upregulation at a specific timepoint of the ADM process. In particular, xCT, GDF15 and CXCL17 were upregulated early in the process, around D1; MXRA5 showed a peak of upregulation around D3-D4, the timepoint at which acinar cells are fully dedifferentiated. Finally, GFRA1 showed a modulation around D5-D6, when acinar cells are starting to transdifferentiate towards a ductal-like phenotype.
Based on the peak in expression at a consistent timepoint (D1) in four out of five patient samples that was also confirmed on protein level, in addition to the valuable tools we had available such as a full knock-out mouse model, xCT was chosen as the main target of this study. Due to the lack of such models for the other four genes of interest, lentiviral particles were purchased to obtain a short-hairpin RNA (shRNA)-mediated knockdown.

Lentiviral transduction of human acinar cells results in a fraction of acinar cells with a knockdown for genes of interest.

Primary human acinar cells are known to be a difficult cell type to genetically manipulate [24]. In this study, lentiviral vectors coding for shRNA sequences targeting one of the genes of interest were used to obtain a knockdown. To exclude possible off-target effects of these shRNAs, four different shRNA sequences were used per gene. GFP was included as a reporter in order to discriminate the transduced from the untransduced cells. As most of the acinar cells should be dedifferentiated at day four of culture, the cells were sorted at this timepoint by FACS (Fluorescence-Activated Cell Sorting), based on their GFP positivity (Fig. 2A).
In Fig. 2B, the different genes of interest are represented at day three after transduction. The transduction efficiency was very variable between conditions, with percentages ranging between 0.62-3.3% (Fig. 2C).

Due to the Covid-19 pandemic during which this study took place, human exocrine fractions availability was scarce. As a result, the further analysis of this experiment has not yet taken place. However, with the aim to get RNAseq data, samples were sent for RNA quality control with the BioAnalyzer, in which the RIN-value (RNA integrity number) will be determined. If this value falls within the suitable range (a minimum of 8) and the concentration is sufficiently high, this will allow us to confirm the knockdown of the gene of interest by RT-qPCR. Thereafter, the most promising samples will be sent for RNA-sequencing. This will provide us with information how and if these cells still underwent ADM after 4 days of suspension culture in the absence of one of the genes of interest.

Human and murine acinar cells form smaller clusters when xCT is inhibited or absent.

Since xCT was chosen as our primary lead, we performed further experiments using pharmacological inhibitors for xCT and mice with a full body knockout for xCT (KO).
Acinar cells *in vivo* are located in small groups of cells, called acini. *In vitro* these cells also live in a grouped formation and clusters of acini form during the first days of suspension culture resulting in spheroid-like structures at day three. In this experiment, we aimed to see if there was a phenotypical difference in the generation of spheroid-like clusters between acinar cells when xCT is inhibited or knocked out. The graphs that are shown are the result of a HALO image analysis in which a 'mask' can distinguish clusters from background (Fig. 3A).
In the case of human acinar cells, two pharmacological inhibitors of xCT Sulfasalazine and Erastin were used. In presence of Sulfasalazine, the acinar cells formed significantly smaller ($P<0.0001$) clusters than their non-treated counterparts on both day 3 and day 4 of culture (Fig. 3B). The same result was obtained with Erastin treatment, where the difference between the control condition and the treated condition was even higher at day three of culture. These results suggest that there might be a phenotypical difference between non-treated and xCT-inhibited human acinar cells.
However, to confirm that this difference is solely caused by the loss of xCT function, xCT KO mice were used (Fig. 3C). A similar result was observed, where the acinar cells originating from xCT KO mice form significantly ($P<0.0001$) smaller clusters than those of WT mice. In this case, the decrease in cluster size from D3 to D4 was smaller in mice than in humans. This could indicate that the presence of the inhibitors had an effect on the capacity of the acinar cells to form clusters.
This smaller cluster size in acinar cells with absent or inhibited xCT might be caused by cell death. Since xCT's unique function is to import the essential amino acid cystine, its absence might cause cells to go into apoptosis. To see if we could obtain a rescue effect of this phenotype, β-mercaptoethanol was added to the medium. The latter reduces cystine to cysteine, whose uptake is xCT independent. However, no increase in cluster size was obtained in xCT KO acinar cells (Fig. 3D). In contrast, xCT WT cells that were treated with β-mercaptoethanol seemed to show a slightly increased cluster size in the treated condition. This observation can be caused by the simultaneous uptake of cystine and cysteine.

Mouse acinar cells seem to go through an impaired ADM process in absence of xCT.

