Expression of human cationic trypsinogen (PRSS1) in murine acinar cells promotes pancreatitis and apoptotic cell death

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Hereditary pancreatitis (HP) is an autosomal dominant disease that displays the features of both acute and chronic pancreatitis. Mutations in human cationic trypsinogen (PRSS1) are associated with HP and have provided some insight into the pathogenesis of pancreatitis, but mechanisms responsible for the initiation of pancreatitis have not been elucidated and the role of apoptosis and necrosis has been much debated. However, it has been generally accepted that trypsinogen, prematurely activated within the pancreatic acinar cell, has a major role in the initiation process. Functional studies of HP have been limited by the absence of an experimental system that authentically mimics disease development. We therefore developed a novel transgenic murine model system using wild-type (WT) human PRSS1 or two HP-associated mutants (R122H and N29I) to determine whether expression of human cationic trypsinogen in murine acinar cells promotes pancreatitis. The rat elastase promoter was used to target transgene expression to pancreatic acinar cells in three transgenic strains that were generated: Tg(Ela-PRSS1)NV, Tg(Ela-PRSS1*R122H)NV and Tg(Ela-PRSS1*N29I)NV. Mice were analysed histologically, immunohistochemically and biochemically. We found that transgene expression is restricted to pancreatic acinar cells and transgenic PRSS1 proteins are targeted to the pancreatic secretory pathway. Animals from all transgenic strains developed pancreatitis characterised by acinar cell vacuolisation, inflammatory infiltrates and fibrosis. Transgenic animals also developed more severe pancreatitis upon treatment with low-dose cerulein than controls, displaying significantly higher scores for oedema, inflammation and overall histopathology. Expression of PRSS1, WT or mutant, in acinar cells increased apoptosis in pancreatic tissues and isolated acinar cells. Moreover, studies of isolated acinar cells demonstrated that transgene expression promotes apoptosis rather than necrosis. We therefore conclude that expression of WT or mutant human PRSS1 in murine acinar cells induces apoptosis and is sufficient to promote spontaneous pancreatitis, which is enhanced in response to cellular insult.

Cell Death and Disease (2014) 5, e1165; doi:10.1038/cddis.2014.120; published online 10 April 2014

Subject Category: Experimental Medicine

Hereditary pancreatitis (HP) is characterised by recurrent episodes of acute pancreatitis (AP) that frequently progress to chronic pancreatitis (CP), with exocrine and endocrine insufficiency.¹⁻³ HP is associated with mutations in the human cationic trypsinogen gene (protease serine 1) PRSS1.¹ Two mutations most frequently found in HP patients are PRSS1 R122H and N29I.⁵ Biochemical studies have demonstrated that both mutations are associated with a ‘gain of function’ caused by an increased propensity for trypsin-mediated trypsinogen autoactivation.⁶,⁷ In addition, R122H mutation renders trypsin resistant to auto-hydrolysis, resulting in increased protein stability.⁸,⁹ Trypsinogen is an inactive precursor secreted by the acinar cells of the pancreas that is activated by duodenal enterokinase into active trypsin.¹⁰ Trypsin has a key role in digestion due to its ability to activate itself and other pancreatic digestive enzyme precursors. This has led to the hypothesis that inappropriate intra-acinar activation of trypsinogen initiates a cascade that can overwhelm the protective mechanisms of the acinar cell, such as PST1 (pancreatic secretory trypsin inhibitor or SPINK1), which can inhibit up to 20% of trypsin activity,¹¹⁻¹⁴ resulting in pancreatitis.¹⁵,¹⁶ Once pancreatitis has been initiated, secondary events such as inflammatory cell infiltration, release of pro-inflammatory mediators and cellular death influence the severity of the disease.¹⁷⁻¹⁹ Experimental models of AP have shown that acinar cell death can occur via both apoptosis and

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Keywords: PRSS1; hereditary pancreatitis; transgenic mouse; apoptosis; necrosis

Abbreviations: α-SMA, alpha-smooth muscle actin; AP, acute pancreatitis; CP, chronic pancreatitis; HA, haemagglutinin; H&E, haematoxylin and eosin; HP, hereditary pancreatitis; IHC, immunohistochemistry; SB, Southern blotting; WB, western blotting

