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Vil-Cre specific Slfn3KO mice exhibit sex-specific differences in lung, stomach, cecum, kidney, and proximal colon differentiation markers and Slfn family members expression levels

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ABSTRACT

The Schlafen (Slfn) family proteins are critical regulators of cell proliferation, induction of immune responses, differentiation, self-restoration, and cell cycle progression. Rodent Slfn3 and human ortholog SLFN12 are critical in the regulation of intestinal epithelial differentiation. Following previous work utilizing Vil-Cre epithelial-specific Slfn3 knockout (VC-Slfn3KO) mice to evaluate Slfn3's role in small intestinal epithelial differentiation, we sought to characterize and distinguish the effects of Slfn3 loss on Slfn family member mRNA expression and differentiation markers for other epithelial cells in the lung, stomach, cecum, and proximal colon. Quantitative PCR analysis of Slfn1, 2, 4, 5, 8, and 9 and multiple differentiation markers were evaluated. We observed gender-specific effects with the loss of Slfn3 on the other Slfn family members and epithelial differentiation markers expression. Lung Slfn4 and 5 were increased only in male VC-Slfn3KO while lung Slfn2 and 8 were decreased only in female VC-Slfn3KO compared to controls. Slfn1, 2, 4, and 9 were increased in the gastric mucosa of male VC-Slfn3KO mice compared to controls. Slfn5 was reduced in female VC-Slfn3KO proximal colonic mucosa compared to controls. Lung AT1 cell differentiation marker Hopx mRNA expression was decreased and Ager was increased in VC-Slfn3KO male mice compared to controls. Lung AT2 differentiation markers and surfactant genes Sftpc and Sftpd were decreased in male VC-Slfn3KO mice. Stomach transcription factors, Lgr5 and Notch1 were increased in male VC-Slfn3KO. Tff1 secretory protein gene was decreased in female VC-Slfn3KO mice. Sucrase isomaltase was greatly increased in male VC-Slfn3KO mice in both cecal and proximal colonic mucosa, but glucose transporter Glut2 was decreased only in the cecum of female VC-Slfn3KO. The changes induced by VC-Slfn3KO in the expression of epithelial differentiation markers and other Schlafen proteins in various target tissues, indicate a complex regulation of gene expression that is sex-dependent.

1. Introduction

The Schlafen (Slfn) protein family is chiefly responsible for controlling cellular processes such as cell differentiation, cell growth, viral replication, and cell cycle progression [1]. The Slfn family is expressed during thymocyte maturation and T cell activation [2,3]. Specifically, Slfn3, 4, 5, and 8 have been shown to influence T cell differentiation, activation, and proliferation [2,3]. This family of growth regulatory genes can be categorized by molecular mass and structure into three groups. The Group I members Slfn1 and Slfn2 are the shortest Slfns (37–42 kDa) [1–4]. The Group II members Slfn3 and Slfn4 are intermediate in mass (58–68 kDa) while Group III members such as Slfn5, Slfn8, Slfn9, and Slfn14 are the longest Schlafen proteins (100–104 kDa) [1–4]. All Slfn family members carry a distinctive Slfn box domain that lies next to a divergent AAA domain. Group II and group III Slfns contain a SWADL (Ser-Trp-Ala-Asp-Leu) domain not present in Group I Slfns [1,3,4]. However, only Group III Slfns possess a C-terminal nuclear targeting sequence (RKRRR), a homolog to DNA/RNA helicase superfamily I. The human Slfn proteins include SLFN5, SLFN11, SLFN12, SLFN13, and SLFN14. The closest ortholog to the murine Group II Slfn3 and Slfn4

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is the human SLFN12 gene [1,3,4].

The lung alveolar epithelium consists of two primary cell types: Alveolar Type 1 (AT1) and Alveolar Type 2 (AT2). We examined the AT1 cell differentiation markers Hopx, Pdpn, and Ager and the AT2 cell differentiation markers Sftpb, Sftpc, and Sftpd [5–10]. In the lung, Hopx acts as a negative regulator of *GATA* gene expression [11,12]. GATA6 and Nkx2.1 interact to activate lung-restricted genes, *SP-C* and *WNT7b*, which are required for differentiation, development, and maturation of airway epithelium [11,12]. If Hopx is lost, then GATA6 and Nkx2.1 are derepressed and abnormal lung development and function results [11, 12]. Pdpn is required for lung development and AT1 maturation [5,6]. The AT1 cells cover most of the lung surface and are necessary for alveolar development [5,7]. Pdpn knockout mice die shortly after birth as they are unable to inflate their lungs [5,6]. Finally, the surfactant proteins (Sftp) are found primarily in AT2 cells and help to form a protein-lipid complex that covers alveolar surfaces and prevents alveoli from collapsing [8].

We also investigated the gastric epithelial markers cytokeratins 8 and 18 (Krt8, Krt18), Atp4b, E-cadherin (Chd1), Epcam, Gif, Tff1, and Pgc. E-cadherin plays a crucial role in epithelial architecture and maintaining the cell polarity and differentiation [9]. If there is dysregulation of E-cadherin, this will lead to tumor invasion and progression [9]. Krt8, Krt18, Epcam and Chd1 epithelial markers are utilized as gastric epithelial markers for stable gastric organoid cultures and in simple epithelial tissues [10]. Atp4b, Tff1, Pgc, and Gif represent terminal differentiation markers of the specific gastric epithelial cells of chief, parietal, pit, mucous neck, or isthmal cells [13].

Sucrase isomaltase (SI), dipeptidyl peptiase (Dpp4) and villin 1 (Vil1) digestive enzymes are canonical markers for intestinal epithelial differentiation [14]. SI hydrolyzes α- 1, 2, α-1,6, and α- 1,4 glucosidic bonds [15]. Dpp4 recognizes the L-proline or L-alanine and cleaves at the NH_2-terminal penultimate position [14]. Vil1 is the major protein associated with actin-binding in the microfilament core in microvilli that is regulated by calcium [14,16]. Glucose transporter, Glut1, is the first identified member of the glucose transporter family and has been utilized as a prognostic marker in colorectal cancer as overexpression of Glut1 is unfavorable in patients [17,18].