As mentioned before, during ADM acinar cells dedifferentiate into a progenitor-like cell, lose the expression of acinar cell markers and acquire progenitor-markers. Meanwhile, they can transdifferentiate into a ductal-like state, thus acquiring the expression of ductal cell markers. For this reason, a gene panel was selected for qPCR analysis. This panel included an acinar and a ductal cell marker, respectively amylase and keratin-19. In addition, CD44v9, a protein which interacts with xCT by stabilizing it on the cell membrane [25] and a known cancer stem

cell marker [26], was also included. Finally, TGFβ, reported in literature to be one of the main regulators of the ADM process [9] and suggested to interact with xCT [27], was also looked into.
As shown in Fig. 4, the peak of xCT expression at day one of the dedifferentiation process, which was earlier observed in human acinar cells (Figure 1B), was confirmed in mouse acinar cells.
In the knockout cells, xCT expression was absent ('undetermined' after 40 cycles). CD44v9 was upregulated at day 1 in WT cells, which is in line with the expression pattern of xCT. A steady expression level was maintained up to day 4. In the KO cells a stronger, yet unsignificant upregulation was observable at day 1, which was again downregulated at day 4 to baselevels. Intriguingly, the expression level of CD44v9 at the day of isolation was approximately 5 times higher in KO cells than in WT cells. This and the stronger upregulation at day 1 might suggest a possible coping mechanism of the KO cells for the absence of xCT. In concordance with what has been described in literature, the ductal cell marker keratin-19 showed an expected upregulation and th acinar cell marker amylase was lost over time in both conditions. Interestingly, we observed that this upregulation of keratin-19 tended to be lower in KO cells, suggesting an impaired or delayed ADM process in absence of xCT. In addition, where TGFβ reached a peak in expression at day 4 of culture in WT cells, it was found significantly downregulated (P=0.0467) in KO. This further suggests the impairment of the ADM process due to the lack of xCT.
Finally, there were two genes that had higher baseline expression levels on the day of isolation when comparing KO to WT cells, being CD44v9 and TGFβ. This indicates that there is a phenotypical difference between xCT WT and KO cells.

Confirmation of upregulation of xCT at day 1 at protein level

To examine whether the upregulation of xCT expression can be found back on protein level, a Western Blot analysis was performed. Fig. 5A shows the clear upregulation of xCT in both day one samples. A higher protein level than the day of isolation was maintained up to day four. In Fig. 5B, it can be seen that the xCT protein levels follow a similar trend as the expression levels of xCT during ADM.

Testing of the effect of xCT inhibitors in an acinar cell context.

As mentioned before, xCT is responsible for the uptake of cystine. The latter is required for the production of glutathione, a reactive oxygen species (ROS) scavenger. Thus, an experiment was conducted to test the effect of xCT inhibition on ROS scavenging. A measurement of intracellular ROS was performed, since the cells should be less efficient in scavenging them absence of xCT.
In Figure 6A, it was observed that inhibition with Sulfasalazine led to significant (P<0.0001) increases of ROS with rising concentrations of the inhibitor. With Erastin, however, a significant (P=0.0003) increase was only obtained with the highest concentration of the compound. This experiment suggests that xCT inhibition did indeed lead to a decreased potential to scavenge ROS.
Additionally, a pilot experiment for cystine-FITC uptake assay was performed, as cystine uptake is a unique function of xCT. Hence, during xCT inhibition, we expected to see lowered or no cystine-FITC uptake. However, it was observed that there was a large number of dying (blebbing) cells, which were aspecifically incorporating the cystine-FITC and consequently resulting in very bright signals causing an evident bias in the results (results not shown).

Discussion

Acinar-to-ductal metaplasia is a natural process for regeneration that occurs in the pancreas upon injury. However, cells are susceptible to oncogenic mutations during this process and can give rise to PDAC.

The process of acinar-to-ductal metaplasia is not yet completely understood, although some important regulators or initiators have already been identified. In this study, five genes of interest that showed to be differentially expressed in dedifferentiated acinar cells were selected for further characterization. xCT has been paid a particular attention in this thesis. The other genes' analysis has not been pursued during this internship due to the limited term of the internship and the COVID pandemic. In the future, these genes will be analyzed by performing RNA-sequencing on human acinar cells with a knockdown of one of the genes of interest. This will allow us to compare their expression profile to that of non-transduced cells. This way, we will see if the transduced cells acquire or lose expression of some genes and if they follow the normal ADM-pattern of the genes in our selected gene panel.

xCT, which has been linked to various cancers, including pancreatic cancer, is being significantly (P=0.0079) upregulated at day 1 of the ADM process in both murine and human cells. The fact that this peak occurs in two different species is promising, since this suggests that xCT-upregulation might be a conserved mechanism for ADM initiation. Although this upregulation can also be expected since xCT is normally upregulated during stress conditions [28]. However we were further encouraged by the fact that xCT is required for another type of metaplasia called spasmolytic polypetide-expressing metaplasia (SPEM) in gastric chief cell *in vivo* [29,30]. In addition, it was shown in literature that there are striking similarities between SPEM and ADM [31].This further supports our hypothesis. Another feature that makes xCT an interesting target is its interactors. CD44v9 stabilizes xCT on the cell membrane [25]. The former is a known and important cancer stem cell marker, which is also upregulated in pancreatic cancer cells and it is also involved in metaplasia in the gastric epithelium [30]. Thus, upregulation of CD44v9 during ADM fits with the progenitor-like phenotype that is acquired. Furthermore, literature has shown that the system xCT-CD44v9 is required for cancer metastasis and is involved in epithelial-to-mesenchymal transition (EMT)[26]. With this information, it could be hypothesized that xCT functions as a sensor for acinar cells to start the reprogramming process and will then interact with other pathways and regulators, including CD44v9. In this perspective, we propose several genes that will be further investigated in the continuation of this study.