Received 16.4.13, revised 17.12.13; accepted 19.12.13; Edited by M Agostini
necrosis, with the latter correlating with a more severe outcome.\textsuperscript{18,20,21} Although intra-acinar activation of trypsinogen has been shown in experimental pancreatitis, the exact mechanism of activation and the role of trypsin in development of pancreatitis remain unclear. In an attempt to investigate the role of trypsinogen \textit{in vivo}, Archer et al.\textsuperscript{22} developed a model based on acinar-specific expression of a mutated mouse trypsinogen gene, equivalent to the human R122H mutation, and reported an early onset of acute pancreatic injury with evidence of fibrotic changes with increased age of animals. Another mouse model based on expression of the R122H mutant of human cationic trypsinogen was also developed; however, these animals failed to develop a spontaneous phenotype possibly due to low transgene expression.\textsuperscript{23} The present study was designed to determine whether expression of wild-type (WT) human cationic trypsinogen, \textit{PRSS1}, in the murine pancreas would be sufficient to result in spontaneous pancreatitis, or whether expression of mutant forms of \textit{PRSS1} (specifically the HP-associate \textit{PRSS1} R122H and N29I) would be necessary to promote disease. We have chosen to base our studies on human trypsinogen (\textit{PRSS1}) for two main reasons: (1) because of its higher propensity to auto-activate\textsuperscript{24,25} compared with other mammalian trypsinogens and (2) because it is unclear which of the known murine trypsinogen genes is the orthologue of \textit{PRSS1}, the main human trypsinogen, mutation of which can cause HP. We show that animals from all three strains are more prone to developing spontaneous pancreatitis and that this is increased following cerulein challenge. Our data demonstrate that expression of the human \textit{PRSS1} gene, whether WT or mutant, predisposes these animals to develop pancreatitis. In addition, our studies suggest that acinar cell apoptosis, rather than necrosis, is associated with transgene expression and thus with the development of pancreatitis in our model system.

**Results**

\textit{PRSS1} transgenic mice express human \textit{PRSS1} in a tissue-specific manner. We generated three transgenic mouse strains Tg(Ela-\textit{PRSS1})\textsuperscript{NV}, Tg(Ela-\textit{PRSS1}*R122H)\textsuperscript{NV} and Tg(Ela-\textit{PRSS1}*N29I)\textsuperscript{NV} that express either WT or one of two mutated forms of \textit{PRSS1} (R122H and N29I) in the acinar cells of the pancreas by using a rat elastase promoter to target transgene expression.\textsuperscript{26} Hereafter, these strains are referred to collectively as \textit{PRSS1} transgenic mice and individually as \textit{PRSS1}, R122H and N29I, respectively. Founder mice for each strain were identified by Southern blotting (SB) (Figure 1b), and analysis of transgene expression by western blotting (WB), using an antibody specific for human \textit{PRSS1} (AF3848), demonstrated tissue-specific expression of a 32-kDa band corresponding to human \textit{PRSS1} that was detectable only in the pancreas and not in other tissues (Figure 1c). Figure 1d shows that all three transgenic strains express comparable levels of the \textit{PRSS1} transgenes in the pancreas. The antibody used for these WBs is specific for human \textit{PRSS1}, showing no cross-reactivity to mouse trypsinogen by WB or immunohistochemistry (IHC) analyses (Supplementary Figure 1). We obtained similar results using an haemagglutinin (HA) antibody to detect the transgene product by WB (for example, see Figure 3f). Figure 1e shows immunohistochemical staining typical of all three strains, obtained using a PRSS1-specific antibody, indicating that the transgene is expressed specifically in acinar cells (see also Supplementary Figure 1D). Expression of \textit{PRSS1} transgenes, as detected by IHC, was heterogeneous, with some acinar cells exhibiting strong staining, whereas others stained relatively weakly (Figure 1e and Supplementary Figure 2), which is consistent with previous reports of transgene expression from the rat elastase promoter.\textsuperscript{27} Interestingly, we did not observe any substantial difference in the overall expression level between animals homozygous or heterozygous for the transgene by WB or by IHC (Supplementary Figure 2). This might be a consequence of the observed heterogenous expression, suggesting either that each individual acinar cell has a differential ability to regulate the transgene expression level, perhaps due to a feedback regulation process that limits the amount of trypsinogen/trypsin expression, or this could be due to destruction of the most highly expressing acinar cells.

Transgene expression is stable and does not appear to change/diminish with age (Supplementary Figure 2B). All three strains are genetically stable and have continued to express the transgenes for more than 10 generations. Transgenic proteins are part of the pancreatic secretory pathway, since they are present in pancreatic juice collected from animals that express the transgene, but not in WT littermates (Figure 1f). We therefore conclude that the expression of each transgenic has been successfully targeted to the acinar cells of the pancreas and, furthermore, that the transgenic proteins are secreted via pancreatic secretory pathways.

