Stimulation of Murine Intestinal Secretion by Daily Genistein Injections: Gender-dependent Differences

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Key Words

Genistein • Intestine • Secretion • CFTR

Abstract

Background/Aims: The effect of daily injections with genistein (naturally occurring phytoestrogen) on intestinal chloride (Cl⁻) secretion was measured with Ussing chamber short circuit current (I_{sc} , $\mu A/cm^2$), in C57BL/6J male and female mice, using 600 mg/kg genistein/day (600G), 300 mg/kg genistein/day (300G), 150 mg/kg genistein/day (150G) or genistein-free vehicle control (0G) for 1- or 2-weeks. **Methods and Results:** Injecting with 600G elicited significant increases in basal I_{sc} in females after 1-week ($\sim 70 \mu A/cm^2$, $n=15$, $p<0.05$) and in males after 2-weeks ($\sim 80 \mu A/cm^2$, $n=5$, $p<0.05$) compared to their 0G counterparts. Chloride-free ringer significantly reduced basal I_{sc} by 65% in 600G males and 72% in 600G females, suggesting that Cl⁻ was the major anion comprising the genistein-stimulated secretion. The forskolin-stimulated ($10 \mu M$) I_{sc} was significantly inhibited by the CFTR chloride channel inhibitors, glibenclamide ($500 \mu M$) and CFTR_{inh}-172 ($100 \mu M$) in 600G males and females, suggesting some contribution by genistein-dependent CFTR-mediated

Cl⁻ secretion. We found no associated changes in intestinal morphology, nor change in total CFTR protein with 600G. There was a 5% increase in apical/subapical ratio in 600G males compared to controls (no change in females). **Conclusion:** These data suggest that male and female mice both exhibit increased Cl⁻ secretion with 600G, however, the mechanisms mediating this are gender-dependent.

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Introduction

Genistein is a naturally occurring isoflavonic phytoestrogen found in high concentrations in soy products [1]. During the last decade there have been numerous studies demonstrating genistein's potent ability to stimulate cystic fibrosis transmembrane conductance regulator, CFTR, chloride (Cl⁻) channel activity in both isolated cells [2-4] and in intact isolated tissues [5-9]. These studies have aided in our still limited understanding of genistein's action and its mechanism of action. Both wild-type (Wt)-CFTR [10] and the most common CF disease-associated mutation, $\Delta F508$ -CFTR, have been shown to be stimulated by genistein [11-12] and thus genistein's role as a potential

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therapeutic treatment in CF has been debated. Despite the fact that genistein appears to have limited importance (if any) in the field of clinically relevant CFTR-activators, a better understanding of its mechanism of action on intestinal function can not only improve our development of future enhanced CFTR activators, but aid our understanding of intestinal function and the mechanisms involved in mediating increases in intestinal transepithelial chloride secretion. Genistein's pluripotent action is likely due to the activation of multiple ion channels (particularly CFTR channels) mediated via the activation of several intracellular signaling pathways.

We have previously shown that mice fed a diet of 600 mg genistein/kg food for a period of 4 weeks yield serum concentrations of $7.73 \pm 3.83 \mu\text{M}$ in Wt female mice and $3.97 \pm 2.14 \mu\text{M}$ in male mice [7], levels that are comparable to a soy milk diet in humans [13]. This exposure to dietary genistein (600 mg/kg, for 4 weeks) stimulated basal Cl^- secretion across freshly isolated segments of jejunum from Wt female mice, but not male mice [7]. Whilst studies involving dietary manipulations have great merit, they often produce variable data, given the lack of control for daily intake per animal. Use of genistein as a pharmacological tool to manipulate tissue function has been described by Noel et al [14]; subcutaneous (sc.) injection of 50 μM genistein (or MPB-07), in the presence of isoprenaline (10 μM), induced salivary secretion in *Cfr^{+/+}* mice. To the best of our knowledge, no previous studies have assessed the effects of daily sc. injections of genistein alone on intestinal function in mice. We predicted that this route of genistein administration would yield less variable and perhaps more sustained elevations in intestinal anion secretion (than our previous murine genistein diet study [7]), presumably we hypothesize, but certainly not limited to, an action on the CFTR Cl^- channel. Indeed, recent evidence suggests that beneficial effects of genistein on CFTR-mediated Cl^- secretion may be a result of a dual action: potentiation of the CFTR Cl^- channel by binding to CFTR and stabilizing the channel in its open state [15] and promotion of its retention in the plasma membrane via inhibition of endocytosis [16].

Furthermore, we predicted that increases in intestinal I_{sc} will likely be detected at earlier time points than observed in our previous 1 month diet study [7]. Here, we initially compared the effects of either 1- or 2-weeks of daily sc. genistein injections, 600 mg/kg body weight/day (600G), 300 mg/kg body weight/day (300G), 150 mg/kg body weight/day (150G), and the vehicle control, 0 mg/kg body weight/day (0G), on small intestinal (jejunum)

epithelial anion secretion (using freshly excised intestinal segments) in Wt female and male mice. Pharmacological inhibitors were utilized to determine genistein's effect on key transporters known to contribute towards the I_{sc} across jejunum epithelia. In addition, we examined genistein's effect on intestinal morphology. We hypothesized that exposure to genistein would generate a dose-dependent increase in Cl^- secretion, without change in intestinal morphology. Moreover, to better understand genistein's mechanism of action, its effects on total intestinal CFTR protein expression and intestinal epithelial apical/subapical localization were assessed.

Materials and Methods

Mice

Male and female C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) at 4-6 weeks of age and housed in an animal care facility with 12:12-hour light-dark cycle. Mice consumed food and water *ad libitum*. Body weight and general health were monitored biweekly. Mice were fed a casein based genistein-free diet throughout the study and randomly assigned to one of the following groups; 600G (600 mg/kg body weight genistein), 300G (300 mg/kg body weight genistein), 150G (150 mg/kg body weight genistein), or 0G (0 mg/kg body weight genistein – genistein free) for either 1- or 2-weeks. At the end of the injection study period, mice were asphyxiated in an atmosphere of 100% CO_2 , followed by surgical thoracotomy to induce pneumothorax. Animal care and treatments were conducted in accordance with established guidelines and all protocols were approved by Midwestern University IACUC. **Diets.** The casein-based diet prepared by Dr. R. S. MacDonald (Department of Nutrition, Iowa State University), contained 0G and had an estimated energy content of 16.28 kJ/g. Diet composition is described previously in Al-Nakkash et al [7].

Serum genistein measurements

At the time of euthanasia, blood samples were obtained by heart puncture, serum was separated by centrifugation and then stored at -80°C . Serum samples were analyzed for genistein level by HPLC using a modification of the methodology of Franke et al. [17]. Values represent means of duplicate serum samples.

Histology and morphology

Freshly isolated pieces of jejunum were embedded and flash frozen in Optimal Cutting Temperature compound (O.C.T., Tissue-Tek, Torrance, CA). Frozen sliced sections (8-10 μm) of murine jejunum were stained with a standard hematoxylin and eosin (H & E) protocol, prior to performing the morphometric analyses to evaluate basic histological measurements. In brief, sections were exposed to the following wash protocol: hematoxylin 30 s, water rinse 10 s, Scott's Solution 5 s, water rinse 10 s, 95% ethanol 5 s, eosin 15 s, rinses with 95% ethanol 10 s, then 100% ethanol 10 s, followed by xylene 15s. Crypt

depth, villi length, along with numbers of goblets cells per crypt and villi, were measured using Image J (NIH), from images of H & E stained jejunum sections. All images were taken at 20x magnification. Averages of measurements were taken from 6 separate slices per frozen section of jejunum (i.e. per mouse) and data are presented as the average of seven mice per group.