Sirtuin-1 has previously been described as a regulator of ADM [4]. Interestingly, it was shown that the knockdown of this gene lead to a downregulation of both xCT and CD44v9 [32]. In addition, overexpression of Sirtuin-1 led to an upregulation of stemness-associated genes, including CD44v9 [33].

Furthermore, osteopontin (OPN), a ductal cell marker which is involved in inflammation, metastasis and tumor progression, has been shown to be a ligand of CD44v9 [34]. Its upregulation is also associated with more metaplasia in the gut [35,36]. We have not yet investigated into this gene, but since it is a ductal marker, it could be possible that it is upregulated during ADM. Due to its interaction with CD44v9, it might be involved in the ADM-initiating pathway.

Literature suggests that TGFβ, one of the main regulators of ADM, negatively regulates xCT [27]. This is also reflected in our results, since xCT expression is downregulated when TGFβ is being upregulated in WT cells. It should be noted that the TGFβ that regulates ADM primarily originates from ductal cells *in vivo* according to literature [8]. Thus, it is logical that the acinar cells acquire its expression at day 4, since they acquire a ductal-like phenotype. However, in the xCT KO cells, this upregulation at day 4 is absent, which suggests that they do not go through the ADM process in the canonical way. To conclude that the ADM process is indeed

impaired or delayed, additional evidence will be required as thus far we did not find a difference between amylase or KRT19 expression in WT versus xCT KO cells. It would also be interesting to look further into markers for SPEM, as there are similarities between SPEM and ADM and xCT has been shown to be involved in the former. There were also interesting differences in baseline expression (at the day of isolation) in CD44v9 and TGFβ between xCT WT and KO mice. This suggests that there might be a phenotypical difference between these genotypes. In the future, RNA-sequencing will be performed to obtain a detailed comparison of the gene expression signatures of xCT wildtype and knock-out mice. The interaction between TGFβ and xCT is also interesting to further investigate into. Future experiments to investigate into this interaction will be performed in the continuation of this study.
In this study, two pharmacological inhibitors for xCT were used. Although there are specificity issues, these inhibitors made a good complementary tool and the effect of their xCT inhibition was evaluated.Since xCT is responsible for the uptake of cystine, which is required for the production of glutathione, a scavenger of reactive oxygen species, we performed a ROS measurement assay and a cystine-FITC uptake assay. A significant accumulation of ROS in the human acinar cells that were treated with xCT inhibitors was indeed observed. However, in the cystine-uptake experiment, more optimization is needed to adjust this protocol to the acinar cells. A bias is introduced in the experiment due to dead or dying cells that have a porous membrane through which the cystine-FITC will flow. We confirmed this with the EVOS-microscope, where we observed large, round cells which showed very strong signal. These dying cells will influence the fluorescence reads and cause a very strong background signal. For this reason, in the future, we will conduct this experiment by flow cytometry for FITC-positivity and a live/dead staining. This way, the dead cells can be separated from the healthy cells.

One could think of using existing inhibitors such as sulfasalazine and other more specific ones under development for targeting ADM, for example for pancreatitis patients to reduce the risk of forming a PDAC. However, since this is a natural mechanism for regeneration, it raises the question if it is beneficial or not to hamper this process. If any therapeutic strategies are developed that target ADM, it could be possible that additional treatment will be required for the patient to treat pancreatitis in the absence of ADM occurring.

For the other four genes of interest, we opted for an shRNA-mediated knockdown model. Literature has already proven that the genetic manipulation of acinar cells is very challenging. However, we did obtain a fraction of transduced human cells which will be used in future experiments.
In Fig.1 it was seen that these genes of interest are upregulated during ADM. However, we do not yet know whether these genes are upregulated because they are modulated through the process, or because they have a regulating function during ADM. For this, additional experiments will be required. Interestingly, three out of four genes of interest, namely GDF15, GFRA1 and MXRA5, have a known interaction with TGFβ. This knowledge will be taken into account for future experiments.

Performing research on the topic of PDAC is a challenging task. Dedifferentiated acinar cells are described as the cells of origin of at least part of the cases of PDAC. Primary acinar cells, due to their elevated plasticity, their susceptibility to stress and reticence to be genetically manipulated, are a very demanding cell type. Additionally, these cells can only survive in a clustered form, thus complicating the manipulation and the application in *in vitro* assays. For these reasons, a large part of this study existed of optimizing assays so that they fit the difficult

profile of the acinar cells. Some experiments were preceded by pilot studies with pancreatic cancer cell lines. However, these results are not shown in this paper.

In conclusion, xCT is a promising gene of interest in the process of ADM, with increasing evidence of its role. Thus, when including CD44v9, three of the other genes of interest and interesting targets from literature, this study has the potential to unravel a (possibly TGFβ-mediated) network of genes that, altogether, are involved in initiating and regulating acinar-to-ductal metaplasia. However, to prove this hypothesis, additional experiments will be required that fall out of the scope of this manuscript. In this perspective, several experiments are lined up, such as a caerulein-induced pancreatitis mouse model to study the role of xCT in pancreatitis.