\textit{PRSS1} transgenic mice display histological and immunohistochemical evidence of pancreatitis. Transgene-expressing animals demonstrated no gross physical or behavioural differences compared to WT littermates. However, histological examination of the pancreas isolated from transgenic animals revealed pathological changes characteristic of pancreatitis, particularly in ageing (>9 month) animals. Interestingly, these changes occurred spontaneously in up to 10% of animals expressing all three transgenes (Figure 2). The earliest anomaly seen was vacuolisation present throughout the pancreas of a majority of transgenic animals, often associated with areas of prominent transgene expression (Figures 2a and b). In addition, infiltration of the pancreas by inflammatory cells associated with the loss of adjacent acinar cells was frequently observed and typical examples are shown in Figure 2c. This inflammatory infiltrate coincided with areas in the pancreas where the transgene expression was most visible in the acinar cells (Figure 2d). Some transgenic animals demonstrated a more severe phenotype with large areas of the pancreas replaced by adipocytes (Figure 2e) and a surrounding fibrotic reaction identified by Sirius red staining (Figure 2f). T and B cells, as well as macrophages, often associated with chronic inflammatory lesions,\textsuperscript{28} were frequently present in pancreatic parenchyma (Figures 3a–c). Increased levels of \(\alpha\)-smooth muscle actin (SMA), an indicator of an active fibrotic process, were observed by IHC in activated pancreatic stellate cells (Figure 3d) and also
by WB in pancreata from animals displaying pancreatitis (Figure 3e). Minor variations in transgene expression observed on WB (Figure 3e) did not correlate with the histological findings of pancreatitis in the transgenic animals. Similar results were obtained for all three transgenic strains and we therefore conclude that transgenic animals...
Figure 2  Histological analysis of PRSS1 transgenic animals. (a) Representative images of H&E-stained tissue sections of pancreatic tissue isolated from 6-month-old heterozygous animals from each transgenic strain, as indicated, showing examples of vacuolisation that was not present in matched WT control. (b) Immunohistochemical analysis of the animals analysed in (a) using a PRSS1-specific antibody (AF3848) showing co-localisation between vacuolisation and transgene expression and absence of staining in WT control. (c) Representative images of H&E-stained pancreatic tissue sections isolated from 16-month-old heterozygous animals from each transgenic strain showing infiltration of the pancreas by inflammatory cells associated with the loss of adjacent acinar cells that was not detected in WT control. (d) Immunohistochemical analysis of the same animals analysed in (c) showing that the inflammatory infiltrate coincides with areas of high transgene expression. (e) Representative images of H&E-stained pancreatic tissue sections isolated from 8-month-old homozygous animals from each transgenic strain showing adipose atrophy that was not detected in WT control. (f) Sirius red staining of pancreatic tissue isolated from 12-month-old heterozygous animals from each transgenic strain, showing collagen deposits (red) in the fibrotic reaction resulting from destruction of acinar tissue that was not detected in WT control. Original magnification × 200
expressing WT human PRSS1 as well as R122H and N29I mutant forms develop pancreatitis characterised by the presence of vacuolisation of the acinar cells, intralobular inflammatory cell infiltrates and fibrosis.

Since not all animals developed pancreatitis spontaneously, we speculated whether increased levels of SPINK1/PSTI expression, an inhibitor of PRSS1, might have a role in the suppression of this phenotype. Analysis of SPINK3 (the murine homologue of human SPINK1) by WB demonstrated that there was no difference in the expression level between transgenic animals that developed pancreatitis and histologically normal and WT littermates (Supplementary Figure 3), suggesting that SPINK3 levels do not influence development of pancreatitis in our model system.