CFTR Western blot

At collection, jejuna were immediately snap frozen in liquid nitrogen and stored at -80°C . Jejuna were later prepared for western blot analysis by homogenization. The western blot protocol was similar to that described previously [18]. Briefly, samples were analyzed for protein content, and ran on 4-12% Bis-Tris gels at 150 volts for ~ 1.5 hours. Transfer was for 2 hours at 30 volts on ice. Gels were incubated with primary antibody to CFTR [CF3] (1:500 dilution) overnight at 4°C . After washing, gels were incubated with secondary antibody (anti-mouse IgG, HRP conjugated, 1:10,000 dilution) for 1 hour at room temperature. To re-probe for actin: gels were incubated with anti-actin primary antibody (1:500 dilution) overnight at 4°C . Gels were washed and then re-incubated with the same secondary antibody. Gels were visualized using ECL (Amersham, Piscataway, NJ). Images were taken and analyzed using the STORM 860 scanner (Molecular Dynamics, Piscataway, NJ) and image quant (Molecular Dynamics, Piscataway, NJ).

CFTR Immunocytochemistry

Freshly isolated pieces of jejunum were embedded and flash frozen in O.C.T. compound (Tissue-Tek, Torrance, CA). Immunocytochemistry was performed using methodology similar to that previously described [19]. Briefly, frozen sliced sections of murine jejunum (8 μm) were fixed in HistochoiceTM (1 hour), and rinsed in PBS. Sections were incubated for 30 min in 2% BSA in PBS with 100 mM glycine to reduce autofluorescence, then rinsed in PBS. Sections were incubated with CFTR primary antibody (CFTR H-182, 1:200 dilution), for 24 hr at 20°C . Slides were rinsed with 2% BSA in PBS to block non-specific binding, and rinsed in PBS. Covered sections were incubated with CFTR secondary antibody (anti-rabbit IgG FITC, 1:400 dilution) at room temperature for 1 hr. Slides were rinsed with 2% BSA in PBS then rinsed in PBS. Sections were incubated in the dark, for 2 hours at room temperature with E-Cadherin primary antibody (Mouse anti-E-Cadherin, 1:200 dilution), then rinsed with 2% BSA in PBS then rinsed in PBS only. Slides were incubated with E-Cadherin secondary antibody (Alexa Flour594 anti-mouse, 1:400 dilution) in the dark at room temperature for 1 hr, then rinsed in PBS. Slides were mounted with Vectashield and CFTR localization was examined using an Olympus IX70 inverted fluorescent microscope. CFTR was quantified using methods described previously [19-21]. The apical domain was considered to be $< 1.5 \mu\text{m}$ from the luminal surface and the subapical domain was considered $> 1.5 \mu\text{m}$ from the luminal surface [20]. CFTR intensity was quantified using Image J (NIH) with a modified EdgeRatio Macro.

Bioelectric measurement of intestinal secretion

Via an abdominal incision, ~ 5 cm of mid-jejunum was

removed and placed in ice-cold oxygenated Krebs bicarbonate ringer (KBR). Each mouse yielded 2-3 jejunum pieces, isolated as described previously [7, 22-24]. Jejunum sections mounted in the Ussing chambers had 0.3 cm^2 exposed surface area. Transepithelial short circuit current (I_{sc} , $\mu\text{A}/\text{cm}^2$) was measured via an automatic voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA) and the experimental conditions and methods were as previously described [25]. Intestinal tissue pieces were maintained in $1 \mu\text{M}$ indomethacin (minimizing tissue exposure to endogenously generated prostanoids due to manipulation and mounting of the tissue, [26]. Glucose (10 mM) was added to the serosal KBR bath and mannitol (10 mM) substituted for glucose in the mucosal KBR bath, to avoid an inward current due to Na^+ -coupled glucose transport [25]. Once mounted, the serosal side was exposed to tetrodotoxin ($0.1 \mu\text{M}$), minimizing variations in intrinsic intestine neural tone [27]. Intrinsic neural tone limits the absorptive capacity of the murine mucosa (decreased I_{sc} denotes neural block).

Experimental protocols

Tissues were exposed to KBR (20 min) and steady-state basal I_{sc} measured at that time. cAMP-dependent anion secretion was assessed by bilateral application of $10 \mu\text{M}$ forskolin (at time 20 min) and the steady-state forskolin response (at time 50 min). At this time point, addition of glibenclamide ($100 \mu\text{M}$ or $500 \mu\text{M}$, mucosal) or 3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone (CFTR_{inh}-172, $10 \mu\text{M}$ or $100 \mu\text{M}$, mucosal) is used as an indicator of the CFTR-mediated Cl^- secretory component. Addition of 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS, $200 \mu\text{M}$, bilateral) and clotrimazole ($10 \mu\text{M}$ or $100 \mu\text{M}$, bilateral) are used to determine the contribution to the anion secretory component by Ca^{2+} -activated Cl^- channels and Ca^{2+} -activated K^+ channels respectively. Addition of acetazolamide ($100 \mu\text{M}$ bilateral), to indicate the secretory component represented by HCO_3^- . Glucose (10 mM, mucosal) was added at the end of each experiment to stimulate Na^+ -coupled glucose transport and assess tissue viability (as denoted by $> 10\%$ increase in I_{sc}). Tissues failing to respond to glucose within this parameter were discarded

Solutions

Cl^- -containing KBR contained the following (in mM): 115 NaCl, 25 NaHCO_3 , 5 KCl, 1.2 MgCl_2 and 1.2 CaCl_2 , pH 7.4. Cl^- -free KBR contained the following (in mM): $0.4 \text{ KH}_2\text{PO}_4$, $2.4 \text{ K}_2\text{HPO}_4$, 115 NaGluconate, 25 NaHCO_3 , 2.4 hemicalcium, 1.2 MgSO_4 , 3 CaSO_4 , pH 7.4.

Chemicals

Forskolin, DIDS and CFTR_{inh}-172 were purchased from Calbiochem (San Diego, CA). Glibenclamide, acetazolamide and clotrimazole were purchased from MP Biomedicals (Solon, OH). Immunocytochemistry antibodies: CFTR (H-182) (Santa Cruz Biotechnology, CA), anti-rabbit IgG FITC (Santa Cruz Biotechnology, CA), mouse anti-E-Cadherin (Invitrogen, Carlsbad, CA) and Alexa Flour594 anti-mouse (Invitrogen, Carlsbad, CA). Western blot antibodies: mAb to CFTR [CF3] (Abcam, Cambridge, MA), anti-mouse IgG, HRP conjugated

Fig. 1. Effect of 600G on basal and cAMP-stimulated I_{sc} in mice. A. Average basal I_{sc} from female and male mice injected over 1- or 2-week durations with either 600G (solid bars) or 0G (open bars). B. Average steady-state forskolin-stimulated (10 μ M, bilateral) I_{sc} from female and male mice injected over 1- or 2-week durations with either 600G (solid bars) or 0G (open bars). Values are means $\pm$ SEM, numbers in parentheses are numbers of experiments. * denotes, $p < 0.05$, ** denotes $p < 0.01$.

