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Hydrokinetic pancreatic function and insulin secretion are moduled by Cl^- uniporter Slc26a9 in mice

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Abstract

Aim: Slc26a9 is a member of the Slc26 multifunctional anion transporter family. Polymorphisms in Slc26a9 are associated with an increased incidence of meconium ileus and diabetes in cystic fibrosis patients. We investigated the expression of Slc26a9 in the murine pancreatic ducts, islets and parenchyma, and elucidated its role in pancreatic ductal electrolyte and fluid secretion and endocrine function.

Methods: Pancreatic Slc26a9 and CFTR mRNA expression, fluid and bicarbonate secretion were assessed in *slc26a9*$^{-/-}$ mice and their age- and sex-matched wild-type (wt) littermates. Glucose and insulin tolerance tests were performed.

Results: Compared with stomach, the mRNA expression of Slc26a9 was low in pancreatic parenchyma, 20-fold higher in microdissected pancreatic ducts than parenchyma, and very low in islets. CFTR mRNA was ~10 fold higher than Slc26a9 mRNA expression in each pancreatic cell type. Significantly reduced pancreatic fluid secretory rates and impaired glucose tolerance were observed in female *slc26a9*$^{-/-}$ mice, whereas alterations in male mice did not reach statistical significance. No significant difference was observed in peripheral insulin resistance in *slc26a9*$^{-/-}$ compared to sex- and aged-matched wt controls. In contrast, isolated *slc26a9*$^{-/-}$ islets in short term culture displayed no difference in insulin content, but a significantly reduced glucose-stimulated insulin secretion compared to age- and sex-matched wt islets, suggesting that the impaired glucose tolerance in the absence of Slc26a9 expression these is a pancreatic defect.

Conclusions: Deletion of Slc26a9 is associated with a reduction in pancreatic fluid secretion and impaired glucose tolerance in female mice. The results underline the importance of Slc26a9 in pancreatic physiology.

KEYWORDS
anion channel, cystic fibrosis, diabetes, electrolyte transport, pancreas

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1 | INTRODUCTION

The SLC26A9 isoform was one of the last of the multifunctional SLC26 anion transporter family to be identified at the molecular level[1] and has been highly controversially discussed at the functional level[2] but is the first mammalian SLC26 isoform whose cryostructure was recently presented.[3] It was identified as a Cl^- uniporter, which locks Cl^- ions in a closed state during the translocation process.

SLC26A9 is strongly expressed in the airways and the gastric epithelium.[1,4] A functional interaction of Slc26a9 with CFTR has been described, although both in an inhibitory and a stimulatory fashion.[5-7] The first indication of a clinically significant function of SLC26A9 in patients was found during the search for modifier genes in a cohort of cystic fibrosis patients, in which polymorphisms in the *SLC26A9* gene were identified as risk genes for the occurrence of meconium ileus.[8] This finding was surprising, because SLC26A9 expression is high in stomach, and low in murine and human intestine, with a sharp decline of Slc26a9 and a sharp increase of CFTR expression in the duodenum.[9] In murine duodenum, CFTR and Slc26a9 were crypt-predominantly expressed, and Slc26a9 deletion by itself altered proximal duodenal fluid transport in young mice. *Cftr*$^{-/-}$/*slc26a9*$^{-/-}$ mice displayed a very strong increase in intestinal mortality in the weaning period, suggesting that the *slc26a9*$^{-/-}$ mouse may serve as a useful model to gain insight into cystic fibrosis as well as SLC26A9 pathophysiology.[9] *Slc26a9*$^{-/-}$ mice also experience a very high postnatal death rate because of asphyxiation. The underlying molecular reason is controversially discussed and not yet published in full (defective fluid reabsorption during lung expansion vs mucus obstruction).[10, 11] Owing to the extremely low generation rate of *slc26a9*$^{-/-}$ pups, the study of their organ-related phenotypes has been slow.

More recently, polymorphisms in SLC26A9 were found to be strong risk modifiers for CF-related diabetes.[12] CF diabetes has been explained as secondary damage of the islets because of inflammation and fibrosis in the wake of ductal dysfunction.[13] In this context, data from CF patients suggest that *SLC26A9* genetic variants associated with age at onset of CFRD affected the expression of SLC26A9. Single cell RNA sequencing of pancreatic cells from various patients revealed a low number of cells that were positive both for CFTR and for SLC26A9, the majority of them of ductal origin.[14] However, recent data also suggest a direct role of CFTR in islet cell function.[15,16]

The present study was undertaken to unravel the expression and function of Slc26a9 in murine pancreatic tissues, as well as the effect of Slc26a9 deletion on pancreatic ductal, exocrine and endocrine function. To this end, pancreatic ducts were microdissected, islets were enzymatically isolated and handpicked, and RNA was extracted. Ductal, exocrine and islet morphology was histologically assessed. Pancreatic fluid and bicarbonate secretory rates were determined in anaesthetized, blood-pressure, acid-base and systemic fluid-controlled *slc26a9*$^{-/-}$ and wt littermates of different age groups and gender. Glucose tolerance as well as insulin sensitivity was studied in vivo, and glucose-stimulated insulin secretion in isolated islets from *slc26a9*$^{-/-}$ mice and wt littermates in short-term culture.