Figure 3 Immunohistochemical analysis of inflammatory lesions observed in pancreatic tissues from PRSS1 transgenic mice. Immunohistochemical analysis of inflammatory infiltrates in 8.5-month-old heterozygous animals from each transgenic strain and age-matched WT control, as indicated, using cell-type-specific marker antibodies for: (a) T cells (CD3), (b) B cells (CD20), (c) macrophages (F4/80) and (d) activated pancreatic stellate cells (PSCs), identified by staining for alpha-smooth muscle actin (α-SMA). Insets represent magnified areas of the photomicrographs (original magnification × 200). (e) WB analysis of pancreatic tissue isolated from littermates derived from an intercross of PRSS1 heterozygous animals using antibodies specific for α-SMA, HA and β-actin as indicated. Please note that the same blot was probed for SPINK3, shown in Supplementary Figure 3. P = animal with pancreatitis observed by histological analysis; N, histologically normal animal; wt, wild-type animal of the same genetic background; M, molecular weight marker (kDa)
Acinar cell architecture is altered by PRSS1 transgene expression. In order to examine the phenotype of acini in more detail, we analysed ultra-thin sections of pancreas tissue by electron microscopy. Marked differences were observed in the appearance of acinar cells from each of the transgenic strains when compared with the WT (Figure 4). The most distinctive feature observed was the presence of enlarged, dilated endoplasmic reticula (DER in Figure 4c). In addition, vacuoles, decondensing zymogen granules and disruption of the apical membranes of acinar cells were observed in each transgenic strain, but not in WT littermates. These data suggest that expression of the WT or mutant human PRSS1 has a detrimental effect on acinar cell architecture.

PRSS1 transgenic mice are sensitised to cerulein-induced pancreatitis. The transgenic mouse strains we generated are prone to developing pancreatitis and therefore we examined whether expression of WT or mutant human PRSS1 would render them more susceptible to chemically induced pancreatitis. Peritoneal injection of cerulein at a supra-physiological dose (typically 50 μg/kg), has been shown to induce an AP-like phenotype in mice. When this dose of cerulein was administered to WT and PRSS1 transgenic strains, we observed no differences in pancreatic response, with comparable numbers of animals developing pancreatitis from each strain (data not shown). However, when these animals were treated with a lower dose of cerulein (20 μg/kg), transgenic animals from each of the three transgenic strains displayed more severe pancreatitis than WT animals, characterised by pancreatic oedema and infiltration of inflammatory cells, determined through histological analysis (Figure 5a). Quantitative analysis of these parameters demonstrated that the extent of oedema and inflammatory infiltration and the overall histopathological score (oedema, inflammation and necrosis) in animals from each of the three transgenic strains were significantly higher compared with WT animals (Figures 5b–e), with \( P \) values for WT versus PRSS1/R122H/N29I being respectively: for oedema: \( P = 0.0064/0.0219/0.011 \), for inflammation: \( P = 0.0029/0.0397/0.0017 \), and for overall histopathological score: \( P = 0.0111/0.0191/0.0038 \). With respect to necrosis, only the R122H strain displayed a statistically significant increase (\( P = 0.0197 \)) following cerulein treatment compared with WT animals (Figure 5d). This suggests that necrosis is not the predominant mode of cell death following the onset of pancreatitis in PRSS1 transgenic mice, since only one strain displays increased necrosis, but all three strains develop...
Figure 5  Analysis of cerulein-induced pancreatitis in PRSS1 transgenic mice. (a) Representative images of H&E-stained tissue sections isolated from animals from each transgenic strain and age-matched WT control, as indicated, in the absence (–CER) and presence (+ CER) of cerulein treatment (20 μg/kg). Original magnification × 200. Histological score examination of WT and transgenic animals for (b) oedema, (c) inflammation, (d) necrosis and (e) overall histopathological score.31 (f) Analysis of trypsin activity from pancreata isolated from wild-type and transgenic animals in the absence and presence of cerulein treatment. All animals analysed were 10 months old and derived from intercross mating of heterozygous animals from each transgenic strain as indicated.
pancreatitis comparably. In addition, amylase levels were not significantly different between transgenic and WT animals, both with and without cerulein treatment (Supplementary Figure 4). There is, however, a statistically significant difference between basal levels of trypsin activity (i.e. without cerulein treatment) between each of three transgenic strains and WT animals ($P = 0.0088/0.0363/0.0153$ for the WT versus PRSS1/R122H/N29I, respectively), which disappears following cerulein treatment as Figure 5f shows. It is not clear why there was a reduction in trypsin activity in the PRSS1 animals treated with cerulein compared with the untreated group. Taking these observations together, we conclude that animals from each of the three transgenic strains express higher basal levels of trypsin and thus are primed to develop more severe pancreatitis upon treatment with cerulein than the WT animals, as indicated by the significantly higher score for oedema, inflammation and overall histopathological score.

**Acinar cells from PRSS1 transgenic mice display increased apoptosis.** There is evidence that the balance between apoptosis and necrosis might determine the severity of AP that occurs in response to acinar cell injury.21 Our studies of cerulein-induced pancreatitis demonstrate that there is very little difference in necrosis between transgenic and WT animals, suggesting that changes in necrosis are unlikely to mediate acinar cell death leading to pancreatitis in these animals.