(Upstate/Millipore, Temecula, CA), Anti-Actin (Millipore, Temecula, CA) and anti-mouse IgG, HRP conjugated (Upstate/Millipore, Temecula, CA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Data are expressed as mean $\pm$ SEM. Numbers in parentheses are numbers of tissues used from separate individual mice. One-way ANOVA with Neuman-Keul's multiple comparison test or t-tests were performed using GraphPad (San Diego, CA). $p < 0.05$ was considered statistically significant.

Results

Mouse weights

During the 1- or 2-week studies, some mice groups exhibited a significant increase in weight gain; 150G female 1-week increased from 16.62 ± 0.24 g to 18.32 ± 0.33 g ($n = 5$, $p < 0.05$), 150G female 2-week increased from 16.15 ± 0.38 g to 18.13 ± 0.50 g ($n = 6$, $p < 0.05$), 300G male 1-week increased from 22.36 ± 0.98 g to 23.21 ± 0.79 g ($n = 5$, $p < 0.05$), and 600G male 2-week increased from 19.54 ± 0.99 g to 21.00 ± 0.76 g ($n = 8$, $p < 0.05$). All other groups maintained a constant weight throughout the study duration.

Serum genistein concentrations

All 0G serum samples produced non-detectable serum genistein levels (i.e. 0 ng/ml, $n = 4-7$ per control 0G group). Serum genistein levels were significantly increased in both 600G female 1- and 2-week (5193 ± 617 ng/ml ($n = 5$) and 8792 ± 2084 ng/ml ($n = 5$) respectively, $p < 0.05$) compared to 0G. Similarly, serum genistein levels were significantly increased in both 600G male 1- and 2-week (5947 ± 815 ng/ml ($n = 4$) and 4161 ± 543 ng/ml ($n = 4$) respectively, $p < 0.05$) compared to 0G. The serum genistein levels of both groups of 600G females and males were analogous. These data demonstrate that daily genistein injections for either 1- or 2-weeks significantly increases serum genistein levels in both male and female mice to similar levels regardless of sex.

Fig. 2. Effect of 600G on average I_{sc} in mice. A. A comparison of the I_{sc} responses from female mice injected over a 1-week duration (600G, $n = 15$; 0G, $n = 6$). B. A comparison of the I_{sc} responses from male mice injected over a 2-week duration (600G, $n = 5$; 0G, $n = 7$). Basal I_{sc} is recorded from time 0-20 min, at which point forskolin is added to induce the cAMP-mediated I_{sc} (10 μ M, bilateral). Values are mean $\pm$ SEM, * denotes significant difference from 0G, $p < 0.05$.

Ussing chamber bioelectric measurements

After 1-week, basal I_{sc} was significantly increased in the female 600G group compared to female 0G mice ($p < 0.05$, Fig. 1A, Fig. 2A, Table 1), and was unchanged compared to 0G in the 1-week female 150G and 300G groups. After 2-weeks, basal I_{sc} was significantly increased in female 600G mice compared to female 0G mice ($p < 0.05$, Fig. 1A, Table 1), and was unchanged compared to 0G in the 2-week female 150G and 300G groups. In contrast to females, basal I_{sc} was unchanged in 1-week males with 600G, 300G and 150G compared to 0G (Fig. 1A, Table 1). After 2-weeks, basal I_{sc} was significantly increased in male 600G mice compared to male 0G mice ($p < 0.05$, Fig. 1A, Fig. 2A, Table 1), and was unchanged compared to 0G in the 2-week male 150G and 300G groups.

Bilateral application of 10 μ M forskolin increased I_{sc} in all mice and all injection groups, which peaked and then remained elevated. In female mice, steady-state forskolin-stimulated I_{sc} was significantly increased in 1-week 600G mice compared to 0G controls ($p < 0.01$, Fig. 1B, Fig 2A, Table 1). Steady-state forskolin-stimulated I_{sc} was unchanged compared to 0G in the 1-week female 150G and 300G groups. In 2-week 600G females, the steady-state forskolin I_{sc} was significantly increased compared to 0G mice ($p < 0.05$, Fig. 1B, Table 1), and was unchanged compared to 0G in the 2-week female 150G and 300G groups. In males, after 1-week the steady-state forskolin I_{sc} was significantly increased in 600G mice compared to their 0G counterparts ($p < 0.05$, Fig. 1B, Table 1), and was unchanged compared to 0G in the 1-week male 150G and 300G groups. After 2 weeks, the steady-state forskolin I_{sc} was significantly increased in both 600G mice ($p < 0.01$, Fig. 2B, Table 1) and 300G mice compared to 0G mice ($p < 0.05$, Fig. 1B, Table 1). Steady-state forskolin-stimulated I_{sc} was unchanged compared to 0G in the 2-week male 150G.

Maximum genistein-mediated increases in basal I_{sc} were observed after only 1-week in female mice and after 2-weeks in male mice (in other words maximum difference from the control, 0G, conditions), thus rather than pooling the data, we maintained the notion that genistein exerts a time-dependent effect that is gender-dependent. The average I_{sc} trace data that yielded optimal genistein-mediated increases in basal I_{sc} are shown in Fig. 2A for females after 1-week and in Fig. 2B for males after 2-weeks. Since we obtained the greatest genistein-stimulated increases in basal and forskolin-stimulated I_{sc} with 600G after 1-week in females and after 2-weeks in males, we chose to use these optimal conditions when

		0G	150G	300G	600G
Basal	Female 1-week	133.2 $\pm$ 8.4	126.7 $\pm$ 16.8	110.2 $\pm$ 15.4	215.9 $\pm$ 13.1*
	2-week	96.5 $\pm$ 7.4	91.3 $\pm$ 8.1	126.9 $\pm$ 15.4	144.9 $\pm$ 11.8*
Male	1-week	139.6 $\pm$ 16.4	122.4 $\pm$ 18.4	154.4 $\pm$ 13.2	175.6 $\pm$ 17.4
	2-week	114.8 $\pm$ 12.4	112.9 $\pm$ 10.5	133.7 $\pm$ 15.9	186.6 $\pm$ 7.6*
SS-Fsk	Female 1-week	165.4 $\pm$ 15.7	165.4 $\pm$ 15.8	178.4 $\pm$ 27.5	316.6 $\pm$ 23.4*
	2-week	165.3 $\pm$ 13.2	137.4 $\pm$ 16.4	186.6 $\pm$ 14.5	218.4 $\pm$ 15.4*
Male	1-week	192.3 $\pm$ 17.2	188.8 $\pm$ 25.4	206.0 $\pm$ 19.2	282.3 $\pm$ 25.2*
	2-week	143.4 $\pm$ 9.7	159.2 $\pm$ 8.3	199.6 $\pm$ 15.7*	259.8 $\pm$ 17.7*

Table 1. Effect of genistein on I_{sc} . The following basal and steady-state forskolin-stimulated (SS-Fsk) I_{sc} (μ A/cm²) measures were taken in both female and male mice, injected with 600G (genistein, highest dose), 300G (genistein, medium dose), 150G (genistein, lowest dose) or 0G (genistein-free). Data is expressed as mean $\pm$ SEM (n=4-15/group). * denotes significant difference to 0G ($p < 0.05$).