Methods

Animals

In this study, xCT wildtype ($xCT^{+/+}$) and xCT knock-out ($xCT^{-/-}$) mice were used. These mice have a C57BL/6J background and were kindly provided by the lab of Prof. Dr. Ann Massie. All animals were housed and maintained in accordance with national guidelines regarding animal experimentation. The experiments involving these animals were approved by the ethical committee for animal experimentation of the Faculty of Medicine and Pharmacy of the Vrije Universiteit Brussel.

Human samples

The human pancreatic exocrine cell fraction was obtained from the UZ Brussels hospital as a by-product of islet transplantation. The cells were first washed with Dulbecco's phosphate buffered saline (DPBS, Gibco, Waltham, Massachusetts, USA) before being cultured in non-adherent petridishes in Advanced RPMI (Gibco), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin solution (P/S), and 1% GlutaMax (Gibco).

Pharmacological compounds

Two pharmacological inhibitors of xCT were utilized: Sulfasalazine (SAS) was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA) and Erastin (Er) from Selleckchem (Houston, USA). SAS was kept in powder form at room temperature. To use this compound, 32 mg of it was dissolved in 8 mL of NaOH. Then, 8 mL of HCl was added to neutralize the pH. Finally, milliQ H_2O was added until the final volume reached 20 mL. This resulted in a stock solution of 4 mM, which was stored at 4 °C. The Erastin was dissolved in DMSO and this 10mM stock was stored at -18 °C.

ADM assay

The ADM assay is an in-house developed method to mimic the process of acinar-to-ductal metaplasia *in vitro*. Acinar cells are cultured in 35 mm petridishes in a volume of 2-4 mL, depending on for which following assay they are intended. Human acinar cells are cultured in Advanced RPMI, supplemented with 10% FBS, 1% P/S and 1% GlutaMax. Mouse acinar cells were cultured in the same medium, but with addition of G418 (Life Technologies, Carlsbad California, USA), which was diluted 1/2000 in order to remove fibroblasts. During experiments involving xCT, the pharmacological xCT inhibitors Sulfasalazine and Erastin were also added

to the medium during suspension culture at different concentrations. Cells were cultured for four or eight days, depending on the experiment. Refreshing of medium was done on day one and then each two days.

Mouse acinar cell isolation

Mice were sacrificed by cervical dislocation and pancreata were injected with 1 mL of chilled collagenase (Sigma-Aldrich). Pancreata were then dissected and processed into small tissue fragments, after which they were digested for 15 minutes at 37 degrees. The pancreas fragments were then washed three consecutive times with a 5% FBS HBSS (Gibco) solution. Thereafter, the resulting cell suspension was pipetted through a 100µM mesh to remove remaining large tissue fragments, including connective tissue and ducts. The resulting fraction consists of acinar cells and was pipetted on top of a 30% FBS HBSS solution, after which it was centrifuged for 2 minutes at 1000 RPM to obtain only the viable acinar cell fraction.

Cluster size analysis

When acinar cells were in culture during the ADM assay, pictures were taken with the Evos microscope (Invitrogen, Carlsbad, California, USA) of the clusters in the petri dishes. This was performed on day three and day four of culture, since the clusters are only properly formed from day three on. Approximately 15 pictures were taken of every dish. Afterwards, pictures were analyzed with the HALO imaging software (Indica Labs, Albuquerque, New Mexico, USA), which was able to distinguish cell clusters from background and single cells and measure their size, enabling us to calculate the mean cluster size for each sample.

RT-qPCR

Cells were collected on the day of isolation (day 0) and on different culture time points (day 1 and day 4). Cells were resuspended in 350 µL of lysis buffer LBP and RNA was extracted using the NucleoSpin RNA extraction kit (Macherey-Nagel, Germany) following manufacturer's instructions. The concentration of the RNA was determined using the Nanodrop-1000 spectrophotometer (Thermo Fisher, Waltham, Massachusetts, USA). Thereafter, 250 ng of RNA was used for retro-transcription to obtain cDNA, using the GoScript Reverse Transcription System (Invitrogen). qPCR was performed using FastSYBRGreen 5× MasterMix on a QuantStudio 6 (Invitrogen). The analysis was done using the ΔΔCt method using HPRT and Gapdh as housekeeping genes. Primers were purchased from IDT (Newark, New Jersey, USA).

Western Blot

Cells were lysed in radioimmunoprecipitation buffer (RIPA buffer, Sigma-Aldrich), supplemented with 1% phosphatase inhibitor (Sigma-Aldrich) and 1% protease inhibitor (Sigma-Aldrich). After centrifugation, to remove cell debris, a Bradford protein assay was conducted to determine the concentration of the protein samples. 20 µg of protein was then transferred to another Eppendorf tube, with a one in five dilution of 5x loading dye. These samples were then loaded into 10% polyacrylamide gels. The Bio-Rad system was used to run the gels at 120 V for 15 minutes and then 165 V, until the loading dye has run out of the gel. Subsequently, the proteins were transferred on nitrocellulose membranes either overnight at 55 V or for 2 hours at 200 mA per gel. The membranes were then blocked for one hour in a 5% w/v milk TBS-Tween (1% Tween, Sigma-Aldrich) solution, to prevent non-specific binding of

the antibodies. Thereafter, the primary antibodies were added and the membranes were kept in the cold room overnight. Primary antibodies include Rabbit anti-SLC7A11 (Cell Signaling Technologies, Danvers, MA, USA) and Mouse anti-αTubulin (T9026, Sigma-Aldrich). Both were diluted 1/1000 in 3% w/v BSA TBS-Tween. The secondary antibodies were then added for 1 hour, diluted 1/5000 in 5% w/v milk TBS-Tween. These antibodies were fluorescent and included Donkey anti-Rabbit (LI-COR, Lincoln, NE, USA) and Donkey anti-Mouse (LI-COR), which showed red and green signal respectively. Finally, the blots were analyzed, using the Odyssey system (LI-COR).