To examine the role of apoptosis in more detail, sections of pancreatic tissue from 9.5-month-old untreated transgenic animals and WT littermates were stained with antibodies specific for a cleaved form of caspase-3, an indicator of apoptosis.32,33 and representative images of the IHC are shown in Figure 6a. Analysis of cleaved caspase-3 staining showed that significantly more cells were stained positively in all three of the transgenic strains when compared with WT animals ($P = 0.0029/0.0014/0.0041$ for the WT versus PRSS1/R122H/N29I, respectively) (Figure 6b). In addition, we have also analysed apoptosis using TUNEL assay on pancreatic tissue sections (Figure 6c). Quantification for these assay data confirmed that there were more apoptotic cells in each of our transgenic strains than in WT animals ($P = 0.0071/0.0014/0.0002$ for the WT versus PRSS1/R122H/N29I, respectively) (Figure 6d). Therefore, using two independent methods, we demonstrated that there is more spontaneous apoptosis in pancreatic tissue from each of our transgenic strains, than in a matched WT control. These results suggest that the expression of human PRSS1 in the acinar cells, regardless of whether it is WT or a mutated form, induces an apoptotic response in pancreatic tissue.

To further investigate the response of pancreatic acinar cells to injury, we utilised an *in vitro* cell death assay in which isolated acinar cells are exposed to the bile acid tauroliothiocholic acid sulphate (TLC-S) that causes caspase activation resulting in apoptosis.34,35 To analyse the apoptotic response, acinar cell clusters isolated from the three transgenic strains or from WT littermate animals were incubated with TLC-S in the presence of R110-asparatic acid amide, a fluorescent indicator-linked caspase substrate.34 Propidium iodide staining was used to identify necrotic cells, while Hoechst staining was used to visualise cell nuclei (Figure 7a). As expected, there is a marked increase in the percentage of necrotic and apoptotic cells upon treatment in cells from WT and transgene-expressing animals. However, analysis demonstrates that, with respect to necrosis, acinar cells isolated from each of the three transgenic strains behave in a similar fashion to acinar cells isolated from WT animals (Figure 7b). In contrast, there is a significant increase in the number of apoptotic cells from the three transgene-expressing strains compared with the WT animals, both with TLC-S treatment ($P = 0.0248/0.0483/0.0186$ for the WT versus PRSS1/R122H/N29I, respectively) and without ($P = 0.0016/0.0253/1.53 \times 10^{-5}$), as shown in Figure 7c. We have also analysed apoptosis in isolated acinar cells using TUNEL assay, and representative images from a typical experiment are shown in Figure 7d. Using this assay, we have observed a statistically significant increase in apoptosis in acinar cells isolated from transgenic animals that were not treated with TLC-S ($P = 0.0367/0.0174/0.0056$ for the WT versus PRSS1/R122H/N29I, respectively) (Figure 7e), but not in treated acinar cells. The difference compared with results obtained using the *in vitro* cell death assay might be a consequence of the different methodology utilised or the fact that animals used for TUNEL assay analysis were young. Regardless, using two independent assays we have demonstrated that there is more spontaneous apoptosis in acinar cells isolated from transgenic animals than in WT ones. Thus we conclude that isolated acinar cells recapitulate the behaviour of *in vivo* acinar cells in terms of response to cellular injury. In both systems, the mode of acinar cell death that distinguishes samples from pancreatitis-prone PRSS1 transgene-expressing mice from WT mice is apoptosis, not necrosis.