Fig. 3. Effect of bilateral chloride-free ring on the basal and forskolin-stimulated I_{sc} in jejuna removed from mice injected with either 600G or 0G. A. Female mice injected with either 600G or 0G for 1-week; I_{sc} responses to forskolin (10 μ M, bilateral), in the presence of chloride-containing KBR (filled symbols) or chloride-free KBR (open symbols). B. Male mice injected with 600G or 0G for 2-weeks; I_{sc} responses to forskolin (10 μ M, bilateral), in the presence of chloride-containing KBR (filled symbols) or chloride-free KBR (open symbols). Values are mean $\pm$ SEM (n=3-5), * denotes significant difference from chloride containing, $p < 0.05$.

performing all further experiments aimed to determine genistein's mechanism of action.

Fig. 4. Effect of pharmacological inhibitors on the forskolin-stimulated jejuna I_{sc} . A. Percent inhibition of steady state forskolin-stimulated I_{sc} by clotrimazole (Clot, 10 μ M and 100 μ M), glibenclamide (Glib, 100 μ M and 500 μ M), and CFTR_{inh}-172 (CFTR_{inh}, 10 μ M and 100 μ M), on genistein-treated (600G, solid bars) and genistein-free (0G, open bars), female and male mice. Values are means $\pm$ SEM (n = 4-12). * denotes significant inhibition of the steady-state forskolin-stimulated I_{sc} ($p < 0.05$). † denotes significant difference of 600G from 0G ($p < 0.05$). # denotes significant difference of 0G males from female 0G counterparts ($p < 0.05$). B. Typical raw trace demonstrating glibenclamide inhibition. Typical I_{sc} trace from 600G male (2-week) showing: increased I_{sc} with forskolin (10 μ M, bilateral) at time 20 min, followed by inhibition with glibenclamide at two concentrations tested at time 50 min (100 μ M) and 60 min (500 μ M). C. Typical raw trace demonstrating clotrimazole inhibition. Typical I_{sc} trace from 600G male (2-week) showing: increased I_{sc} with forskolin (10 μ M, bilateral) at time 20 min, followed by inhibition with clotrimazole at two concentrations tested at time 50 min (10 μ M) and 60 min (100 μ M).

Contribution of Cl^- and HCO_3^- to the I_{sc}

Bathing jejuna from female mice injected with 600G (1-week) in bilateral Cl^- -free KBR significantly decreased the magnitude of the basal I_{sc} by 62.3% (from $192.66 \pm 15.24 \mu A/cm^2$ to $72.63 \pm 17.84 \mu A/cm^2$ (n = 5), $p < 0.05$, Fig. 3A), and significantly reduced the steady-state forskolin-stimulated I_{sc} by 60.7% (from $325.68 \pm 53.31 \mu A/cm^2$ to $127.95 \pm 16.32 \mu A/cm^2$ (n = 5), $p < 0.05$, Fig. 3A). To determine whether 600G selectively increased Cl^- secretion in female mice, comparable experiments were performed with 0G controls. For 0G control female mice (1-week), basal I_{sc} and steady-state forskolin-stimulated I_{sc} were unchanged by Cl^- -free KBR (Fig. 3A). Bathing jejuna from male mice injected with 600G (2-weeks) in bilateral Cl^- -free KBR significantly decreased the magnitude of the basal I_{sc} by 65.9% (from $193.99 \pm 11.22 \mu A/cm^2$ to $66.14 \pm 12.13 \mu A/cm^2$ (n = 3), $p < 0.05$, Fig. 3B), and significantly reduced the steady-state forskolin-stimulated I_{sc} by 49.1% (from $246.77 \pm 21.12 \mu A/cm^2$ to $125.66 \pm 34.21 \mu A/cm^2$ (n = 3), $p < 0.05$, Fig. 3B). In the corresponding male vehicle control mice, basal I_{sc} and steady-state forskolin-stimulated I_{sc} were also significantly decreased by Cl^- -free ringer, albeit to a lesser extent; basal I_{sc} decreased by 46.8% (from $131.93 \pm 17.99 \mu A/cm^2$ to $70.16 \pm 8.35 \mu A/cm^2$ (n = 3), $p < 0.05$, Fig. 3B) and steady-state forskolin-stimulated I_{sc} was decreased by 37.5% (from $161.87 \pm 1.04 \mu A/cm^2$ to $101.12 \pm 14.04 \mu A/cm^2$ (n = 3), $p < 0.05$, Fig. 3B). These data suggest that in the absence of Cl^- , I_{sc} 's are decreased and that the magnitude of the decreases in Cl^- -free conditions are greater in the 600G groups than in the 0G

groups, indicating a genistein-dependent Cl^- component. We performed a series of experiments aimed to determine the contribution of HCO_3^- to the forskolin-stimulated I_{sc} utilizing acetazolamide (bilateral, 100 μ M). The forskolin-stimulated I_{sc} was similarly inhibited by acetazolamide in both 0G and 600G female mice (% inhibition was 12.9 ± 3.4 (n = 7) and 11.9 ± 4.1 (n = 5) respectively) and male mice (% inhibition was 18.2 ± 4.1 (n = 8) and 14.7 ± 2.2 (n = 6) respectively). These data suggest that genistein treatment did not selectively increase the HCO_3^- -sensitive contribution to the forskolin-stimulated I_{sc} .

Pharmacological profile of the genistein-stimulated I_{sc}

We examined the effects of four pharmacological inhibitors to assess the role of key epithelial ion channels that may contribute towards the steady state forskolin-stimulated I_{sc} . CFTR_{inh}-172, a known inhibitor of CFTR at 100 μ M [28] significantly inhibited the I_{sc} in both 600G and 0G females and males (Fig. 4A). CFTR_{inh}-172, at the higher concentration tested showed a significantly greater % inhibition in 0G males (12.16 ± 1.95 , n = 5) versus 0G females (6.72 ± 2.27 , n = 6, $p < 0.05$), indicating a genistein-independent gender difference. The 100 μ M CFTR_{inh}-172-sensitive I_{sc} was significantly increased ($p < 0.05$), comparing 600G to 0G in both females and males,

Fig. 5. Effect of 2-week genistein treatment on total CFTR protein expression in murine jejunum. A. Typical western blot demonstrating CFTR and actin expression in jejunum from 3 samples of both 600G and 0G female mice. CFTR and actin bands were observed at ~170 KDa and 43KDa respectively. B. Typical western blot demonstrating CFTR and actin expression in jejunum from 3 samples of both 600G and 0G male mice. C. Average CFTR/actin ratio comparing 600G (solid bars) and 0G (open bars) in male and female mice (n=10/group). Values are means $\pm$ SEM.

indicating a genistein-dependent component in both sexes (Fig. 4A). The sulfonylurea, glibenclamide, a widely used inhibitor of CFTR [29] significantly decreased the forskolin-stimulated I_{sc} in both 0G and 600G females and males at both concentrations tested (100 μ M and 500 μ M, Fig. 4A and B). Glibenclamide, at the higher concentration tested showed a significantly greater % inhibition in 0G males (23.48 ± 1.58 , n = 12) versus 0G females (16.52 ± 3.93 , n = 5, $p < 0.05$), indicating a genistein-independent gender difference. The 500 μ M glibenclamide-sensitive I_{sc} was significantly increased ($p < 0.05$), comparing 600G to 0G in both females and males, indicating a genistein-dependent component in both sexes (Fig. 4A). Clotrimazole, a Ca^{2+} -activated K^+ channel blocker [30-31] inhibited the forskolin-stimulated I_{sc} similarly in both 600G and 0G females and males (Fig.