Bradford protein assay

A standard dilution series was prepared with increasing bovine serum albumin (BSA) concentrations. Subsequently, solutions A' and B were prepared, following manufacturer's instructions (Bio-Rad, Hercules, California, USA). 25 µL of solution A' was added to each well of a 96-well plate, after which 5 µL of the standards and samples were added to the corresponding wells (in duplicates). 200 µL of solution B was then pipetted into every well. Finally, the plate was incubated in the dark at room temperature (RT) for 15 minutes. The absorbance could then be read, using the xMark^{TM} microplate absorbance spectrophotometer (Bio-Rad).

Lentiviral transduction

Human acinar cells were washed with DPBS. Thereafter, 30 µL of the cell pellet was plated in a 35 mm petri dish for every condition. These cells were cultured in 850 µL of Advanced RPMI with 10 % FBS and 1 % P/S, to which 150 µL of the solution containing the virus was added to obtain a Multiplicity of Infection of 5. Polybrene was also added to the medium at a final concentration of 5 µg/mL to improve the transduction efficiency. The dishes were incubated overnight, after which the cells were resuspended in fresh medium and transferred to a new 35 mm dish. At the fourth day of incubation, the cells were dissociated with NeuroCult (Stemcell Technologies, Vancouver, Canada). Finally, the cells were FACS-sorted based on GFP-positivity and collected in 350 µL of the RNA RLT extraction buffer (Macherey-Nagel).

ROS-measurement

Human acinar cells were cultured in a 96-well black plate in presence or absence of xCT inhibitors Sulfasalazine and Erastin at a density of approximately 20,000 cells per well. The cells were incubated at 37 °C for 24 hours. Thereafter, 100 µL of the master reaction mix of the ROS detection kit (Sigma-Aldrich) was added to each well. The plate was then again placed in the incubator for 1 hour. The medium was then removed and the cells were washed with DPBS three times. Finally, the fluorescence intensity was measured with following settings: λex= 490 nm and λem= 525 nm and the viability was determined using Cell titer glo (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. The ROS value was normalized to the viability value, which resulted in the ROS index.

Cystine-FITC uptake measurement

Human acinar cells were cultured in a 96-well black plate in presence or absence of xCT inhibitors Sulfasalazine and Erastin at a density of approximately 20,000 cells per well. The cells were incubated at 37 °C for 24 hours. The cystine-FITC was then added in medium without cystine (RPMI 1640, Sigma-Aldrich) and the plate was incubated for 1 hour. The medium was then removed and the cells were washed with DPBS three times. Finally, the

fluorescence intensity was measured with following settings: λex= 450 nm and λem= 525 nm and the viability was determined using Cell titer glo according to the manufacturer's instructions. The cystine-uptake value was normalized to the viability value, which resulted in the cystine-uptake index.

Statistical analysis

Statistical analysis was performed using Prism v9.0 (GraphPad Software, La Jolla, CA, USA). The statistical significance of differences between groups was tested using a Mann-Whitney test, a one-way ANOVA, a Dunnett's Multiple Comparison test and a Bonferroni post hoc test to correct for multiple comparisons. The number of independent repeats is mentioned in each figure in the legenda. Results are presented as mean ± sem. $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