2 | RESULTS

2.1 | Slc26a9 and CFTR mRNA expression in pancreatic and other upper GI epithelial tissues

To delineate the distribution of SLC26A9 mRNA expression and compare it with that of CFTR mRNA expression at the epithelial level, tissues were harvested by a microdissection or enzymatic digestion followed by manual selection, as described in the method section, and either immediately processed for RNA extraction, or short-term cultured for higher purity. A relatively low level of Slc26a9 mRNA expression was observed in pancreatic compared to gastric epithelium (Figure 1A). In contrast, CFTR mRNA expression levels were higher in pancreatic parenchyma compared to gastric epithelium (Figure 1B). Figure 1C shows the mRNA expression of Slc26a9 and CFTR in pancreatic parenchyma and microdissected pancreatic ducts, with a ~20 fold higher expression level for Slc26a9, and a ~10 fold higher level for CFTR in the latter. Figure 1D demonstrates the expression levels for Slc26a9 and CFTR in the islets, which showed low, but discernable levels of both CFTR and Slc26a9. The results demonstrated that pancreatic ducts express relatively high levels of both Slc26a9 and CFTR, but that even in the pancreatic islets, where CFTR mRNA expression levels are low, very low amounts of Slc26a9 mRNA expression are also found. Although Slc26a9 has been discussed primarily as a CFTR modulator, the results in the gastric epithelium suggest that Slc26a9 is also expressed independently of CFTR and has other functions that modulates CFTR function.

2.2 | Pancreatic ductal fluid and bicarbonate secretion

Basal and secretin-stimulated pancreatic fluid and bicarbonate secretion was assessed in both young adult female and male (6-8 weeks), and in mature (8-14 months) adult

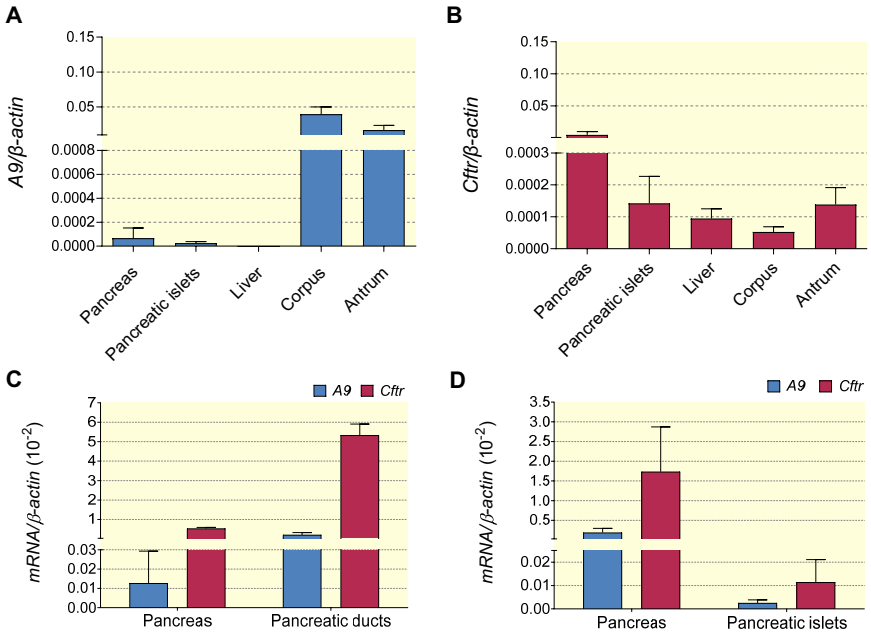

FIGURE 1 Slc26a9 and CFTR mRNA expression in pancreatic and other upper GI epithelial tissues. A, Slc26a9 mRNA expression in upper GI organs was assessed in 12 wk old S129svj mice, and demonstrates a relatively low expression in pancreatic compared to gastric epithelium. B, CFTR expression levels in the same organs. C, mRNA expression of Slc26a9 and CFTR in pancreatic parenchyma and microdissected pancreatic ducts. D, mRNA expression levels for Slc26a9 and CFTR in pancreatic parenchyma and pancreatic islets, which showed low, but discernable levels of both CFTR and Slc26a9. The results from C and D are from different groups of mice. n = 6

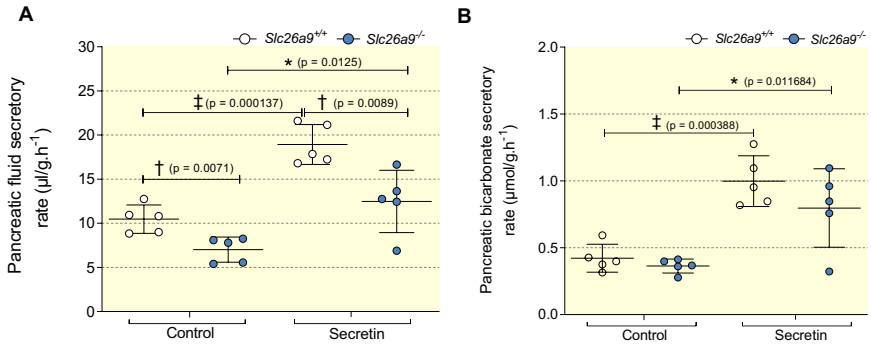

FIGURE 2 Pancreatic ductal fluid and bicarbonate secretion in young *slc26a9*$^{-/-}$ females. A, Significantly different basal and secretin-stimulated pancreatic fluid secretory rates in young (6-8 wk) female *slc26a9*$^{-/-}$ and wt littermates. B, The bicarbonate secretory rates were not different, indicating that the bicarbonate concentration is slightly higher in the *slc26a9*$^{-/-}$ pancreatic fluid. n = 5, *$P < .05$, $^{\dagger}P < .01$, $^{\ddagger}P < .001$

female and male *Slc26a9*$^{-/-}$ and wt mice (Figure 2A-D). A significant difference in both basal and secretin-stimulated pancreatic fluid secretory rates was observed between young female *slc26a9*$^{-/-}$ and wt littermates. The bicarbonate secretory rates were not different, indicating that the bicarbonate concentration is slightly higher in the *slc26a9*$^{-/-}$ pancreatic fluid (Figure 2A,B). No significant difference was observed in males, as well as in mature females (Table 1). However, the match was less perfect in males, which could not be cohoused, and aged females, which in part had been in part used for breeding.