Fig. 6. Effect of 2-week genistein treatment on CFTR localization in sections of male murine jejunum. A. Typical image of a control section showing lack of CFTR fluorescence in the absence of primary antibody (x60 mag). B. Typical image showing CFTR fluorescence from a 0G section (x60 mag). C. Typical image showing CFTR fluorescence from a 600G section. Arrows indicate regions of high CFTR localization (x60 mag). D. Average apical/subapical ratios with 600G (solid bars) or 0G (open bars) in both females and males (n = 250 cells/group). Values are means $\pm$ SEM, *denotes significant difference from controls, $p < 0.0001$.

4A and C). Clotrimazole, at the higher concentration tested showed a significantly greater % inhibition in 0G males (16.33 ± 1.89 , n = 9) versus 0G females (8.87 ± 2.06 , n = 7, $p < 0.05$), indicating a genistein-independent gender difference. These data suggest some contribution of Ca^{2+} -activated K^+ channels towards the I_{sc} that is genistein-independent and gender-dependent. The Ca^{2+} -activated Cl^- channel inhibitor, DIDS (bilateral, 200 μ M), had no effect on the forskolin-stimulated I_{sc} in either females (n = 9) or males (n = 9), suggesting a lack of contribution of Ca^{2+} -activated Cl^- channel towards the I_{sc} .

Total CFTR protein expression

To better assess the potential mechanism(s) involved in generating the 600G mediated increase in basal I_{sc} we

quantified total CFTR protein expression, utilizing standard western blot techniques on freshly isolated jejunum removed from 600G or 0G female and male mice. Methods were similar to those described previously [18, 32-33]. As with these previously reported studies, we demonstrated a single band with the expected molecular weight of ~170 kD for CFTR. Total CFTR protein expression was normalized to total actin protein expression. The CFTR/actin ratio was similar for all groups; 0G and 600G both males and females (Fig. 5). These data suggest that the 600G-mediated increases in intestinal I_{sc} are not mediated via changes in total intestinal CFTR protein expression.

CFTR Immunocytochemistry

In the absence of primary CFTR antibody, fluorescence was minimal (Fig. 6). Using 60x magnification images, fluorescent intensity of CFTR was measured in crypt cells at both apical and sub-apical (the area 1.5 μm beneath the surface of the cell membrane) regions in randomly assigned cells in each group, according to methods previously described [20]. Measures were taken from 5 animals per group, and 50 cells per mouse (i.e. $n = 250$ cells per group). In females after 1 week, there was no difference in the apical/subapical ratio of genistein treated (600G, 1.17 ± 0.01) versus control group (0G, 1.17 ± 0.01). In males, after a 2-week duration, 600G significantly increased the apical/subapical ratio to 1.17 ± 0.01 ($p < 0.05$) compared to the 0G counterparts (1.11 ± 0.01 , Fig. 6), a modest 5% genistein-dependent increase in apical localization. These data suggest that the trafficking of CFTR to the plasma membrane is in small part genistein-dependent in males (not females) and could account for (at least in part) the 600G-stimulated increases in intestinal I_{sc} in males.

Jejunum histology

To determine whether genistein-mediated effects on intestinal I_{sc} could be correlated to changes in murine jejunum morphology, histological sections were stained using H & E and analyzed for villi length, crypt depth, villi and crypt goblet cell numbers using Image J software (NIH). We found no changes in these parameters in sections analyzed from jejunum removed from female or male 600G mice compared to their 0G counterparts (Table 2). Of note, villi length was significantly greater in the male 0G group compared to their female counterparts ($p < 0.05$), which likely would have no bearing on the genistein-mediated increases in Cl⁻ secretion, given that villi are the sites of absorption.

	Female 0G	Female 600G	Male 0G	Male 600G
Villi length (μm)	765.3 ± 21.9	815.1 ± 26.9	$851.5 \pm 17.8^*$	834.6 ± 35.8
Crypt depth (μm)	161.9 ± 12.1	164.9 ± 8.1	174.7 ± 10.1	161.2 ± 3.9
Goblet cells/villi	2.6 ± 0.5	3.1 ± 0.7	1.97 ± 0.6	2.0 ± 0.6
Goblet cells/crypt	0.6 ± 0.2	0.4 ± 0.1	0.29 ± 0.1	0.6 ± 0.2

Table 2. Effect of genistein on jejunum morphology. The following measures were taken in both female and male mice, injected with 600G (genistein) or 0G (genistein-free). Data is expressed as mean $\pm$ SEM ($n=7/\text{group}$). * denotes significant difference to 0G ($p < 0.05$).

Discussion

Our recent finding demonstrating that dietary genistein elevates basal and forskolin-stimulated I_{sc} in female mice [7], prompted our investigation to determine whether an alternative route of genistein administration (daily subcutaneous injections) could produce more effective increases in intestinal Cl⁻ secretion, over shorter time courses in both male and female mice. One of the greatest difficulties with dietary studies remains the variability; large sample sizes are needed, there is limited control of when or indeed the amount of genistein-containing food animals consume prior to the experimental date, unless of course force-feeding is part of the protocol. Our previous diet studies have shown that Wt mice fed 600 mg genistein/kg food for a period of 4 weeks yield serum concentrations of $7.7 \pm 3.8 \mu\text{M}$ ($n = 6$) in female mice and $3.9 \pm 2.1 \mu\text{M}$ ($n = 5$) in male mice [7]. These changes in serum levels of genistein translated into a significant genistein-mediated increase in basal I_{sc} in females ($\sim 36 \mu\text{A}/\text{cm}^2$, $n = 11$, $p < 0.001$) but not males ($\sim 13 \mu\text{A}/\text{cm}^2$, $n = 8$).