The drug transporter proteins on tubular cells, Oat1, Oat3, and Oct2, are a characteristic of cellular maturity in the kidney [19]. These transporters are key contributors to the chemotherapy drug, cisplatin, nephrotoxicity [19]. There is a correlation of increased Ppara and Esrra expression with the terminal differentiation of proximal tubule cells in the kidney [20]. Ppara is a regulator of oxidative phosphorylation while Essra regulates fatty acid oxidation [20].

We previously investigated the role of Slfn3 in the regulation of intestine differentiation, development, and maturation in Slfn3 knockout (Slfn3KO) and Vil-Cre epithelial-specific Slfn3KO mice models (VC-Slfn3KO) [21,22]. We demonstrated direct correlation between the expression of Slfn3 and the expression of epithelial differentiation markers in Slfn3KO mice but an increase in these markers in only the female VC-Slfn3KO mice [21,22]. In addition to differentiation markers, the other Slfn family members, Slfn1, 2, 4, and 8 were increased in RNA expression and Slfn5 and 9 were decreased in the VC-Slfn3KO mice based on the small intestinal region and in a sex-dependent manner [22]. Since Slfn family members are expressed broadly in the epithelial cells of other organs, we sought to determine if Slfn3 has a functional role in regulating the mRNA expression of the differentiation markers and Slfn family members in the lung, kidney, gastric mucosa, cecal mucosa, and proximal colonic mucosa tissue of male and female VC-Slfn3KO compared to control mice.

2. Material and methods

2.1. Generation and genotyping of VC-Slfn3KO mice

Animal breeding and procedures were approved by the University of North Dakota institutional animal use and care committee under protocol number 1807-7C. Under this protocol, the mice were euthanized by being subjected to CO_2 inhalation followed by cervical dislocation. The Vil-Cre specific Slfn3KO mice were created as previously described [22] The DNeasy Blood and Tissue kit and the Qiacube from Qiagen (Valencia, CA) was utilized for isolating DNA from 2 to 4 mm collected tails. qPCR genotyping was performed as previously described [22].

2.2. RNA isolation and qPCR

RNA isolation was performed after sacrifice of 8-17-week-old male and female control and VC-Slfn3KO mice. Lung, gastric mucosa, cecal mucosa, kidney, and proximal colonic mucosa tissue samples were collected and RNA isolation was performed on tissue samples using the RNeasy Lipid Kit and the Qiacube instrument per specified manufacturers' protocols (Qiagen, Valencia, CA). cDNA was synthesized from isolated RNA samples using the SMARTScribe Reverse Transcription kit (Takara Clontech, Mountain View, CA). The synthesized cDNA samples underwent qPCR analysis which was analyzed using the BioRad CFX96 Touch Real-Time PCR Detection System and the PrimeTime Gene Expression Master Mix from Integrated DNA Technology (IDT, Coralville, IA). RNA expression levels were established from the threshold cycle (Ct) values using the $2^{-\Delta\Delta Ct}$ method which uses RPLP0 or Actb as the reference control genes. Primer/probe sets are listed in S1, S2, and S3 Tables and qPCR cycle conditions were 1 cycle of 2 min at 95 °C, 50 cycles of 10 s at 95 °C and 45 s at the annealing temperature of 55 °C.

2.3. Protein expression by flow cytometry

Lung tissue was homogenized through a 70 μm sieve with a 3 ml syringe plunger. The single cell suspension was centrifuge at 500×*g* and then red blood cells were lysed with red blood cell lysis buffer (Genesee Scientific, El Cajon, CA). Cell suspensions were washed with PBS, followed by staining with Zombie Aqua viability dye (Biolegend, San Diego, CA). Cells were pretreated with TruStain FcX (Biolegend) and then fixed and permeabilized with the FoxP3/Transcription factor fixation/permeabilization kit from eBioscience (San Diego, CA). The following antibodies were all purchased from Bioss Antibodies (Woburn, MA) and used for intracellular staining: Alexa 488 anti-mouse Hopx/Lagy, Alexa 594 anti-mouse Ager, Alexa 647 anti-mouse Sftpc, and APC/Cyanine7 anti-mouse Sftpd (see S4 Table for antibody catalog number and dilutions used).

Single cells were dissociated from either stomach, cecum, or proximal colon tissue with 15 ml of 1 mM DTT, 5 mM EDTA, 2 % FBS, 100 U/mL penicillin, 100 μg/mL streptomycin in HBSS (Fisher Scientific, Hampton, NH) for 30 min, 400 rpm shaking, at 37 °C. Cells were collected through a 70 μm sieve, centrifuged at 500×*g*, washed with 5 ml complete media (RPMI, 10 % FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Fisher Scientific)), centrifuged again, resuspended in complete media and stored on ice. The remaining whole tissue was transferred out of the sieve into a new 50 ml tube and washed with 10 ml 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin in HBSS (Fisher Scientific) for 10 min, 400 rpm shaking, at 37 °C. Cells were collected through a 70 μm sieve into the previous tube described above, centrifuged and washed as above. The tissue was then digested with 7 ml Liberase solution (0.2Wunsch unit/ml Liberase TM (Roche Diagnostics, Indianapolis, IN), 200K/ml DNase I, 10 mM HEPES, 100 U/mL penicillin, and 100 μg/mL streptomycin in HBSS (Fisher Scientific)) for 45 min, shaking at 400 rpm at 37 °C. Following digestion, 4 ml complete media was added. Then cells were collected through a 70 μm sieve into the previous tube again, centrifuged and again washed as above. Cell suspensions were washed with PBS, stained with Zombie Aqua viability dye, pretreated with TruStain FcX, and then fixed and permeabilized. The following antibodies were all purchased from Bioss Antibodies and used for intracellular staining of gastric cells: Alexa 488 anti-mouse Lgr5, Alexa 594 anti-mouse Notch1, and APC anti-mouse

Tff1 (S4 Table). FITC-*anti*-mouse SI was used for cells from the cecum and proximal colon and was purchased from LifeSpan BioSciences (Seattle, WA) (S4 Table).

2.4. Statistics

The qPCR analysis of all gene markers for the lung, gastric mucosa, cecal mucosa, kidney, and proximal colon were assessed by 2-way ANOVA, multiple comparisons in GraphPad Prism (San Diego, CA). Flow cytometry data was assessed by 2-way ANOVA. Data are represented as mean ± SE.