2.3 | Slower decline in blood glucose levels after an iv glucose bolus in *slc26a9*$^{-/-}$ females

The stomach of *slc26a9*$^{-/-}$ mice is morphologically strongly altered,[17] affecting the gastric transit time which is different from wt. After pilot experiments for oral vs iv glucose tolerance test, we chose to perform blood glucose measurements after iv glucose bolus injection given by an infusion pump. We sampled blood glucose levels before and after a 6h fast, then 20 min after anaesthesia induction and catheter placement, and then at the time intervals given in (Figure 3A,B).

TABLE 1 Pancreatic fluid and HCO_3^- secretion in male and aged female slc26a9$^{-/-}$ and WT mice were not significantly different. P = 5-6

	Fluid secretion of pancreas juice (μl/g·h^{-1})			HCO_3^- secretion of pancreas juice (μmol/g·h^{-1})		
	Control	Secretin	*P*	Control	Secretine	*P*
Slc26a9$^{+/+}$ young male mice	0.41 ± 0.08	0.78 ± 0.12	<.01	0.0185 ± 0.005	0.044 ± 0.006	<.01
Slc26a9$^{-/-}$ young male mice	0.39 ± 0.05	0.78 ± 0.16	<.01	0.018 ± 0.002	0.048 ± 0.089	<.01
Slc26a9$^{+/+}$ old female mice	0.40 ± 0.01	0.64 ± 0.16	<.05	0.021 ± 0.003	0.036 ± 0.009	<.05
Slc26a9$^{-/-}$ old female mice	0.40 ± 0.13	0.49 ± 0.20	>.05	0.019 ± 0.006	0.028 ± 0.014	>.05
Slc26a9$^{+/+}$ old male mice	0.34 ± 0.05	0.56 ± 0.12	<.01	0.016 ± 0.002	0.032 ± 0.006	<.01
Slc26a9$^{-/-}$ old male mice	0.37 ± 0.07	0.52 ± 0.04	<.01	0.018 ± 0.003	0.029 ± 0.002	<.01

The blood glucose levels were not significantly altered after the fasting period, as well as after the anaesthesia, in both *slc26a9*$^{-/-}$ mice and wt littermates of both genders (Figure 3A,B). However, the decline in blood glucose was significantly delayed in adult (10-20 weeks) female *slc26a9*$^{-/-}$ mice compared to wt littermates, while the delay in blood glucose levels did not reach significance in male mice.

2.4 | Peripheral insulin resistance was not different in *slc26a9*$^{-/-}$ mice and wt littermates

An attenuated decline in blood glucose levels may also be related to decreased insulin-dependent glucose uptake into glucose-utilizing tissues. We therefore investigated the insulin sensitivity of *slc26a9*$^{-/-}$ and wt male and female mice the were in the same age range as the mice for the glucose-tolerance test had been (12-28 wk/age). No significant difference was observed between *slc26a9*$^{-/-}$ mice and wild-type littermates of either gender in the insulin tolerance tests (ITTs) (Figure 4A,B). The corresponding area under curve (AUC) for *slc26a9*$^{-/-}$ females (281.7 ± 90.4) and male mice (271.6 ± 61.2) vs wild-type female (231.5 ± 91.8) and male (258.8 ± 66.7) was also not significantly different (Figure 4B,D).

2.5 | The decreased glucose tolerance in female *slc26a9*$^{-/-}$ mice is not because of pancreatic inflammation or damage

Slc26a9$^{-/-}$ and wt pancreatic tissues were carefully inspected for signs of inflammation (infiltrates by

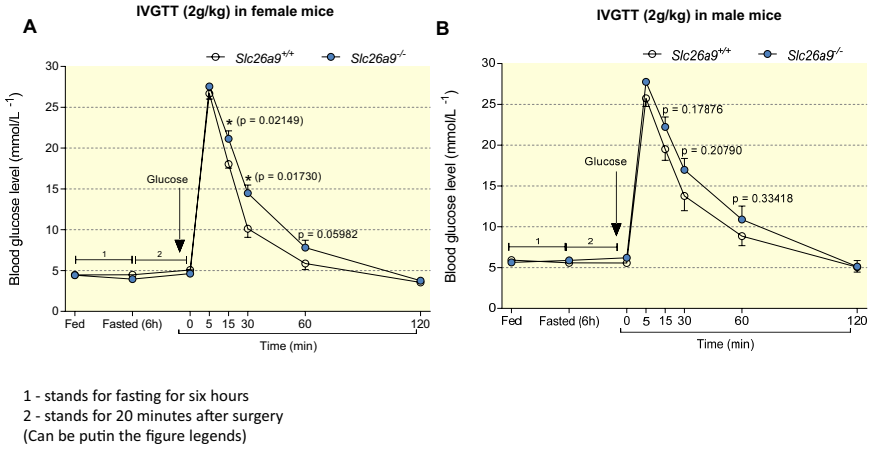

FIGURE 3 Impaired glucose tolerance in *slc26a9*$^{-/-}$ females. Peripheral blood glucose levels were determined in female (A) and male (B) mice in the fed state, after a 6 h fast, after anaesthesia induction and stabilization, and during the indicated time points after an iv glucose bolus. Basal blood glucose levels were not different in the absence of Slc26a9 expression. A significant delay in blood glucose normalization was observed in female *slc26a9*$^{-/-}$ mice compared to their wt female littermates. n = 5, *P < .05, n.s, not significant