Our hypothesis of administering genistein via an alternate route, one more readily controlled, (sc. genistein injections), to elicit effective increases in serum genistein and thus intestinal I_{sc} (Cl⁻ secretion) in female and male mice, was verified in this current study. Here we provide the first evidence that a period of daily sc. injections of genistein (600G) ameliorates both basal and cAMP-stimulated I_{sc} (compared to 0G controls) in freshly isolated jejuna segments from mice after 1-week (females) or 2-weeks (males) with concomitant increases in serum genistein levels ($\sim 4\text{-}9 \mu\text{M}$, after treatment). Serum genistein levels were significantly increased in both 600G females (1-week = $8.8 \pm 2.1 \mu\text{M}$ ($n = 5$) $p < 0.05$) and 600G males (2-weeks = $4.2 \pm 0.5 \mu\text{M}$ ($n = 4$) $p < 0.05$) compared to their corresponding 0G controls. These changes in serum levels of genistein translated into significant genistein-mediated increases in basal I_{sc} in both females ($\sim 82 \mu\text{A}/\text{cm}^2$, $n = 15$, $p < 0.01$) and males ($\sim 86 \mu\text{A}/\text{cm}^2$, $n = 5$, $p < 0.01$). In other words, this route

of administration of genistein (sc. injections versus diet) produced similar potentiative effects on basal jejuna I_{sc} in both female and male mice, and moreover, that these increases were considerably greater than via the diet route (2.3-fold increase in I_{sc} in females and 7.8-fold increase in I_{sc} in males). Moreover, we observed maximal genistein-mediated increases in basal I_{sc} compared to 0G control groups after different time points of genistein-treatment; after 1-week in females and after 2-weeks in males. Whilst the overall genistein-mediated increases in basal I_{sc} were comparable in both females and males, these time-dependent effects may be due to either the presence of endogenous sex hormones (genistein is structurally similar to estrogen) or differences in clearance rates and pharmacokinetics between the sexes [34]. Future studies will determine the possible hypotheses/mechanisms involved in generating the sex-dependent, time-dependent differences in genistein's action on basal I_{sc} .

Typical secretion from intestinal crypts involves the following: Cl^- entering the cells via the bumetanide-sensitive $Na^+/K^+/2Cl^-$ co-transporter, activation of apical Cl^- channels, activation of basolateral K^+ channels (to both recycle the K^+ across the basolateral membrane and to maintain a driving force for Cl^- exit across the apical membrane), and the presence of the Na^+/K^+ -ATPase to maintain Na^+ and K^+ concentration gradients across the membrane. The CFTR Cl^- channel is thought to provide the major route for Cl^- exit across the apical membrane in the normal murine intestine [35–37]. Acute application of genistein has previously been shown to stimulate electrogenic Cl^- secretion across male murine jejunum in a dose-dependent manner [6]. Indeed, given the evidence from the literature and our own previous studies, demonstrating genistein's action on CFTR *in vitro* [10, 15, 38], we predicted that at least one pathway that could be involved in mediating genistein's stimulatory action on the I_{sc} would be a potentiative action on the CFTR Cl^- channel itself; either increasing channel activity or channel localization, or perhaps both [16].

This study determined that at least part of the 600G-stimulated increases in I_{sc} in both female and male murine jejunum, involved the CFTR Cl^- channel, since glibenclamide and CFTR_{inh}-172 significantly inhibited the I_{sc} by up to ~35%. Our data is consistent with other studies utilizing glibenclamide at similar concentrations in isolated rat distal colon [31], human intestinal epithelial cells [39] and isolated murine ascending colon [29]. Recent evidence suggests the importance of an alternative Cl^- channel to CFTR that is present in intestinal epithelia which is regulated by a PKA-independent, Epac-mediated

pathway [40]. Whether this novel Cl^- conductance is of importance in our genistein-mediated increased I_{sc} , remains to be seen. Interestingly, we found gender-dependent differences in the % inhibition by CFTR-inhibitors in the control (0G) groups, where males had significantly greater inhibition compared to females; glibenclamide % inhibition in 0G was 23.5 ± 1.6 (n=12) and 16.5 ± 3.9 (n=5) respectively and CFTR_{inh}-172 was $12.2 \pm .9$ (n=5) and 6.7 ± 2.3 (n=6) respectively, indicating that males had a greater resting or basal CFTR-mediated Cl^- secretion. We found no role for Ca^{2+} -activated Cl^- channels in mediating the genistein-stimulated increases in I_{sc} . Our data are in accord with previous evidence suggesting that Ca^{2+} -activated Cl^- channels do not play a role in mediating isoflavone stimulated intestinal mucosal secretion [41]. Certainly, there is evidence in the literature demonstrating the role of non-CFTR channels towards murine intestinal Cl^- secretion. CIC-2 and CIC-4 are thought to be responsible for the lack of severe intestinal impaction observed in a subset of CF mice [42]. CIC-4 has been shown to co-localize with CFTR in intestinal crypts in mouse and human tissues [43]. Members of the family of Ca^{2+} -activated Cl^- channels (CLCA) have been implicated as modifiers of the phenotype in CF; both mCLCA2 and mCLCA3 are thought to be involved in intestinal function of both CF and Wt mice [44].

Epithelial basolateral K^+ channels are known to be involved in recycling of K^+ , required to establish the favorable electrical potential for Cl^- secretion. Given that previous studies have introduced the importance of the Ca^{2+} -activated K^+ (K_{Ca}) channel in intestinal transport, and described its blockade by the nonpeptidyl inhibitor, clotrimazole [45–46], we aimed to determine its contribution under our experimental conditions. We found a K_{Ca} -mediated component to the I_{sc} that was not affected by genistein. The K_{Ca} -mediated basal I_{sc} was greater in 0G males (% inhibition was 16.3 ± 1.9 , n=9) than 0G females (% inhibition was 8.9 ± 2.1 , n=7), indicating a gender effect. These gender-dependent differences in ion channel contribution towards tissue function ought not to be surprising, given evidence by other investigators of similar observations in other tissues, e.g. a role for voltage-dependent K^+ channels in adenosine-mediated relaxation of coronary arterioles from male but not female swine [47].

The ability of any agonist to increase CFTR localization to the plasma membrane is interesting, and those that correct processing of mutated CFTR offer a usefulness as a potential pharmacotherapy for cystic fibrosis. One of the earliest studies to provide evidence

of a genistein-mediated increase in CFTR trafficking in intact epithelium, used the rectal gland of the spiny dogfish shark; data suggested that specific secretagogues that stimulate Cl⁻ secretion have multiple actions including the acute recruitment of CFTR from intracellular sites to the apical membrane and phosphorylation-mediated regulation of channels in the plasma membrane [48]. Ameen et al. [20] demonstrated a cAMP-dependent shift of CFTR localization (mediated by vasoactive intestinal peptide), from the subapical compartment to the apical compartment in rat jejunum. More recently, Schmidt et al. [4] provided evidence that prolonged treatment (24 hour but not 2 hour) of baby hamster kidney cells with genistein (30 μ M) augmented CFTR maturation and thus cell surface expression and channel function. To our knowledge, we are providing the first evidence that sc. administration of genistein in mice, can also augment CFTR translocation to the apical compartment (from the subapical compartment), however, in our experimental conditions this was a mediocre 5% increase in CFTR localization to the plasma membrane in males (not females) which provided one potential mechanism responsible for the 600G-mediated increases in basal I_{sc} in the 2-week males. The fact that this observation was, (a) so slight (5% increase), and (b) limited to male mice only, suggests that this effect has minimal impact on the overall genistein-mediated increases in I_{sc} . Clearly other mechanisms are participating in the mediating genistein increase in I_{sc} in both male and female mice.

From a morphological standpoint, a potential hypothesis to explain increases in Cl⁻ secretion attributed to the genistein-treatment could be an increase in the overall size of the crypts, the sites of intestinal secretion (i.e. an increased crypt depth, would yield a greater number of secretory epithelial cells, and thus increased Cl⁻ secretion). Our data suggests that genistein-induced increases in basal I_{sc} are not a result of changes in the intestinal morphology, i.e. are not related to changes in crypt depth or number of goblet cells in crypts. Evidence for drug-induced changes in intestinal morphology have been described previously in rats, in a model of short bowel syndrome [49], whereby treatment with benzalkonium chloride was shown to significantly increase both villus height (by ~33%) and crypt depth (by ~26%). The discrepancy between our result and those of Michopoulou et al. [49] could be attributed to one or all of the following: different species (mouse, versus rat), different test drug (genistein versus benzalkonium chloride), different test drug treatment duration (1-2 weeks, versus 30 days post treatment).