3. Results

3.1. Slfn3 influences Slfn family member expression

Previously, we validated the Vil-Cre specific knockout of Slfn3 by single-molecule RNA in situ hybridization [22]. We demonstrated that the loss of Slfn3 in the intestinal epithelial cells of VC-Slfn3KO mice affected the expression of Slfn family members in the duodenal, jejunal, and ileal mucosa [22]. We continued this work by examining the expression levels of Slfn family members 1,2,4,5,8 and 9 in lung, gastric mucosa, cecal mucosa, kidney, and proximal colonic mucosa tissue of male and female VC-Slfn3KO compared to control mice. Slfn1 is a part of the Group I Slfn family and was increased in expression in gastric mucosa of male VC-Slfn3KO mice but not in female mice compared to control mice (Fig. 1A). Slfn2, the other murine member of Group I, was increased in expression in gastric mucosa of only male VC-Slfn3KO mice compared to control mice and decreased in expression in lung of only female VC-Slfn3KO mice but not in male mice (Figs.. 1B and 2B). Slfn4, a Group II Slfn closely related to Slfn3, was increased in expression in both the gastric mucosa and lung specific to male VC-Slfn3KO mice but remained unchanged in female VC-Slfn3KO in comparison to the control mice (Figs.. 1C and 2C). The Group III Slfn member Slfn5 displayed an increase in expression in only male VC-Slfn3KO mice in the lung and gastric mucosa region while it exhibited a decrease in proximal colonic mucosa of only female VC-Slfn3KO compared to control mice (Figs.. 1D, 2D and 3D). Slfn8 presented a decrease in the lung of female VC-Slfn3KO mice and no change in male VC-Slfn3KO mice (Fig. 2E). Finally, Slfn9, another Group III Slfn member, displayed no expression differences between VC-Slfn3KO and control mice across all of the tissues tested (Figs. 1–3, S1 & S2 Figure). No significant changes in Slfn family member expression were exhibited in kidney and cecal mucosa of male and female VC-Slfn3KO (S1 & S2 Figure).

3.2. Slfn3 epithelial cell loss epithelial differentiation marker expression in a sex dependent manner

The lung alveolar epithelium consists of two primary cell types: Alveolar Type 1 (AT1) and Alveolar Type 2 (AT2). We studied the AT1 cell differentiation markers Hopx, Pdpn, and Ager as well as the AT2 cell differentiation markers Sftpb, Sftpc, and Sftpd [11,5,8,23–25]. In the lung, male-specific VC-Slfn3KO mice exhibited increased Ager and a decreased Hopx expression compared to controls, but female knockouts did not (Fig. 4A, C). Pdpn was not significantly different between the VC-Slfn3KO and control samples (Fig. 4B). A similar male-only decrease in both AT2 differentiation markers, Sftpc and Sftpd was observed in the lung of the male VC-Slfn3KO mice compared to control mice, but Sftpb only showed a trending decrease (Fig. 4D–F). The protein expression of Hopx, Ager, Sftpc, and Sftpd were then evaluated by flow cytometry since they exhibited significant changes in RNA expression. However, there was no significant difference in the protein expression for Hopx, Ager, Sftpc, or Sftpd between control and VC-Slfn3KO mice (S3 Figure).

In the gastric mucosa, we evaluated the expression of the gastric epithelial markers Krt8, Krt18, Atp4b, Chd1, Epcam, Gif, Tff1, and Pgc [9,10,13,26,27]. We also analyzed the expression of the gastric transcription factors Lgr5, and Notch1, which are involved in gastric epithelial differentiation [28]. The transcription factors Lgr5 and Notch1 were increased in only male VC-Slfn3KO mice compared to controls. This was not observed for female VC-Slfn3KO mice (Fig. 5A and B). The secretory protein Tff1 was the only gastric epithelial differentiation marker that was significantly reduced in expression in female VC-Slfn3KO mice compared to control mice (Fig. 5C). We then analyzed the protein expression of Lgr5, Notch1, and Tff1. VC-Slfn3KO male mice had higher protein levels of Notch1 compared to control, similar to what we had observed at the RNA level. We also observed similar trends in protein expression for Lgr5 and Tff1 to those identified for RNA expression, although these did not achieve statistical significance (S4 Figure). The other gastric epithelial markers Krt8, Krt18, Atp4b, Chd1, Epcam, Gif, and Pgc did not differ at the protein level among groups (S5

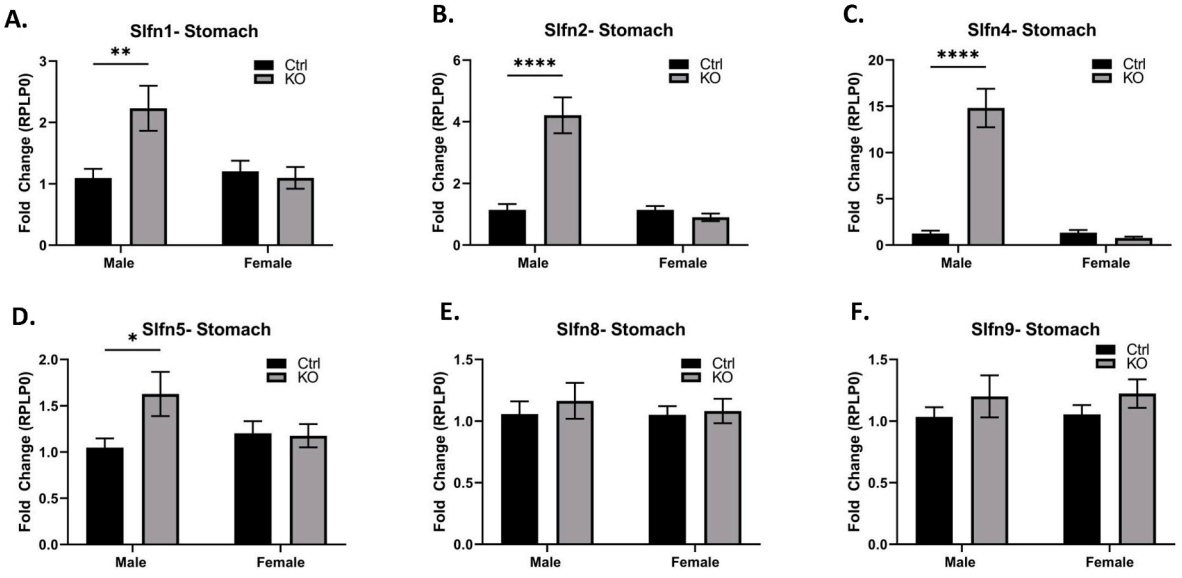

Fig. 1. Slfn1, 2, 4, and 5 are increased in male VC-Slfn3KO gastric mucosa. Total RNA was isolated from gastric mucosa of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) Slfn1, (B) Slfn2, (C) Slfn4, (D) Slfn5, (E) Slfn8, and (F) Slfn9 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–20, *p < 0.05 to respective control).