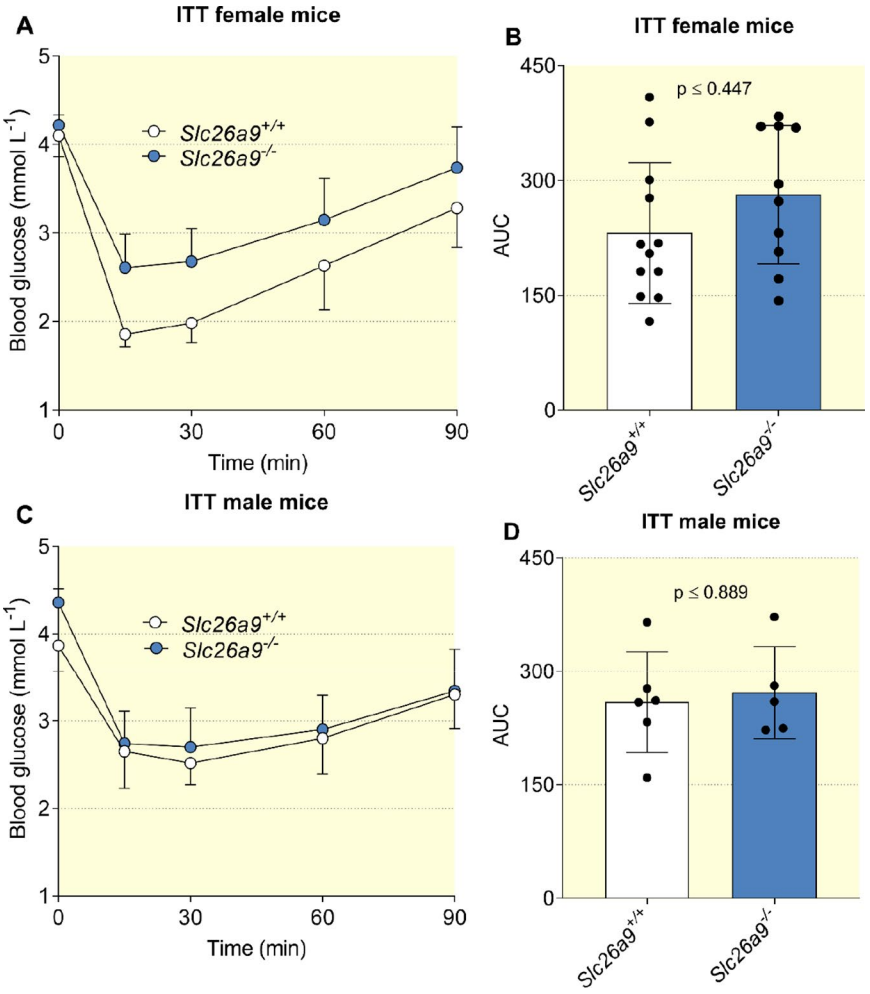

FIGURE 4 No difference in peripheral insulin sensitivity in *slc26a9*$^{-/-}$ mice and wt littermates was observed. Sequential blood glucose measurements after a bolus insulin revealed no significant difference between *slc26a9*$^{-/-}$ mice and wild-type littermates of either gender in ITT and their corresponding area under curve (AUC) (n = 5-12). Values represented as mean ± SD, and differences were considered statistically significant when $P < .05$. The jitter plots show the individual data of mice in each group

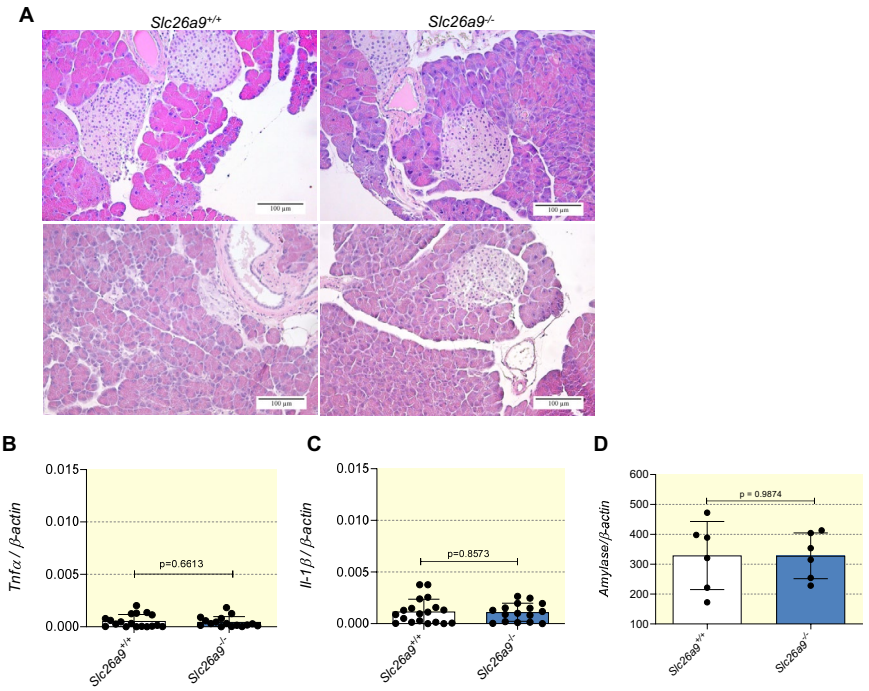

FIGURE 5 No histological and biochemical evidence of pancreatic inflammation and no indication of reduced parenchymal mass or function in *slc26a9*$^{-/-}$ mice. A, H and E staining was performed in pancreatic sections from wt (left panels) and *slc26a9*$^{-/-}$ mice (right panels). No pathological alterations were observed in ductal, islets and parenchyma. B, mRNA expression levels for TNFα and IL-1ß were low and not different between wt and *slc26a9*$^{-/-}$ pancreatic tissue. n = 17 for the cytokines and 6 for amylase. n.s, not significant

haematopoetic cells) or degeneration (tissue fibrosis), but we did not find differences between *slc26a9*$^{-/-}$ and wt pancreas either in the ductal system, the islets or the parenchyma (Figure 5A). We also measured the expression

of the proinflammatory cytokines TNFα and IL-1β in pancreatic tissues, and observed low levels of proinflammatory cytokines both in *slc26a9*$^{-/-}$ and wt pancreas (Figure 6B-D).