The usefulness of genistein as a compound of interest that can increase intestinal chloride secretion is described here and supported by previous studies that have tested its effects *ex vivo*. Mall et al. [5] meticulously examined genistein's effect on CFTR in both *Xenopus* oocytes and human rectal biopsies. Their observations suggested that genistein activated both Wt- and Δ F508-CFTR in oocytes and non-CF human tissue, thus concluding a lack of support for genistein's use as a pharmacological tool in CF. Given that our current study is using Wt-mice, at this point in time, we agree with the findings of Mall et al. [5]. Despite the fact that genistein appears to have limited importance (if any) in the field of clinically significant CFTR-activators, a better understanding of its mechanism of action on intestinal function can not only improve our development of future enhanced CFTR activators, but improve our understanding of intestinal function and the mechanisms involved in mediating increases in transepithelial chloride secretion. Indeed, in recent years, several labs have worked diligently to test the efficacy of various CFTR activators. Small molecule correctors (aminoarylthiozoles, quinazolinylaminopyrimidinones and bisaminomethylbithiazoles) have been shown to have varying effects on CF epithelia dependent upon the mutation [50]. The idea of mutation specific correctors was verified by Caputo et al. [51] whose results suggested that felodipine and the phenylglycine PG-01, exerted a wider pharmacological effect (acting on CFTR mutations E193K, G970R and G551D) compared to the lesser potentiative sulfonamide SF-01. More recently, the newest CFTR corrector, VX-770, has been described *in vitro* to increase CFTR channel open probability in recombinant cells expressing mutated CFTR (Δ F508 and G551D) and to increase chloride secretion in human CF bronchial epithelia [52]. Even more promising, oral application of VX-770 has been shown to be associated with improvements in lung function in patients with at least one G551D-allele, in a study aimed to determine the safety and adverse event profile of the drug [53].

In the non-CF research community the potential beneficial effects of genistein on intestinal function remain relevant and timely, given the fact that (a) genistein is found in soy-related products that many individuals consume to varying degrees daily, and perhaps more importantly, (b) the fact that post-menopausal women are utilizing this compound as an alternative to estrogen [54], both of which in the absence of knowledge of its effects on the long term function of various organs such as the intestines. The goal of this study was therefore to shed

insight as to the role that dietary compounds, such as genistein, can exert on intestinal tissue function.

In conclusion, this study provides the first evidence that sc. injections of genistein (600G) over a period of 1-week in female mice and 2-weeks in male mice is sufficient to significantly increase basal and cAMP-stimulated intestinal anion secretion (with concomitant increases in the level of serum genistein). Whilst the full mechanism(s) of action of genistein mediating [55] this effect are unclear, we can attribute a small part of this effect to an increase in CFTR localization to the plasma membrane (in males only) which thus contributes towards an increase in the measured CFTR-mediated I_{sc} . The majority of the observed 600G-mediated increase in intestinal I_{sc} appears due to an increase in chloride secretion, as determined by the significant decrease in I_{sc} in Cl⁻-free medium or in the presence of key CFTR inhibitors. However, the cellular pathways responsible for

generating the genistein-mediated increases in basal I_{sc} remain unclear. Future studies will determine mechanism(s) that play a role in mediating the genistein-stimulated increase in I_{sc} in either or both male and females.

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References

- Murphy PA: Phytoestrogen content of processed soybean products. *Food Technol* 1982;36:60-64.
- Al-Nakkash L, Hu S, Li M, Hwang TC: A common mechanism for cystic fibrosis transmembrane conductance regulator protein activation by genistein and benzimidazolone analogs. *J Pharmacol Exp Ther* 2001;296:464-472.
- Moran O, Zegar-Moran O: A quantitative description of the activation and inhibition of CFTR by potentiators: Genistein. *FEBS Letters* 2005;579:3979-3983.
- Schmidt A, Hughes LK, Cai Z, Mendes F, Li H, Sheppard DN, Amaral MD: Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator. *Br J Pharm* 2008;153:1311-1323.
- Mall M, Wissner A, Seydewitz H, Huber M, Kuehr J, Brandis M, Greger R, Kunzelmann K: Effect of genistein on native epithelial tissue from normal individuals and CF patients and on ion channels expressed in *Xenopus* oocytes. *Br J Pharm* 2000;30:1884-1892.
- Baker MJ, Hamilton KL: Genistein stimulates electrogenic Cl⁻ secretion in the mouse jejunum. *Am J Physiol* 2004;287:C1636-C1645.
- Al-Nakkash L, Clarke LL, Rottinghaus GE, Chen YJ, Cooper K, Rubin LJ: Dietary genistein stimulates anion secretion across female murine intestine. *J Nutr* 2006;136:2785-2790.
- Chao PC, Hamilton KL: Genistein stimulates electrogenic Cl⁻ secretion via phosphodiesterase modulation in the mouse jejunum. *Am J Physiol* 2009;297:C688-698.
- Tuo B, Wen G, Seidler U: Differential activation of the HCO₃⁻ conductance through the cystic fibrosis transmembrane conductance regulator anion channel by genistein and forskolin in murine duodenum. *Br J Pharm* 2009;158:1313-1321.
- Illek B, Fischer H, Santos GF, Widdicombe JH, Machen TE, Reenstra WW: cAMP-independent activation of CFTR Cl channels by the tyrosine kinase inhibitor genistein. *Am J Physiol* 1995;268:C886-C893.
- Al-Nakkash L, Hwang TC: Activation of CFTR by pharmacological modulators. *Pediatric Pulmonology* 1997;14:71-352.
- Hwang TC, Wang F, Zeltwanger S, Yang I, Reenstra W: Genistein potentiates wild-type and $\Delta F508$ -CFTR channel activity. *Am J Physiol* 1997;273:C988-998.
- Xu X, Wang H, Murphy P, Cook L, Hendrich S: Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* 1994;124:825-832.
- Noel S, Strale PO, Dannhoffer L, Wilke M, DeJonge H, Rogier C, Mettrey Y, Becq F: Stimulation of salivary secretion in vivo by CFTR potentiators in *Cfr^{+/+}* and *Cfr^{-/-}* mice. *J Cyst Fibros* 2008;7:128-133.
- Wang F, Zeltwanger S, Yang I, Nairn A, Hwang TC: Actions of genistein on CFTR channel gating. *J Gen Physiol* 1998;111:477-490.
- Lim CH, Bijvelds MJ, Nigg A, Schoonederwoerd K, Houtsmuller AB, De Jonge HR, Tilly BC: Cholesterol depletion and genistein as tools to promote $\Delta F508$ -CFTR retention as the plasma membrane. *Cell Physiol Biochem* 2007;20:473-482.
- Franke AA, Custer LJ, Wang W, Shi CY: HPLC analysis of isoflavonoids and other phenolic agents from human fluids. *Proc Soc Exp Biol Med* 1998;217:263-273.
- Bajwa PJ, Lee JW, Straus DS, Lytle C: Activation of PPAR γ by rosiglitazone attenuates intestinal Cl⁻ secretion. *Am J Physiol* 2009;297:G82-89.
- Ameen NA, Martensson B, Bourguignon L, Marino CR, Isenberg J, McLaughlin GE: CFTR channel insertion to the apical surface in rat duodenal villus epithelial cells is upregulated by VIP in vivo. *J Cell Sci* 1999;112:887-894.
- Ameen NA, Marino CR, Salas PJI: cAMP-dependent exocytosis and vesicle traffic regulate CFTR and fluid transport in rat jejunum in vivo. *Am J Physiol* 2002;284:C429-C438.
- Golin-Bisello F, Bradbury N, Ameen N: STA and cGMP stimulate CFTR translocation to the surface of villus enterocytes in rat jejunum and is regulated by protein kinase G. *Am J Physiol* 2005;289:C708-C716.