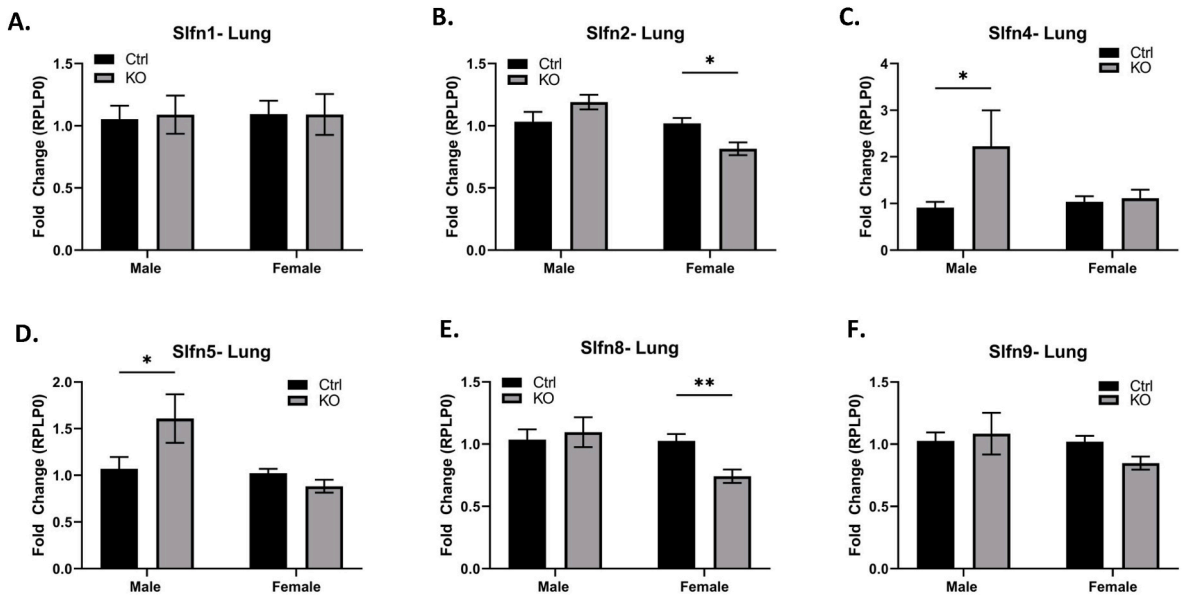

Fig. 2. Slfn2 and 8 were decreased in female VC-Slfn3KO lung while Slfn4 and 5 were increased in male VC-Slfn3KO. Total RNA was isolated from lung tissue of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) Slfn1, (B) Slfn2, (C) Slfn4, (D) Slfn5, (E) Slfn8, and (F) Slfn9 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–20, *p < 0.05 to respective control).

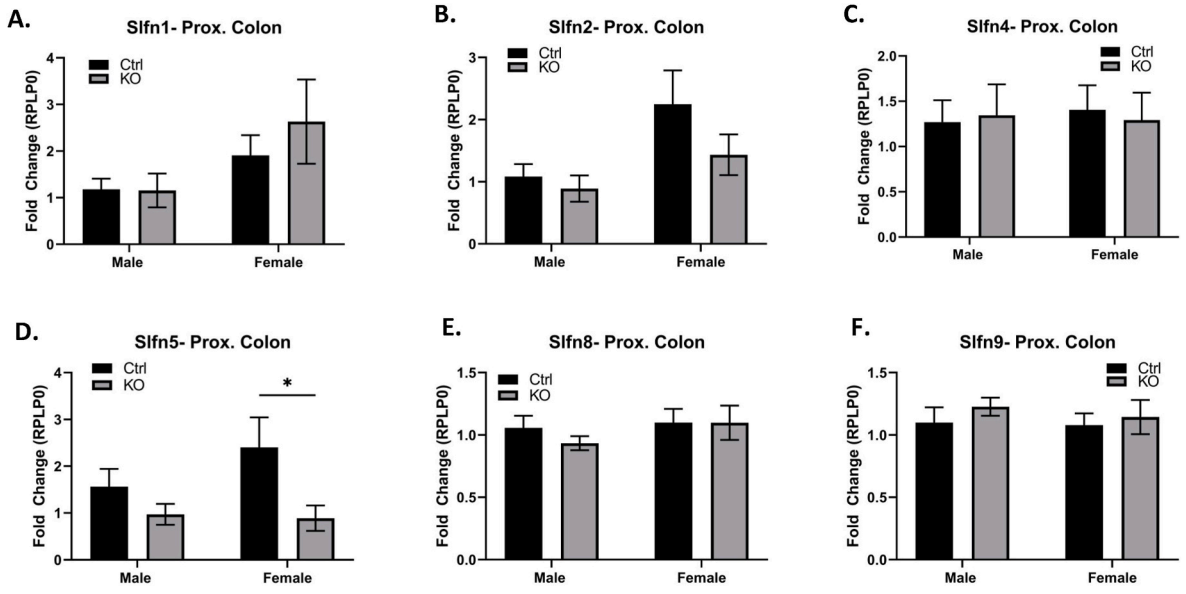

Fig. 3. Slfn5 was decreased in female VC-Slfn3KO proximal colonic mucosa. Total RNA was isolated from proximal colonic mucosa of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) Slfn1, (B) Slfn2, (C) Slfn4, (D) Slfn5, (E) Slfn8, and (F) Slfn9 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–23, *p < 0.05 to respective control).

Figure).