2.6 | Insulin content and glucose-stimulated insulin secretion in isolated Langerhans islets from female *slc26a9*$^{-/-}$ and wt mice

In order to determine whether the impaired glucose-tolerance observed in female *slc26a9*$^{-/-}$ mice was related to an impaired pancreatic insulin secretion in response to glucose, isolated and handpicked islets from female *slc26a9*$^{-/-}$ mice and cohoused wt littermates from 8 to 17 wk/age were taken into short-term culture, insulin content was assessed in the non-stimulated state (Figure 6A), and insulin release was measured in response to three different glucose concentrations (Figure 6B). While the insulin content did not differ (Figure 6A), and insulin release in 5.5 mM glucose was minimal and not different between *slc26a9*$^{-/-}$ and wt islets (Figure 6B, left bars), a significant difference in insulin release in response to elevated glucose concentrations was observed, which became more marked with increasing glucose concentrations (Figure 6B, middle and right bars). These findings demonstrate a secretory defect in the Langerhans islets of Slc26a9-deleted female mice.

3 | DISCUSSION

The present study demonstrates that similar to human pancreas,[14] Slc26a9 is weakly expressed in murine pancreas in relation to CFTR. Nevertheless, its deletion causes a significant reduction in pancreatic ductal fluid secretion, but surprisingly only in young female mice. An altered glucose tolerance was also observed in these mice. Insulin sensitivity was not different in *slc26a9*$^{-/-}$ and wt mice of either gender, indicating that an increased peripheral insulin resistance was not the reason for the reduced glucose tolerance. Surprisingly, a substantially reduced glucose-stimulated insulin secretion was observed in *slc26a9*$^{-/-}$ compared to sex-matched WT littermates. The results demonstrate that the absence of Slc26a9 causes a reduction in ductal and endocrine function in pancreatic tissue that is morphologically indistinguishable from normal pancreas. The mRNA expression levels for Slc26a9 were an order of magnitude lower than those of the CFTR anion channel, and the expression of both channels was found both in isolated pancreatic ducts and isolated pancreatic islets, albeit at manifold lower levels in the islets than in the ducts.

In a previous study, we had also observed a secretory defect in the duodenum of young *Slc26a9*$^{-/-}$ mice.[9] The mRNA expression levels for Slc26a9 were also much lower than for CFTR, but interestingly, in contrast to other members of the SLC26 family, namely Slc26a6 and Slc26a3, Slc26a9 was predominantly expressed in the crypts, similar to CFTR. CFTR/Slc26a9 double knockout mice had an extremely increased lethality during the weaning phase, suggesting that the lack of Slc26a9 increases the propensity to develop intestinal obstructions of CFTR-deficient mice. Since the lack of CFTR per se causes a complete ablation of the anion secretory response to FSK + IBMX in duodenal mucosa, these results did not give further insight into the function of Slc26a9 and its interaction with CFTR. In contrast, the current study suggests that Slc26a9 has a significant impact on pancreatic ductal electrolyte and fluid secretory, as well as on islet insulin secretory function despite its low expression level, particularly in the islets. In both pancreatic ducts and islets, CFTR was coexpressed with Slc26a9.

It is not entirely clear how a loss of Slc26a9 results in defects of cellular secretory functions, such as gastric acid

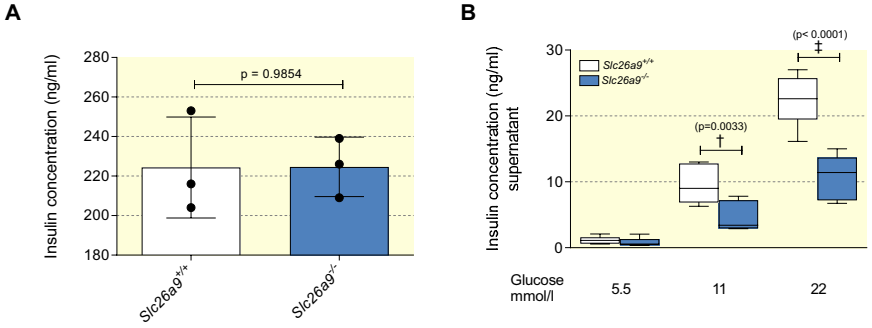

FIGURE 6 Insulin content and glucose-stimulated insulin secretion in isolated Langerhans islets from female *slc26a9*$^{-/-}$ and wt mice. A, Isolated and short-term cultured islets did not reveal differences in the insulin content of non-stimulated *slc26a9*$^{-/-}$ and wt islets, n = 3. B, However, the insulin release in response to glucose was significantly decreased in *slc26a9*$^{-/-}$ compared to wt littermate islets, n = 7-9, $^{\dagger}P < .01$, $^{\ddagger}P < .001$, n.s, not significant

secretion, duodenal bicarbonate secretion and pancreatic fluid and insulin secretion. Slc26a9 is a Cl^- uniporter,[3] and appears to be constitutively active,[5,7] while CFTR channel gating is agonist-regulated.[18,19] Possibly, Slc26a9 is membrane-resident in contrast with CFTR, which traffics to the membrane in response to agonist stimulation.[20-23] This may explain why low levels of Slc26a9 mRNA expression are nevertheless associated with significant transport activity.