**Discussion**

Pancreatitis, whether acute or chronic, is a complex inflammatory disease, with details of the earliest cellular events still to be delineated.36-40 While premature, intra-acinar activation of trypsinogen is considered to be one of the initiating events in the pathogenesis of pancreatitis, the *in vivo* consequences of PRSS1 gene mutations remain unclear. In the present study we have generated three transgenic strains of mice: Tg(Ela-PRSS1)NV, Tg(Ela-PRSS1*R122H)NV and Tg(Ela-PRSS1*N29I)NV, that express a WT human PRSS1, R122H or N29I mutants, respectively, in the acinar cells of the pancreas. This is the first time that transgenic animals expressing the WT and the two most frequently mutated forms of human cationic trypsinogen detected in HP patients have been generated and analysed. Our data show that each transgene is expressed in a tissue-specific manner restricted to the pancreas, is localised to acinar cells and is part of the pancreatic acinar cell secretory pathway. Moreover, transgene expression in all three transgenic strains analysed does not diminish with the age of animals and has been stable for more than 10 generations. Histological analysis of pancreata isolated from PRSS1 transgene-expressing animals demonstrates that all three strains develop pancreatitis spontaneously, whereas none of their WT littermates do. The pancreatitis that is observed displays major hallmarks of AP and CP, including vacuolisation, intralobular inflammatory...
infiltrates containing macrophages, T cells and B cells and fibrosis characterised by the presence of activated pancreatic stellate cells. Examination of pancreatic tissue sections by EM shows that the architecture of the apical region of the acinar cell is disrupted in transgenic animals. These data suggest that pancreas-specific expression of the human PRSS1 gene, regardless of whether it is WT or mutated, promotes pancreatitis and this is the first time that this effect has been observed in an animal model. Clearly, while all of the transgene-expressing animals display pancreatitis following challenge with low-dose cerulein, the incidence of spontaneous pancreatitis indicated incomplete penetrance. One of the possible explanations for the incomplete penetrance seen in transgenic mice might be due to activation of inhibitory or protective mechanisms in acinar cells upon expression of transgenic trypsinogen. It has been suggested that SPINK/PSTI can inhibit up to 20% of the trypsin activity in the pancreas and transgenic studies have demonstrated that PSTI-I expression can reduce the severity of cerulein-induced pancreatitis. 10,12,29 In our model system, however, we see no evidence that SPINK has a role in determining the pathophysiology, as the levels of SPINK3 expression are the same in...

Figure 6 Analysis of cell death in PRSS1 transgenic mice pancreatic tissue sections. (a) Representative images of immunohistochemical analysis of pancreatic tissue sections isolated from animals from each transgenic strain and matched wild-type (WT) control, as indicated, using antibodies specific for cleaved caspase-3. Original magnification ×200. (b) Quantification of immunohistochemical detection of cleaved caspase-3. (c) Representative images of TUNEL analysis of pancreatic tissue sections isolated from animals from each transgenic strain and matched WT control, as indicated. Insets represent magnified areas of the photomicrographs showing punctuate TUNEL-positive staining characteristic of an apoptotic cell (original magnification ×200). (d) Quantification of TUNEL assay data.
Figure 7  Analysis of cell death in acinar cells isolated from PRSS1 transgenic mice. (a) Representative images from a typical experiment using an in vitro cell death assay\textsuperscript{34,35} to examine isolated acinar cells showing bright field image of cells (i), green fluorescent protein (GFP)-labelled cells with cleaved caspase substrate (ii), nuclei stained with Hoeschst (iii) and necrotic cells stained with propidium iodide (iv). Quantification of necrosis (b) and apoptosis (c) observed in acinar cells isolated from WT and transgenic animals. All animals analysed were approximately 8.5 months old and derived from intercross mating of heterozygous animals from each transgenic strain as indicated. WT animals are littermates negative for the presence of the transgene. (d) Representative images from a typical TUNEL assay performed on isolated acinar showing bright-field image of cells (i), fluorescein-labelled cells using TUNEL assay kit (ii), nuclei stained with Hoeschst (iii) and an overlay of the images shown in ii and iii (iv). (e) Quantification of apoptosis using TUNEL assay performed on isolated acinar cells. All animals analysed were approximately 7 months old and derived from intercross mating of heterozygous animals from each transgenic strain as indicated. WT animals are littermates negative for the presence of the transgene.
transgenic animals with pancreatitis and in histologically normal and WT littermates. An alternative possibility might be the heterogeneous nature of transgene expression driven by the elastase promoter, observed by us and others, which, combined with the overall comparable steady-state expression observed in homozygous and heterozygous animals, may suggest either that each individual acinar cell has the ability to regulate the transgene expression levels, or that the pancreas can tolerate only certain levels of trypsinogen expression. This might be due either to some form of feedback regulation or to destruction of the most highly expressing acinar cells, and further studies will be required to examine these questions.

Animals from all three PRSS1 transgenic strains also develop more severe pancreatitis in response to treatment with a low dose (20 μg/kg) of cerulein, and basal trypsin activity, in the absence of cerulein treatment, is significantly higher in the transgene-expressing strains compared to WT littermates. It seems likely that this raised basal trypsin activity may breach a threshold level above which pancreatic damage ensues and is therefore responsible for the increased severity of pancreatitis seen in transgenic animals. This accords with in vivo studies that showed that intra-acinar activation of trypsinogen plays an important role in the initiation of pancreatitis, although a recent study has questioned this in chronic pancreatitis.