The intestinal differentiation markers SI, Vil1, Dpp4, Glut1, and Glut2 were studied in the cecal and proximal colonic mucosa [21,29]. SI was increased in male VC-Slfn3KO cecal and proximal colonic mucosa compared to control mice but not in female VC-Slfn3KO mice (Figs.. 6A and 7A). The protein expression of SI was similarly increased in the proximal colon; however, we observed an increase in SI protein in the cecum of the female VC-Slfn3KO compared to control rather than the male mice (S6 Figure). Glut2 was significantly decreased in the female VC-Slfn3KO cecal mucosa compared to control (Fig. 6E) and Glut2 was not expressed in the proximal colon. Vil1, Dpp4, and Glut1 were not significantly different between the control and VC-Slfn3KO mice (Fig. 6B, C, D and Fig. 7B, C, D).

In the kidney, we investigated the drug transporter genes Oat1, Oat3, and Oct2 [19,20] as well as Ppara and Esrra, regulators of oxidative phosphorylation and fatty acid oxidation [19,20]. No significant changes were observed in the expression of these genes, indicating that Slfn3 does not appear to be involved in the regulation of these kidney epithelial differentiation markers (S7 Figure).

4. Discussion

This study investigated the distinct influence of Slfn3 over the expression of other Slfn family members and of epithelial cell differentiation markers in the lung, stomach, cecum, kidney, and proximal colon. The loss of Slfn3 affected the expression of other Slfn family

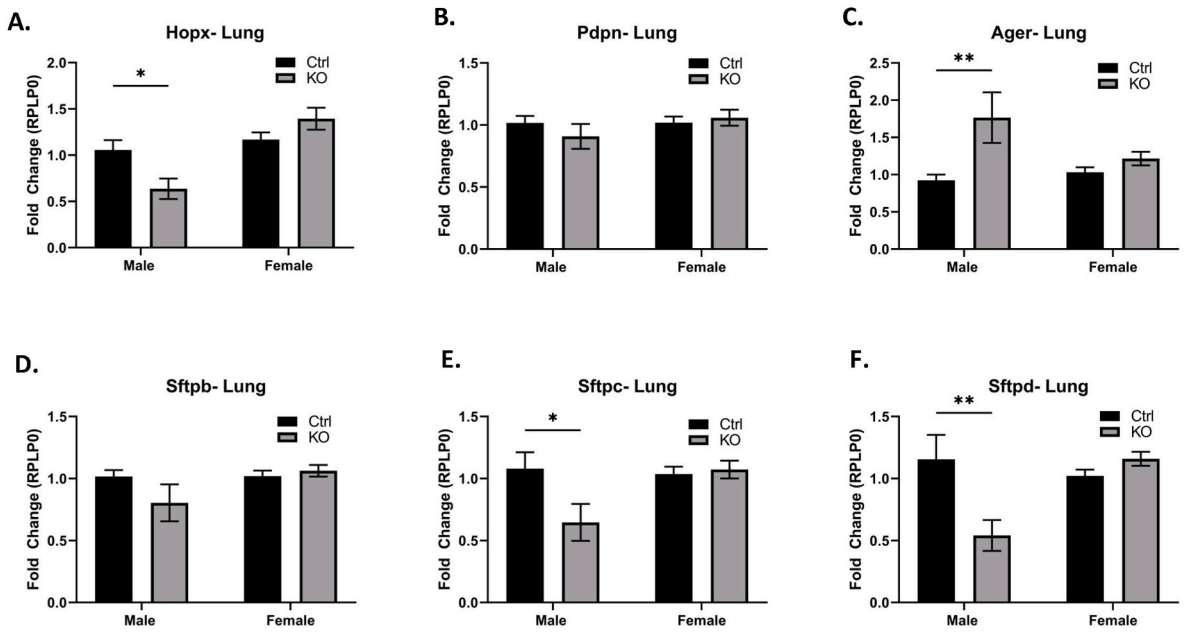

Fig. 4. Lung differentiation marker Hopx, Sftpc, and Sftpd were decreased in male VC-Slfn3KO mice while Ager was increased. Total RNA was isolated from lung tissue of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) Hopx, (B) Pdpn, (C) Ager, (D) Sftpb, (E) Sftpc, and (F) Sftpd was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–20, *p < 0.05 to respective control).

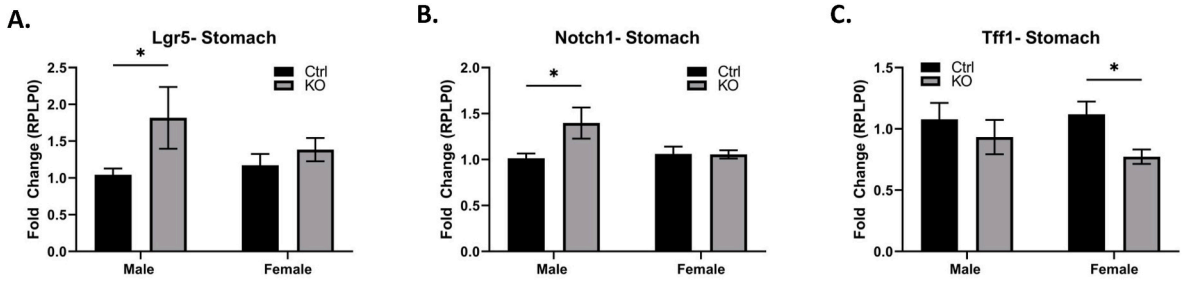

Fig. 5. Gastric transcription factors Lgr5 and Notch1 were increased in male VC-Slfn3KO gastric mucosa, while Tff1 secretary protein was decreased in the female VC-Slfn3KO gastric mucosa. Total RNA was isolated from gastric mucosa of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) Lgr5, (B) Notch1, and (C) Tff1 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–20, *p < 0.05 to respective control).

members in lung, stomach, and colon. Pulmonary and gastric mucosa differentiation markers exhibited the most notable changes due to Slfn3 loss. Expression of both Slfn family members and organ-specific epithelial differentiation markers were impacted by sex.