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Figures

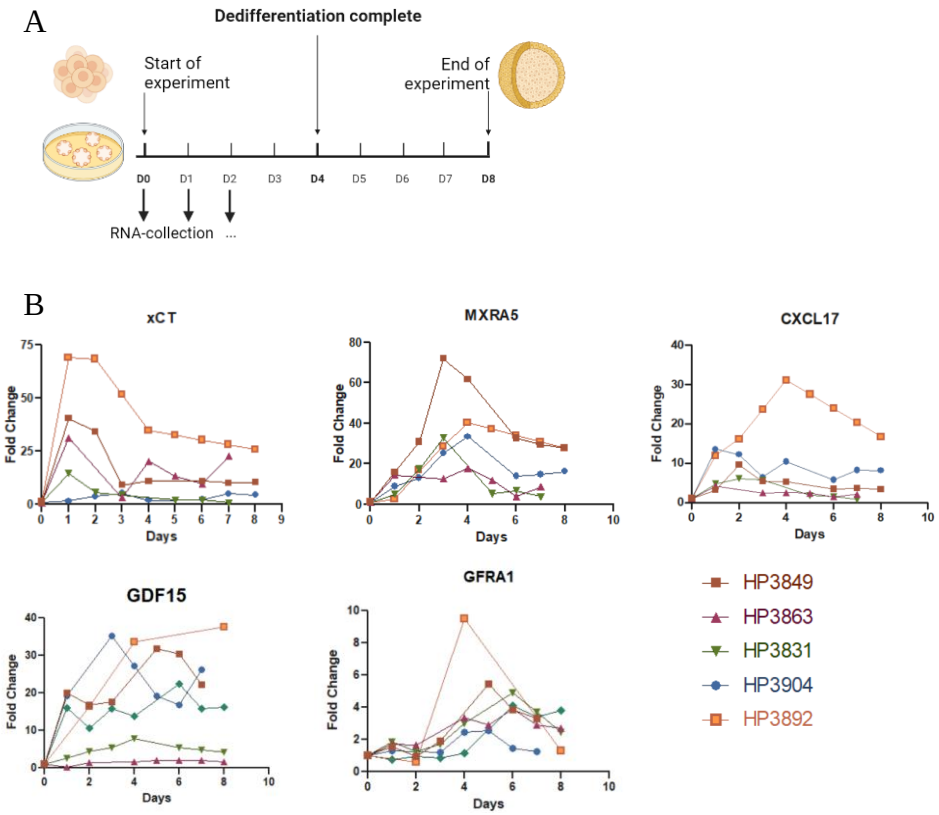
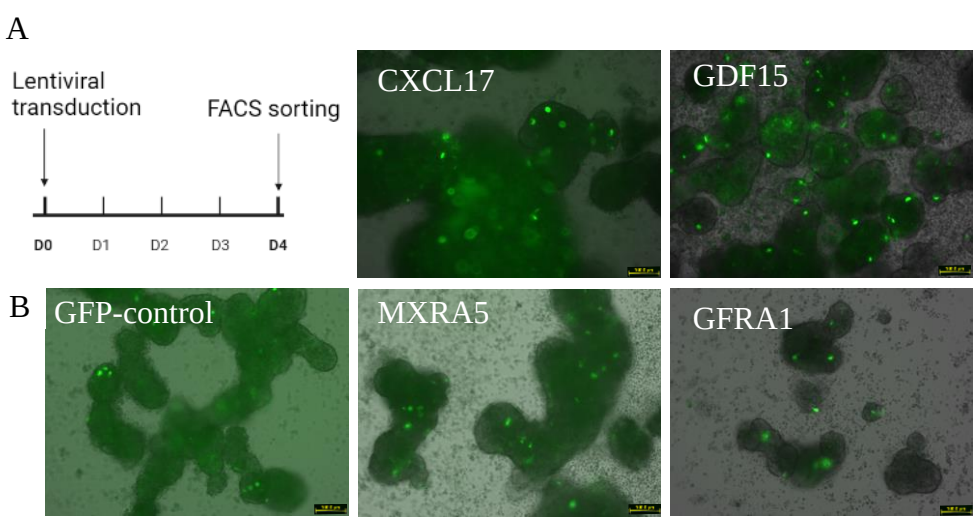


Figure 1: Expression changes of genes of interest during acinar-to-ductal metaplasia. (A) Schematic overview of the *in vitro* ADM assay. D= day. (B) Fold changes of five genes of interest during the process of acinar-to-ductal metaplasia. Legenda represents the different human samples. Fold changes are relative to day 0. N=5



C

	GFP-	GFP+	Efficiency
Control	100,000	6,722	6.7%
CXCL17 sh1	100,000	3,219	3.2%
CXCL17 sh2	70,000	620	0.9%
CXCL17 sh3	100,000	1,200	1.2%
CXCL17 sh4	100,000	1,600	1.6%
MXRA5 sh1	100,000	1,880	1.9%
MXRA5 sh2	100,000	1,728	1.7%
MXRA5 sh3	100,000	2,156	2.2%
MXRA5 sh4	100,000	1,060	1.1%
GFRA1 sh1	100,000	1,412	1.4%
GFRA1 sh2	100,000	2,805	2.8%
GFRA1 sh3	100,000	1,996	2.0%
GFRA1 sh4	100,000	911	0.9%
GDF15 sh1	100,000	3,300	3.3%
GDF15 sh2	100,000	1,621	1.6%
GDF15 sh3	100,000	1,609	1.6%
GDF15 sh4	100,000	1,346	1.3%

Figure 2: Lentiviral transduction of human acinar cells to knock down genes of interest. (A) Timepoints of manipulation during the experiment. (B) GFP-positive, and thus transduced cells for each gene of interest, this with varying efficiency at day three. (C) The numbers of transduced and non-transduced cells in the different conditions, with different shRNA sequences (sh= shRNA, GFP= Green Fluorescent Protein). Multiplicity of infection= 5.