Clinically, most cases of AP are mild, self-limiting events that resolve without long-term consequences for the patient; however, 20% of patients will develop severe AP with evidence of pancreatic necrosis. The underlying mechanism that drives an episode of AP towards pancreatic necrosis in humans remains poorly understood, though animal models have demonstrated that the apoptosis/necrosis ratio varied significantly depending on the type of model studied. We observed significantly higher spontaneous apoptosis in pancreata and in isolated acinar cells isolated from PRSS1 transgenic strains compared with WT animals, suggesting that the expression of human WT or mutated PRSS1 in the acinar cells promotes apoptosis. In contrast, we observed almost no differences in necrosis associated with PRSS1 transgene expression. Therefore, in our model system, apoptosis provides a more likely mechanism of acinar cell death in response to PRSS1 expression. Apoptosis can be a protective mechanism and it is conceivable that this may be a reason we observed the pathological hallmarks of spontaneous AP in only 10% of the transgenic animals. A similar protective mechanism may exist in patients with HP, who, despite carrying the disease-causing mutation in PRSS1 from birth, typically exhibit episodes of AP during adolescence. On the other hand, there is evidence that active intra-acinar trypsin may have a protective role in AP. However, more recent studies based on genetically modified mouse models accord with our findings showing that intra-acinar trypsinogen activation is implicated in acinar cell death and that this contributes to pancreatic tissue injury that leads to pancreatitis. Please note that, when using an antibody that crossreacts with human and mouse trypsinogen, the major reactivity detected is the endogenous mouse signal and thus comparatively low levels of human trypsinogen are apparently expressed compared with the endogenous mouse trypsin (Supplementary Figure 1A). This argues against the idea that high levels of transgenic protein, per se, cause pancreatic damage in this system. However, future studies will be needed to address the mechanism in more detail. Thus, it seems most likely that the small, but significant increase in basal trypsin activity that we have observed (Figure 5) is responsible for the phenotype observed in transgenic animals.

It is intriguing that we do not see any difference in phenotype between the three transgenic strains. PRSS1 mutations are associated with HP in humans and it would be expected that expression of mutant forms of human PRSS1 in mouse acinar cells would cause pancreatitis. It was more surprising that transgenic mice expressing wt human PRSS1 also developed pancreatitis spontaneously and are sensitised to low-dose cerulein-induced pancreatitis. One possible explanation for this might be a higher propensity of human cathionic trypsinogen to auto-activate compared to trypsinos- gens from other species. As mouse acinar cells have evolved to regulate a less intrinsically auto-activate form of trypsinogen, it is likely that expression of wt human PRSS1 in murine acinar cells leads to an imbalance in the normal regulation of trypsinogen activity. This conclusion is supported by a significant increase in basal trypsin activity that we have observed in animals expressing wt PRSS1 (Figure 5).

In conclusion, our findings shed a new light on the role of PRSS1 in acinar response to cellular injury and development of pancreatitis. We have established that expression of human PRSS1 in mouse acinar cells, regardless of whether in WT or mutated form, can promote spontaneous pancreatitis and also increase sensitivity to cerulein-induced pancreatitis. The PRSS1 transgenic strains we have generated may therefore provide useful models for studying early events in pancreatitis, as well an opportunity for developing a pre-clinical testing platform for novel therapeutic approaches.