Previous observations suggest that Slfn proteins may influence each other's expression [22,30,31]. One of the earlier studies of Slfn family members by Schwartz et al. found that Slfn3/6 and Slfn4/7 had an increase in mRNA expression while Slfn1 and 2 had a decrease in expression after T cells were activated with anti-CD3 with or without CD28 co-stimulation [3]. Neumann et al. then showed that Slfn family members had specific subcellular localizations. In transiently transfected HEK 293T cells, Slfn1 was observed in the cytoplasm and nucleus, while Slfn2 and Slfn4 were found only in the cytoplasm and Slfn5, 8, and 9 were only in the nucleus [32]. Geserick et al. infected $RAG^{-/-}$, $TNFRI^{-/-}$, IRF-$1^{-/-}$ and C57Bl/6 mice with *L. monocytogenes* and evaluated the change in Slfn1, 2, 4, 5, 8, 9, and 10 RNA expression in splenocytes [33]. An infection with *Listeria* upregulated all these Slfns in the splenocytes of $RAG^{-/-}$, $TNFRI^{-/-}$, and C57Bl/6 mice. However, IRF-$1^{-/-}$ mice had significantly less upregulation of Slfn1, 4, 5, 6, 9, and 10, but Slfn2 was not affected [33]. This indicated a role for IRF-1 in the transcriptional control of these Slfn genes after a *Listeria* infection [33]. We have previously evaluated Slfn family member expression in the small intestinal mucosa of VC-Slfn3KO mice [22]. Table 1 summarizes present and previous observations of changes in Slfn family member expression in the small intestinal mucosa [22,31], thymus, spleen [31], lung, gastric mucosa, and proximal colonic mucosa and illustrates the sex-dependence of these effects. Indeed, our distinct exploration of Slfn family member expression in both the Slfn3KO and VC-Slfn3KO mice suggests a mechanism of family member regulation that is tissue- and sex-dependent [22,31]. This awaits further exploration. However, this apparent family member-dependent regulation with tissue- and sex-specificity could be linked to the similarities and differences in the *Slfn* sequences, all of which are on the same chromosome (human clustered on chromosome 17 and mouse clustered on chromosome 11 [34]). Additionally, a diverse group of chemicals, including sex-hormone, 17-β-estradiol, anagrelide, nauclefine and DNMDP, have been termed "molecular glues" or "velcrins", and aid in the complex formation of phosphodiesterase PDE3A to SLFN12 (human ortholog to Slfn3) which in turn leads to a cytotoxic response in cancer cells [35–38]. The binding of SLFN12 to PDE3A increases SLFN12 RNase activity to rRNA [35–38]. Further research is needed to determine the extent to which velcrins and the SLFN12-PDE3A complex may influence the regulation of Slfn family member expression and epithelial differentiation marker expression.

In the present study, we found that the loss of Slfn3 had the most substantial impact on the RNA expression of differentiation markers in

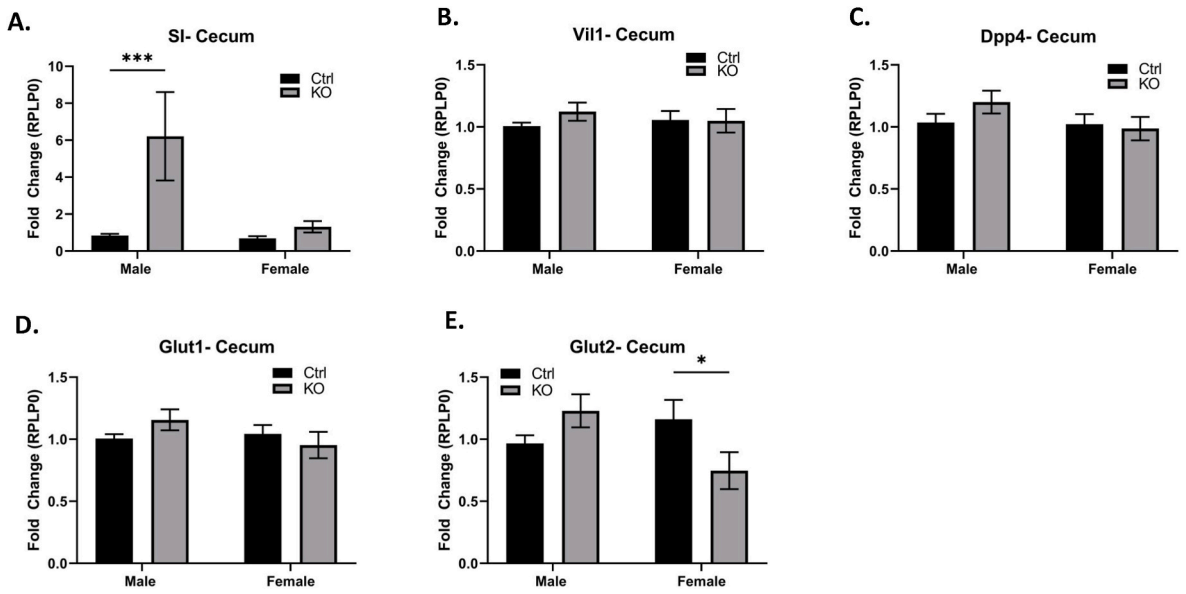

Fig. 6. Sucrase isomaltase (SI) was greatly increased in male VC-Slfn3KO cecal mucosa, whereas glucose transporter Glut2 was decreased in female VC-Slfn3KO. Total RNA was isolated from cecal mucosa of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) SI, (B) Vil1, (C) Dpp4, (D) Glut1, and (E) Glut2 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–20, *p < 0.05 to respective control).