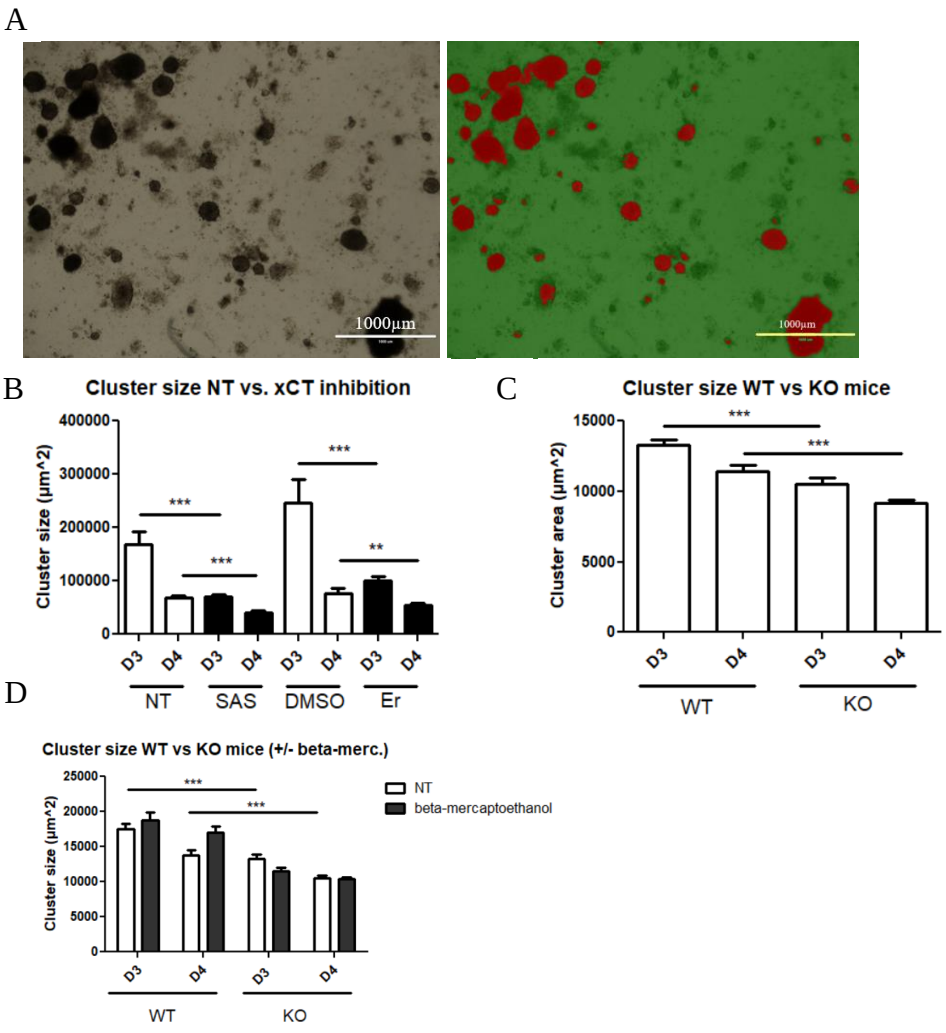


Figure 3: Cluster size analysis of human and mouse acinar cells. (A) Representation of HALO 'mask' to recognize clusters of acinar cells. (B) Cluster size analysis of human acinar cells during suspension culture, this with treatment with xCT inhibitors. This was performed at day three and day four of culture. Technical replicates: 2. (C) Cluster size analysis xCT wild type and knock-out mice during suspension culture. N=6 (D) Cluster size analysis with addition of β-mercaptoethanol in xCT KO and WT mice. N=3, NT= non-treated, SAS= Sulfasalazine, Er= Erastin. Both graphs are the result of two independent experiments. Data represent means ± sem, *p<0.05, **p<0.01, ***p<0.001, two-tailed p value. Mann-Whitney test and post hoc Bonferroni's Multiple Comparison test.