The recent study of pancreatic expression of SLC26A9 in humans utilized a different method.[14] Human pancreatic tissue from a paediatric individual with early chronic pancreatitis in the absence of CF was enzymatically digested to single cells, and single-cell RNA-Sequencing (scRNA-Seq) was performed for the approx 3000 single cells obtained. Of the 2999 pancreatic single cells, CFTR was expressed in 531 cells (86.5% ductal and ductal/acinar), SLC26A9 was expressed in 15 cells, and 11 cells expressed both SLC26A9 and CFTR (100% ductal and ductal/acinar). Data from similar single cell studies with pancreatic tissues from two adults and two children of various disease statuses were available in databases, and reevaluated by Lam et al.[14] The fraction of ductal cells that expressed CFTR ranged from 35.7% to 96.9% across studies. SLC26A9 expression was detected in a lower fraction of ductal cells, ranging from 1.4% to 17%. The authors interpret the data as a proof for an exclusive ductal expression of SLC26a9, as well as co-expression of CFTR and Slc26a9 only in ductal cells. However, mRNA decay during enzymatic tissue digestion is rapid, and a threshold exists for mRNA detection by RNAseq, which may be higher than that for quantitative PCR. It seems mechanistically highly surprising that very few Slc26a9-expressing cells in pancreatic ducts have such a profound effect on pancreatic fluid secretion. On the other hand, the jejunal epithelium of humans and rats expresses so called "CFTR high expressor cells", which are singly interspersed between the absorptive enterocytes, express a unique ion transporter pattern, and respond to agonist stimulation with trafficking of vesicle-resident CFTR into the apical membrane.[24] Highly specific anti-Slc26a9 antibodies are not available to us, but their generation may be accelerated by the recent eludication of its molecular structure.[3] Pancreatic organoid generation and differentiation may be another model to better understand the cellular expression profile and influence on ductal function of pancreatic Slc26a9 over time.[25-27]

The presence of peripheral insulin resistance was ruled out in *slc26a9*$^{-/-}$ mice of both genders. Pancreatic islets were isolated, RNA extracted and studied for the expression of CFTR, Slc26a9, and marker genes for islets and acini (to rule out contamination). Ductal contamination of our hand-picked islet cells was not present (see image in suppl. Files). Although the expression levels for CFTR and Slc26a9 were low (which we had expected for CFTR, based on current literature), nevertheless the amplification curves suggested specificity for both amplification products. In order to evaluate whether the deletion of Slc26a9, despite its low expression levels, may have an effect on ß-cell physiology, we measured glucose-stimulated insulin release from isolated *slc26a9*$^{-/-}$ and wt islets and found a significantly reduced insulin secretion in *slc26a9*$^{-/-}$ islets compared to those isolated from sex-matched littermates. CFTR is also expressed in very low levels in ß cells and appears to nevertheless have a distinct function on insulin exocytosis and granule pH, discovered by the study of isolated pancreatic islets from F508del mutant mice.[16] Furthermore, short-term inhibition of CFTR in mice by CFTRinh172 (small molecule containing 2-thioxo-4-thiazolidinone core that acts to inhibit CFTR Cl^- channel function by binding to the first nucleotide-binding domain of CFTR and stabilising closed channel state) for 8 consecutive days resulted in a reduction in islet cell area, without inducing chronic inflammation.[28]

Lam et al[14] suggested that the cells that express SLC26A9 may have other key roles in the pancreas, such as those reported for centroacinar cells (CACs), a specialized ductal cell type found near acini that express CFTR in foetal and adult pancreas.[29-31] We found Slc26a9 expression in microdissected mid-size pancreatic ducts and in handpicked islets, suggesting that a role for Slc26a9 in murine pancreas is not restricted to progenitor cells, but found in mature epithelium.

It is believed that the pathogenesis of CF-related diabetes on islets is secondary to of chronic inflammation related to the primary ductal pathology that is a direct cause of absent CF function.[13,32] The effect of more Slc26a9 expression, which appears to affect pancreatic exocrine function in CF patients already in utero,[33] could then be explained by better ductal function. The unique finding in our study is an impaired glucose tolerance in female *Slc26a9*$^{-/-}$ mice in the absence of pancreatic inflammation and of CFTR mutations/deletion. The ductal dysfunction is not associated with pancreatic pathological changes by histology, or an increase in proinflammatory cytokines, which is a very sensitive marker for inflammatory events, as validated in the intestine.[34] Thus, the results delineate a ductal and islet secretory defect in the isolated absence of Slc26a9 expression.

A puzzling observation was the female and juvenile preponderance of the pancreatic endocrine and exocrine dysfunction in the absence of Slc26a9 expression. The fact that Slc26a9 deletion causes a stronger phenotype in young compared to aged mice has been observed for the intestinal phenotype.[9] Slc26a9 is functional in the human embryo, because SNPs in SLC26A9 are associated with

prenatal exocrine pancreatic damage in CF as measured by newborn screened (NBS) immunoreactive trypsinogen (IRT) levels,[33] as well as with newborn meconium ileus.[35] In mice, the deletion of Slc26a9 is associated with a very high mortality in the hours after birth because of a lung phenotype. The lungs of the dead pups are fluid filled and show no air in the alveoli. We believe that the pups die because of the inability to clear fluid from the lungs and fill the alveoli with air, similar to the situation in ENaC mice, and that Slc26a9 may serve as an apical influx mechanism for Cl^- in the immediate postpartum period.[10] However, observations by others favour the mucus obstruction as a reason for the early death.[11] In our study we found that the survival rate of female pups is more than twice that of male pups, when born to heterozygote parents, but is overall very low (4.9% female *slc26a9*$^{-/-}$ mice and 1.9% male mice among 580 survivors from breeding with heterozygote parents). Thus, it was excessively difficult to raise male *slc26a9*$^{-/-}$ mice. An additional problem with the male cohorts was that our animal facility is absolutely opposed to having mice sit in isolation for longer periods of time, and since male mice on the S129svj background start to fight, even when littermates, male mice in the older age group were likely to have been separated and cohoused with females from other litters, making the male littermates subject to microbiome alterations and the effects of mating possibly less ideally matched than the female littermates, who remained cohoused from birth. Owing to the above named issues, we also could not raise a perfect cohort for male *slc26a9*$^{-/-}$ islet cell studies. It would be interesting to study isolated islets from perfectly matched male mice, but this may be difficult indeed.