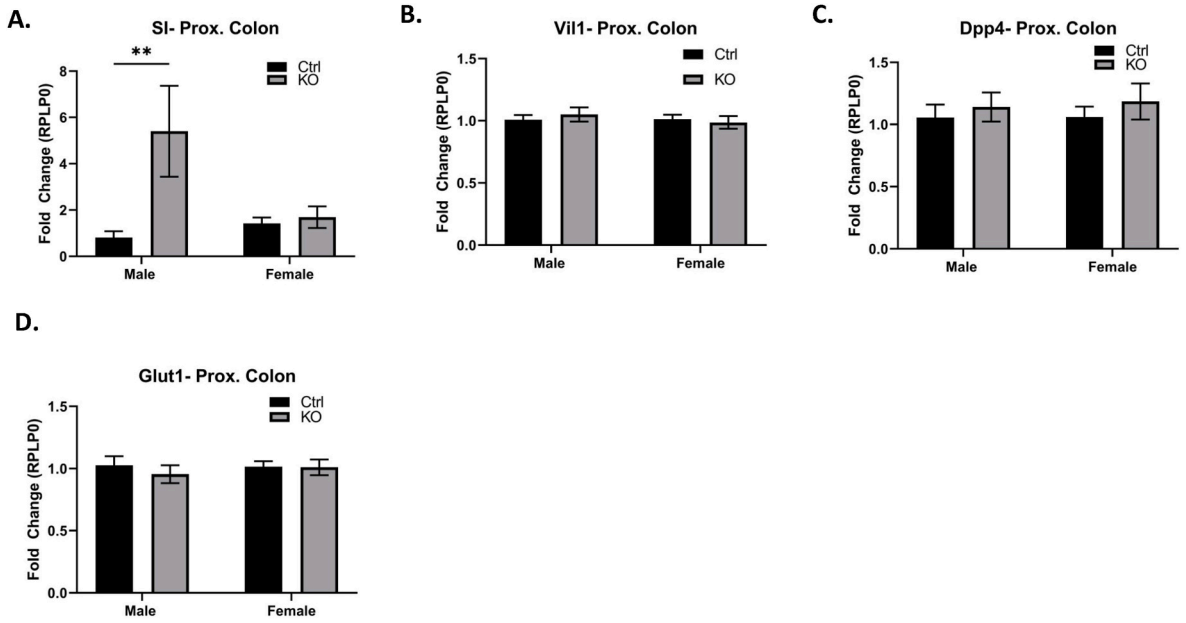

Fig. 7. Sucrase isomaltase (SI) was increase in male VC-Slfn3KO proximal colonic mucosa. Total RNA was isolated from proximal colonic mucosa of control (Ctrl) and VC-Slfn3KO (KO) mice. The mRNA expression of (A) SI, (B) Vil1, (C) Dpp4, and (D) Glut1 was analyzed and determined by qPCR using RPLP0 as a reference control gene. (n = 10–19, *p < 0.05 to respective control).

Table 1
Comparison of Slfn family expression in pan-Slfn3KO vs. VC-Slfn3KO mouse tissues. Arrows indicate an increase (↑) or decrease (↓) in male, female, or both sexes. N.D. indicates no difference between Slfn3KO and WT mice or sex.

	Slfn3KO [23]			VC-Slfn3KO mice			
	Ileal Mucosa	Thymus	Spleen	Small Intestine [12]	Lung	Gastric mucosa	Proximal colon
Slfn1	↑ Both	N.D.	N.D.	↑ Both	N.D.	↑ Male	N.D.
Slfn2	N.D.	N.D.	N.D.	↑ Female	↓ Female	↑ Male	N.D.
Slfn4	↓ Both	↓ Both	N.D.	↑ Both	↑ Male	↑ Male	N.D.
Slfn5	↑ Both	N.D.	N.D.	↓ Both	↑ Male	↑ Male	↓ Female
Slfn8	↓ Both	N.D.	↑ Both	↑ Female	↓ Female	N.D.	N.D.
Slfn9	↓ Both	↑ Both	↑ Both	N.D.	N.D.	N.D.	N.D.

the lung and gastric mucosa. There are two major lung epithelial cells that cover the gas exchange compartment; these are AT1 and AT2. Hopx and Ager are AT1 cell protein-coding genes whose knockdown suppresses AT2-AT1 *trans*-differentiation and activating cellular proliferation [39]. We found that Slfn3 loss resulted in a decrease in Hopx and a converse increase in Ager in the male mice. In the lungs, Hopx acts as a negative regulator of GATA6 gene expression [11]. GATA6 and Nkx2.1 synergistically interact to activate *SP*-C and WNT7b lung-restricted genes in order to regulate differentiation, development, and maturation of the airway epithelium [11]. When Hopx expression is lost there is de-repression of the Nkx2.1-GATA6 complex which then results in abnormal lung development and function [11]. AGER (also known as RAGE) is the receptor of advance glycation end products and is a critical regulator of lung physiology [40]. AGER expression and activation are greatly elevated during stress conditions. This results in chronic inflammation and can lead to epithelial malignant transformation [40]. This overexpression of AGER in normal AT1 cells is followed by a rapid downregulation after malignant transformation and an association with increased cancer aggressiveness [40]. AGER is expressed at low levels in H1299 lung cancer cells and in non-small cell lung cancer tissue [41]. AGER overexpression reduces the proliferation, invasion, and migration abilities of H1299 lung cancer cells [41]. Our results raise the possibility that Slfn3 may be an upstream regulator of Hopx in AT1 epithelial cell differentiation, similarly to Slfn3's role in regulating intestinal epithelial cell differentiation. Slfn3 may also act as a possible regulator of Ager for maintaining normal vs. malignant AT1 cells. This may be a fruitful subject for further investigation.

In the gastric mucosa, loss of Slfn3 increased the Lgr5 and Notch1 transcription factors in male VC-Slfn3KO and decreased Tff1 gastric secretory gene expression in female VC-Slfn3KO mice. The signaling pathway of Notch1, a protein-coding gene that promotes progenitor cell proliferation and absorptive cell differentiation in the adult intestine and gastric mucosa has been shown to be strongly associated with Lgr5 stem cell regulation and function [28]. Several papers have linked alterations in NOTCH1 expression to either promoting or reducing gastric cancer by increasing apoptosis or decreasing proliferation and invasion in gastric tumors and cell lines, although such effects have been paradoxically reported for both NOTCH1 overactivation and NOTCH1 silencing [42–52]. Barker et al. identified $Lgr5^{+ve}$ cells at the gastric gland base that are multipotent stem cells, which contribute to the self-renewal of the glandular units in the pyloric region [53]. While Gifford et al. have shown that Notch1 and Notch2 control the proliferation of Lgr5 stem cells and epithelial cell proliferation [54]. Therefore, since male VC-Slfn3KO mice have an increase in Lgr5 and Notch1, it could be intriguing in the future to study how this may affect routine growth of the primary gastric epithelium between males and females, which could offer new insights into the molecular causes of gastric cancer [53]. TFF1 inhibits cell proliferation by delaying G1-S phase transition but not by inducing apoptosis [55]. TFF1 is frequently downregulated in human gastric cancers, suggesting that TFF1 may be a candidate tumor suppressor [56]. Since SLFN5, SLFN11, SLFN12, SLFN12L, SLFN13, and SLFN14 are overexpressed in human gastric cancer [4,57], future studies could evaluate the potential role of mouse Slfn3 (or the human ortholog, SLFN12) in regulating gastric epithelial cells through Notch1, Lgr5, and Tff1 and how this dysregulation contributes to gastric cancer.