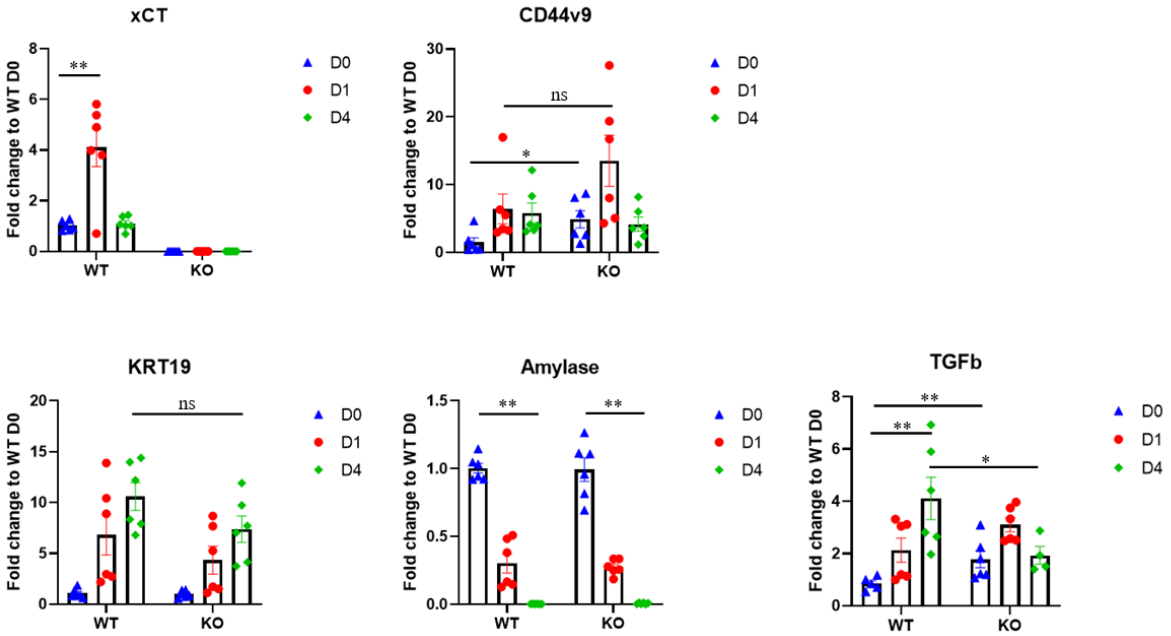


Figure 4: Expression changes in xCT wild type versus knock-out acinar cells during acinar-to-ductal metaplasia. qPCR results for a selected gene panel for acinar-to-ductal metaplasia. In this panel, amylase is the acinar cell marker and keratin-19 the ductal cell marker. TGFβ is the main initiator of the dedifferentiation process. WT= wild type, KO= knock-out. Graphs are the result of two independent experiments. N= 6. Data represent means ± sem, ns= not significant, *p<0.05, **p<0.01, two-tailed p value. Mann-Whitney test and post hoc Bonferroni's Multiple Comparison test.

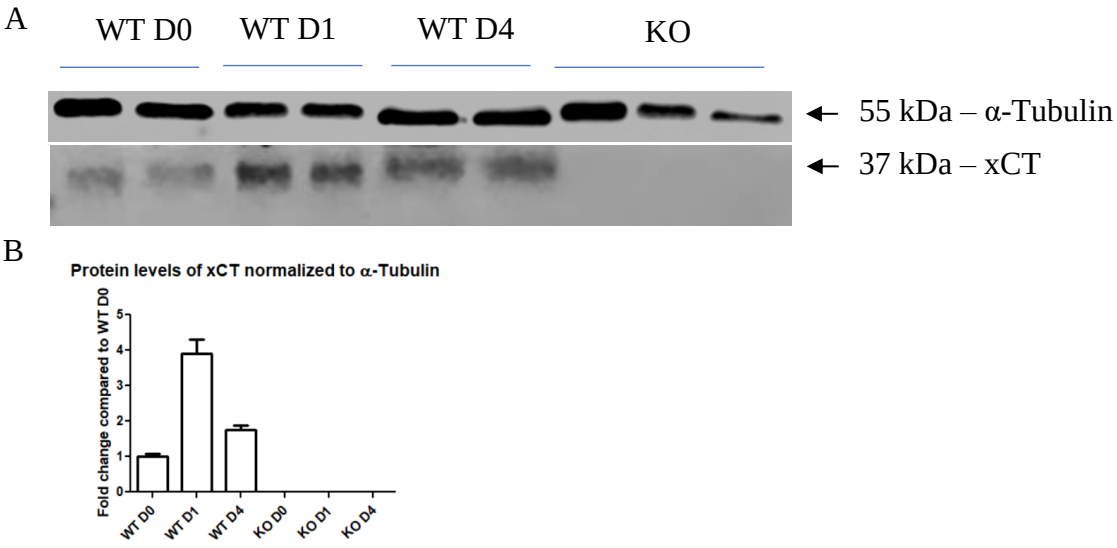
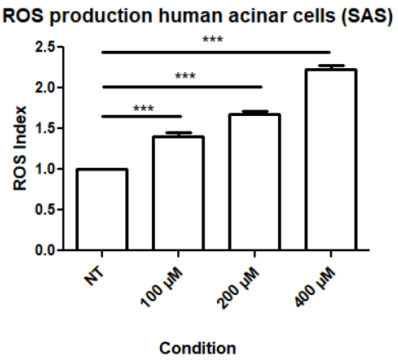


Figure 5: Western Blot of xCT in xCT WT and KO mice. (A) α-Tubulin in the upper blot and xCT in the lower blot for three time points of 2 different wildtype mice and 1 knock-out mouse. (B) Measurement of xCT protein levels normalized to α-Tubulin.

A



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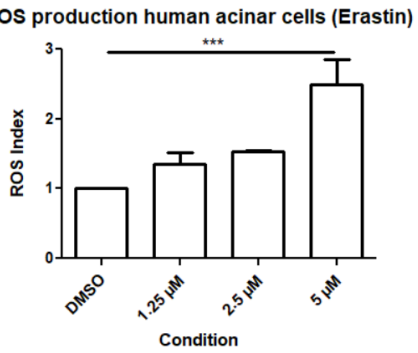

Figure 6: Testing of the efficiency of xCT-inhibitors Sulfasalazine (SAS) and Erastin. Measurement of intracellular reactive oxygen species in human acinar cells during xCT-inhibition with (A) Sulfasalazine or (B) Erastin. Graphs are the result of two independent experiments. Technical replicates: 2. Data represent means ± sem, ns= not significant, *p<0.05, **p<0.01, ***p<0.001, two-tailed p value. One-way ANOVA and post hoc Dunnett's Multiple Comparison test.

Supplementary information

It should be noted that this internship took place during the Covid-19 pandemic. Due to this, the frequency at which human exocrine samples were received was low. As a result, the results of the RNA-sequencing experiment were not yet obtained and are thus not included in this manuscript.