In general, differences between males and females are challenging to explain but have been reported in mouse embryonic cells, before sexual differentiation and attributed to chromosomal rather than hormonal differences. In females, one of the X chromosomes is transcriptionally silenced early in gestation. However, X-chromosome inactivation is often incomplete and may vary according to tissue type and differentiation stage, which sometimes results in higher expression levels for X-linked genes escaping inactivation. Sex-difference in epigenetic mechanisms regulating gene transcription, such as DNA methylation, may also play a role. The mechanisms explaining why female bone marrow cells are more responsive to the lack of CFTR than male cells in vitro are unknown but possibly relate to epigenetic processes.[36]

In summary, the present work demonstrates significantly reduced pancreatic fluid secretory rates and impaired glucose tolerance in female *slc26a9*$^{-/-}$ mice, particularly at young age. Because of the extremely high, male-predominant, excess perinatal mortality, the above-noticed female preponderance of the pancreatic functional defects may be in part explained by breeding-difficulty-related less perfectly matched conditions for breeding of male than female mice, and needs to be re-investigated. The juvenile age preponderance, however, is a feature of Slc26a9 deletion, and has been observed in other epithelial phenotypes of the *slc26a9*$^{-/-}$ strain before.[9]

4 | MATERIAL AND METHODS

4.1 | Animals

The *Slc26a9*-deleted mouse strain, whose establishment and characteristics have been described elsewhere,[17] was congenic on the S129/svj background and was bred and genotyped in accordance with the Institutional Animal Care and Use Committee (IACUC) at Hannover Medical School. Animals had free access to food and water and were maintained at 21°C in a 12/12 hr light/dark cycle. The mice were age- and sex-matched, randomly selected and used between 6 weeks and 14 months of age (studied at different ages). All experiments involving animals were approved by the Hannover Medical School committee on investigations involving animals and an independent committee assembled by the local authorities.

4.2 | Isolation and culture of pancreatic ducts and of Langerhans islets for RNA extraction

Intra-/interlobular pancreatic ducts from adult S129svj mice were isolated and cultured overnight at 37°C in a humidified atmosphere containing 5% CO as described previously.[37] Islets of Langerhans from adult Sw129 mice were isolated and cultured overnight as previously described.[38] In brief, 12 wt mice (10-12 wk/age 6m, 6f) were used. After anaesthesia and laparotomy, the common bile duct was branched off above duodenal entry and cannulated. Immediately, the pancreas was perfused with cold (4°C) collagenase (Roche, Mannheim, Germany) in Hank's solution (Sigma-Aldrich, Steinheim, Germany). After aseptic harvesting of the pancreas, digestion for 9 minutes in 37°C water bath in Hank's solution with collagenase was performed. Subsequently, digestion was stopped by means of cooling down in Hank's solution on ice. For ductal isolation, the pancreatic tissue was spread out on a glass petri dish, placed under a dissecting microscope, and the pancreatic ducts were microdissected with fine tweezers and a microsurgery scissor as described.[37]

For islet cell isolation, the pancreatic suspension was centrifugated at 250× *g* for 15 seconds and washed twice in Hank's solution. Cell suspension was plated on petri dishes, and pancreatic islets were collected under microscopic control. Isolated pancreatic ducts or pancreatic islets were washed with phosphate buffered saline, and used for RNA extraction.

4.3 | Isolation of pancreatic islets for insulin secretion measurements

Pancreatic islets from 8-17 wks/age female *slc26a9*$^{-/-}$ mice and their wild-type littermates (9 mice per group) were isolated as described above and cultured in RPMI medium containing glutamine (Gibco, NY, USA) with 10% foetal calf serum (FCS), 1% penicillin/streptomycin (P/S) and 5.5 mmol/L glucose for islets (all from Serva, Heidelberg, Germany) and maintained in culture (37°C 21% O2, 5% CO2) for at least 24 hours prior to the following experiments.

4.4 | Determination of insulin content in and insulin secretion from *slc26a9*$^{-/-}$ and wt littermate pancreatic islets

For determination of insulin content, five pancreatic islets were collected for each measurement, and the experiments were repeated three times. The islets were resuspended in IRI-buffer (40 mmol/L NaH_2PO_4, 100 mmol/L NaCl, 0.3% BSA, pH 7.4, all from Roth, Karlsruhe, Germany) and then lysed with sonic homogenisator (Sonoplus, Bandelin, Berlin, Germany) for 30 seconds at 20% intensity and 4°C. Aliquots were frozen at −20°C until insulin measurements.

For detection of insulin secretion from pancreatic islets, we determined insulin levels in the supernatant of the latter. Islets were kept in RPMI medium with low (1.5 mmol/L) glucose for 2 hours, then transferred to medium containing 5.5, 11 or 22 mmol/L glucose in RPMI with 10% FCS and 1% P/S for 2 hours in an incubator (37°C, 21% O_2, 5% CO_2). After stimulation, either 100 μL of supernatant were collected for analysis of insulin secretion, or islets were resuspended in 500 μL IRI-buffer followed by the above mentioned homogenization. IRI buffer with 0.1% BSA without glucose was used as a control.