After we evaluated protein expression for lung, stomach, cecum, and proximal colon markers that differed significantly at the RNA level in the VC-Slfn3KO mice, we observed that the protein expression of the lung markers, Hopx, Ager, Sftpc, Sftpd, and cecum SI protein expression did not change similarly to the RNA expression. Previously, we have shown that RNA expression of SI was increased in the duodenum and ileum of VC-Slfn3KO mice but then we only observed a decrease in SI protein of the jejunum of VC-Slfn3KO mice compared to control mice and no protein differences in the duodenum and ileum [22]. We have observed analogous regulation in the human ortholog, SLFN12. In triple negative breast cancer cells, overexpression of SLFN12 lead to an increase in ZEB1 and Slug RNA expression but then a decrease in ZEB1 and Slug protein and the vice versa was seen when SLFN12 was decreased by siRNA [58]. Indeed, SLFN12 regulates ZEB1 post-transcriptionally in terms of rates of both translation and proteasomal degradation, explaining the discrepancy between reduced ZEB1 protein but not ZEB1 mRNA in that setting [58]. Overexpression of SLFN12 also led to the increase in c-myc mRNA expression in lung adenocarcinoma cells but protein expression was decreased due to SLFN12 modulation of c-myc translation [59]. Therefore, these differences between RNA and protein expression in the VC-Slfn3KO mice could indicate a possible translational level regulation that could be evaluated in future studies.

Our laboratory has validated the first example of a sexually antagonistic gene, *Slfn3*, with effects on villus length in three independent studies [21,22,30,60]. We have continued to observe gender-related differences in Slfn family members [22,30,31], intestinal differentiation markers [21,22,30], proliferation markers and epithelial cell-shedding regulators after bowel resection [30], and now in the effects of Slfn3 on differentiation markers of the lung and gastric mucosa (Fig. 8). Waldhorn et al. EBV-immortalized a B cell line with a karyotype of 47,XXY/46,XY/46,XX from a single Klinefelter syndrome patient in order to produce isogenic human induced pluripotent stem cells (hiPSCs) with different sex chromosome complements. These hiPSCs were reprogrammed to create three 46,XX, three 46,XY, two 47,XXY, and one 45,X0 cell lines. This model allowed for the study of sex differences and the effects of X and Y chromosomes in autosomal gene expression of undifferentiated and early neural differentiated hiPSCs. Gene expression in undifferentiated isogenic hiPSCs were evaluated by RNA sequencing. SLFN13 was detected as one of 313 genes that are differentially expressed in XY vs. XX samples and was specifically downregulated in XX compared to XY [61]. (Mouse Slfn8 and SLFN13 are functional homologs and the human SLFN11/13 sequence is most similar to mouse Slfn8/9 [34]). SLFN13 was also one of 46 genes that were related to the dose of X [61]. This is important as sex differences can arise from the X dose or the presence of the Y chromosome [61]. It would be interesting to reprogram these hiPSCs to early differentiated intestinal, lung, or gastric epithelial cell lines to see if our results could be replicated to show that Slfn3 sex-dependence is from the X dose or Y chromosome. These sex-specific observations demonstrate the importance of evaluating both sexes in studies of gene expression, especially if the pathobiology being explored might itself display a gender bias.

5. Conclusion

Overall, our targeted deletion of Slfn3 using a Vil-Cre mouse model allowed us to evaluate the effects of Slfn3 loss on Slfn family members and differentiation markers in the lung, kidney, stomach, cecum, and proximal colon mucosal cells. Although there are no apparent physiological changes in these animals, the loss of Slfn3 did influence the expression of other Slfn family members as well as different organ-specific epithelial differentiation markers. Our results suggest a complex regulation by Slfn3 that is sex-dependent. Further characterization of Slfn3 pathways may illuminate the parallel functional pathway of the human ortholog, SLFN12.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

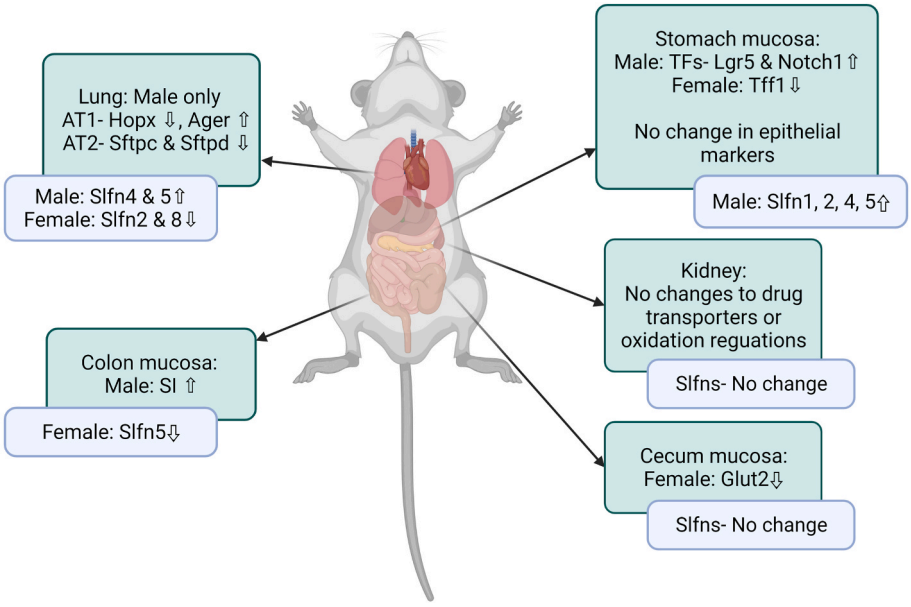

Fig. 8. Summary graphic of sex-specific changes in Slfn family member and epithelial marker expression.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2023.101552.

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