Materials and Methods

Construction of transgenes. The first-strand cDNA of WT human PRSS1 (OMIM #276600) was generated by reverse transcription polymerase chain reaction from total human pancreas RNA (Ambion/Life Technologies Ltd., Paisley, UK) using the primer 5′-ACCATGATCCATCTCCTGGTCTC-3′. This strand was used as the template for PCR synthesis of human PRSS1 cDNA using the following set of primers: 5′-GAGAGATCCACCATGAATCCTCCTGGTCTC-3′ and 5′-GAGAGATCCATCTCAAAAGAGCGTAATCTGGAACATCGTATGGGTAGC-3′. This strand was used as the template for PCR synthesis of human PRSS1 cDNA using the following set of primers: 5′-GAGAGATCCACCATGAATCCTCCTGGTCTC-3′ and 5′-GAGAGATCCATCTCAAAAGAGCGTAATCTGGAACATCGTATGGGTAGC-3′. This strand was used as the template for PCR synthesis of human PRSS1 cDNA using the following set of primers: 5′-GAGAGATCCACCATGAATCCTCCTGGTCTC-3′ and 5′-GAGAGATCCATCTCAAAAGAGCGTAATCTGGAACATCGTATGGGTAGC-3′. This strand was used as the template for PCR synthesis of human PRSS1 cDNA using the following set of primers: 5′-GAGAGATCCACCATGAATCCTCCTGGTCTC-3′ and 5′-GAGAGATCCATCTCAAAAGAGCGTAATCTGGAACATCGTATGGGTAGC-3′. This strand was used as the template for PCR synthesis of human PRSS1 cDNA using the following set of primers: 5′-GAGAGATCCACCATGAATCCTCCTGGTCTC-3′ and 5′-GAGAGATCCATCTCAAAAGAGCGTAATCTGGAACATCGTATGGGTAGC-3′.
Proteins were extracted from tissue samples using water. They were fed standard laboratory chow with access to a 12:12 h light–dark cycle. Each transgenic line was established for each strain, PCR analysis was used for routine screening of the founder animals for each transgenic strain (Tg(Ela-PRSS1)NV, Tg(Ela-PRSS1)R122HNV and Tg(Ela-PRSS1)N291I(NV)) were identified by SB analysis, performed essentially as described previously,50 using a probe specific for the human PRSS1 sequence (indicated in Figure 1a). The transgene copy number for each founder was estimated using SB by comparison with the appropriate transgene copy number controls as shown in Figure 1b. The founders that displayed only a major band, the size of the transgene and only one additional band in SB, suggestive of a single integration site (for example, number 9 for PRSS1 strain, number 7 for R122H strain and number 11 for N291I strain), were chosen for further breeding. SB analysis performed on animals from several consecutive generations displayed the same banding pattern, indicating that each transgenic line was genetically stable and suggesting that the integrations occurred at single loci. We maintained heterozygous inter-crosses for each strain throughout the protocol, normal serum in PBS and a fluorescent indicator-linked general caspase substrate. Plasma membrane rupture, characteristic of necrosis, was detected by staining with propidium iodide (PI; 1 μg/ml), while nuclei were visualised with Hoechst 33342 (5 μg/ml). Fluorescence of R110 and an indirect method described previously. Briefly, for each parameter (oedema, inflammatory cell infiltration and necrosis) ten fields were randomly chosen, scored and the average score was used for data analysis. The overall histopathological score was calculated as the sum of the three parameters, with each contributing equally to the total score.

**In vitro cell death assay.** In vitro cell death assay was performed essentially as described previously. Briefly, fresh pancreatic acinar cells were isolated with collagenase as described previously,48 either treated or not with TCL-S for 30 min and activation of the apoptotic pathway was detected with rhodamine 110/ aspartic acid amide (20 μmol/l), a fluorescent indicator-linked general caspase substrate. Plasma membrane rupture, characteristic of necrosis, was detected by staining with propidium iodide (PI; 1 μg/ml), while nuclei were visualised with Hoechst 33342 (5 μg/ml). Fluorescence of R110/aspartic acid amide (excitation 488 and emission >505 nm), PI (excitation 488 and emission 630–693 nm), and Hoechst 33342 (excitation 364 and emission 405–450 nm) was recorded using a 63x C-Apochromat objective (Carl Zeiss Jena GmbH, Jena, Germany). Thirty high-power fields from each experiment were scored, repeated in triplicate. The total number of necrotic or apoptotic cells counted per field was then used to calculate the percentage of necrosis/apoptosis for each field, which was then averaged across all fields.
 Statistical analysis. Results were expressed as mean ± standard error of the mean from multiple separate experiments. Statistical analysis was performed using t-tests with SPSS version 16 software (IBM, Portsmouth, UK). Statistical significance was set at P < 0.05.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements. We are extremely grateful to Professor Mark Boyd for numerous helpful discussions and also thank Mark Boyd, Drs Carlos Rubbi and Eithne Costello, and Professor Graham Dockray FRS for critically reviewing the manuscript. We are very grateful for the technical support from Dr Kerrynane Crawford and members of the Liverpool BSU, especially Barry Cotterill. This work was partly funded by a grant to NV and JNP from the Royal Liverpool University Hospital R and D Fund, National Institute for Health Research grant to the NIHR Liverpool Pancreas Biomedical Research Unit; MC has been supported by an MRC grant to Professor A. Tepkin.

Author Contributions

TA and WH acquisition, analysis and interpretation of data and preparation of manuscript; RM, DL and MC: acquisition, analysis and interpretation of data; RC and KS: technical support for the study; PT: analysis and interpretation of data; CM: acquisition of data; DC: student supervision; RS: study concept and design; JN: study concept and design, material support and preparation of manuscript; NV: study concept and design, acquisition, analysis and interpretation of data, preparation of manuscript and supervision of the study.

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