Insulin levels were measured by using an ultrasensitive mouse ELISA Kit (BioCat (Alpco), NH, USA) following instructions of the manufacturer. Experiments were repeated at least thrice.

4.5 | Messenger RNA Expression of transporter, enzyme, proinflammatory cytokine and control genes

Tissues and cultured pancreatic ducts and islets were homogenized in lysis buffer and RNA was isolated with a NucleoSpin RNA XS Total RNA Isolation Kit (Machery & Nagel, Düren, Germany). Reverse transcription was performed using Superscript III RT (Invitrogen Corporation, Carlsbad, CA), as described previously.[9] The primer sequences are given in Table S1.

4.6 | In vivo pancreatic fluid and bicarbonate secretion

Male and female *slc26a9*$^{-/-}$ mice and wt littermates were anaesthetised by isoflurane (Forene, Abbott Germany, Wiesbaden, Germany) via tracheal intubation connecting to the mechanical ventilator (MiniVent Type 845, Hugo Sachs Electronik, March Hugstetten, Germany). Respiration rate and TV (tidal volume) depended on the weight of mice according to the recommendations from the *Operating Instructions for the Mouse Ventilator MiniVent Type 845*. Normally, the respiration rate and TV ranged from 140/min to 150/min, and 200 μl to 250ul respectively. Mice were placed on a heating pad to maintain the body temperature at 37.5°C, which was monitored by a rectal thermistor probe. The depth of anaesthesia was tested by probing the pedal withdrawal reflex. After the reflex disappeared, the concentration of isoflurane in the anaesthetic gas mixture was reduced from 5% to 2.5% for maintaining. The left carotid artery was cannulated for monitoring blood pressure and for the infusion of an isosomolar Na_2CO_3 solution at 100 μL/h to prevent acidosis during surgery. Another catheter was placed into the femoral vein for infusing Ringer´s solution at a rate of 0.3 ml/h to maintain hematocrit levels at pre-surgery values, and to infuse secretin (porcine secretin at 17 nmol/kg in a bolus infusion) for stimulation of pancreatic fluid and bicarbonate secretion.

The method for collecting pancreatic secretions were adapted from a description for rat pancreatic juice collection.[39] The mouse was placed under the operating microscope (Leica, Wild M3Z, Wetzler, Germany). The abdominal cavity was opened, the gallbladder was ligated, the common bile duct and the major pancreatic duct isolated, two ligatures were placed but not yet tied, one very close to the ampulla, the other about 5-7 mm distal in the common bile duct, and the common bile duct was ligated between the two ligatures. The bile duct was cut twice with scissors, the first cut was near the entrance to the duodenum

(for pancreatic juice drainage), the second one was distal to the ligated point (for biliary duct drainage). Small and soft 1/32" PE tubes, made thinner at the tip by pulling above an open flame, were placed into the pancreatic duct and the distal bile duct and secured. This maneuver prevented biliary obstruction during the procedure, because bile flow was possible. The mice were left to stabilize for about 30 minutes with continuous fluid and base administration via the carotid artery and the femoral vein catheter for a stable equilibrium, before the start of the experiment. Basal and secretin-stimulated pancreatic juice was collected at 20 minutes time intervals. After the basal 2 collections periods, porcine secretin (Abbiotech, Escondido, CA 92025, USA) with a concentration of 17 nmol/kg,[40] was rapidly injected into the femoral vein, and 2-3 additional collection periods were performed.

4.7 | Glucose tolerance test

The procedure was adapted from Dudele et al[41] After comparing oral, i.p. and iv glucose tolerance tests, we chose the described procedure for best reproducibility. Peripheral blood glucose levels were measured by making a tiny cut in the tail and measuring blood glucose concentration using the Freestyle Lite glucometer (Abott Laboratories, IL, USA). Blood glucose was assessed before and after 6 hours fasting, then male and female *slc26a9*$^{-/-}$ and wt littermates between 12-20 weeks of age were anaesthetized by i.p. injection of ketamine (50 μg/g) and xylazine (100 μg/g), the left carotid artery and jugular vein was cannulated. The carotid artery was used for blood pressure measurements and infusion of base and electrolyte solution, as described above, while the jugular vein was used to administer the glucose bolus. The mouse was left to stabilize, and another peripheral glucose measurement was taken before the bolus injection of 2 mg/g body weight glucose was rapidly injected via a catheter in the jugular vein. Peripheral blood glucose was measured at 5, 15, 30, 60 and 120 minutes points, taking a blood drop via the tail.

4.8 | Insulin tolerance test

Insulin tolerance test was performed as previously described.[42] Adult male and female *Slc26a9*$^{-/-}$ and wt mice between 12 and 28 wk/age were fasted for 4 hours and basal blood glucose was measured using glucometer with 5ul blood samples from the saphenous vein. Immediately after basal blood glucose determination, human insulin Actrapid (Novo Nordisk, Switzerland) 0.30 IU/kg body weight was injected intraperitoneally (i.p) in a volume of 200 μl saline. Blood glucose was measured at 15, 30, 60 and 90 minutes after insulin injection with glucometer strips Freestyle Lite (Abott Laboratories, IL, USA), as described above. Immediately after insulin tolerance test food was given to all animals.

4.9 | Statistical analysis

For GTT and ITT, analysis of variance (two-way ANOVA) was used to analyse for statistical difference between the groups using GraphPad Prism, version 7 (GraphPad Software, Inc, CA, USA). Area under curve for ITT was calculated using trapezoidal method and "*t*" test was used to analyse the difference between the groups. Values represented as mean ± SD, and differences were considered statistically significant when $P < .05$.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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