- 22 Clarke LL, Harline MC: Dual role of CFTR in cAMP-stimulated HCO³⁻ secretion across murine duodenum. *Am J Physiol* 1998;274:G718-G726.
- 23 Goddard CA, Evans M, Colledge W: Genistein activates CFTR-mediated Cl⁻ secretion in the murine trachea and colon. *Am J Physiol* 2000;279:C383-C392.
- 24 Grubb BR: Ion transport across the jejunum in normal and cystic fibrosis mice. *Am J Physiol* 1995;268: G505-G513.
- 25 Clarke LL, Grubb BR, Gabriel SE, Smithies O, Collier BH, Boucher RC: Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science* 1992;257:1125-1128.
- 26 Clarke LL, Harline MC: CFTR is required for cAMP inhibition of intestinal Na⁺ absorption in a cystic fibrosis mouse model. *Am J Physiol* 1996;270:G259-G267.
- 27 Sheldon RJ, Malarchik ME, Fox DA, Burks TF, Porreca F: Pharmacological characterization of neural mechanisms regulating mucosal ion transport in mouse jejunum. *J Pharmacol Exp Ther* 1988;249:572-582.
- 28 Robert R, Norez C, Becq F: Disruption of CFTR chloride channel alters mechanical properties and cAMP-dependent Cl⁻ transport of mouse aortic smooth muscle cells. *J Physiol* 2005;568:483-495.
- 29 Noel S, Faveau C, Norez C, Rogier C, Mettey Y, Becq F: Discovery of pyrrolo[2,3-b]pyrazines derivatives as submicromolar affinity activators of wild type, G551D, and F508del cystic transmembrane conductance regulator chloride channels. *J Pharmacol Exp Ther* 2006;319:349-359.
- 30 Hamilton KL, Kiessling M: DCEBIO stimulates Cl⁻ secretion in the mouse jejunum. *Am J Physiol* 2006;290:C152-C164.
- 31 Wu D, Hu Z: Rutaecarpine induces chloride secretion across rat isolated distal colon. *J Pharmacol Exp Ther* 2008;325:256-266.
- 32 Chen M, Du J, Jiang W, Zuo W, Wang F, Li M, Wu Z, Chan H, Zhou W: Functional expression of cystic fibrosis transmembrane conductance regulator in rat oviduct epithelium. *Acta Biochim Biophys Sin* 2008;40:864-872.
- 33 Lamhonwah AM, Bear CE, Huan LJ, Chiaw PK, Ackerly CA, Tein I: Cystic fibrosis transmembrane conductance regulator in human muscle dysfunction causes abnormal metabolic recovery in exercise. *Ann Neurol* 2010;67:802-808.
- 34 Doerge DR, Twaddle NC, Banks EP, Jefferson WN, Newbold RR: Pharmacokinetic analysis in serum of genistein administered subcutaneously to neonatal mice. *Cancer Lett* 2002;184:21-27.
- 35 Anderson MP, Welsh MJ: Calcium and cAMP activate different chloride channels in the apical membrane of normal and cystic fibrosis epithelia. *Proc Natl Acad Sci USA* 1991;88:6003-6007.
- 36 Clarke LL, Grubb BR, Yankaskas JR, Cotton CU, McKenzie A, Boucher RC: Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in Cftr(-/-) mice. *Proc Natl Acad Sci USA* 1994;91:479-483.
- 37 Grubb BR: Ion transport across the normal and CF neonatal murine intestine. *Am J Physiol* 1999;277:G167-G174.
- 38 Al-Nakkash L, Reinach PS: Activation of a CFTR-mediated chloride current in a rabbit corneal epithelial cell line. *Invest Ophthalmol Vis Sci* 2001;42:2364-2370.
- 39 Krueger D, Gruber L, Buhner S, Zeller F, Langer R, Seidl S, Michel K, Scheimann M: The multi-herbal drug STW 5 (Iberogast) has presecretory action in the human intestine. *Neurogastroenterol Motil* 2009;21:1203-e1110.
- 40 Hoque KM, Woodward OM, van Rossum D, Zachos NC, Chen L, Leung GPH, Guggino WB, Guggino SB, Tse CM: Epac1 mediates protein kinase A-independent mechanisms of forskolin-activated intestinal chloride secretion. *J Gen Physiol* 2010;135:43-58.
- 41 Cermak R, Kuhn G, Wolfrum S: The flavonol quercetin activates basolateral K⁺ channels in rat distal colon epithelium. *Br J Pharm* 2002;135:1183-1190.
- 42 Gyomai K, Garami E, Galley K, Rommens JM, Bear CE: Non-CFTR chloride channels likely contribute to secretion in the murine small intestine. *Pflugers Arch* 2001;443:S103-S106.
- 43 Mohammed-Panah R, Ackerly CA, Rommens JM, Choudhury M, Wang Y, Bear CE: The chloride channel ClC-4 colocalizes with cystic fibrosis transmembrane conductance regulator and may mediate chloride flux across the apical membrane of intestinal epithelia. *J Biol Chem* 2002;277:566-574.
- 44 Leverkoehne I, Holle H, Anton F, Gruber AD: Differential expression of calcium-activated chloride channels (CLCA) gene family members in the small intestine of cystic fibrosis mouse models. *Histochem Cell Biol* 2006;126:239-250.
- 45 Devor DC, Singh AK, Gerlach AC, Frizzell RA, Bridges RJ: Inhibition of intestinal Cl⁻ secretion by clotrimazole: direct effect on basolateral membrane K⁺ channels. *Am J Physiol* 1997;273:C531-C540.
- 46 Hamilton KL, Meads L, Butt AG: 1-EBIO stimulates Cl⁻ secretion by activating a basolateral K⁺ channel in the mouse jejunum. *Pflugers Arch* 1999;439:158-166.
- 47 Heaps C, Bowles D: Gender-specific K⁺-channel contribution to adenosine-induced relaxation in coronary arterioles. *J Appl Physiol* 2002;92:550-558.
- 48 Lechrich RW, Aller SG, Webster P, Marino CR, Forrest JN: Vasoactive intestinal peptide, forskolin and genistein increase apical CFTR trafficking in the rectal gland of the Spiny Dogfish, *Squalus acanthias*. *J Clin Invest* 1998;101:737-745.
- 49 Michopoulou A, Triggas B, Hra E, Spyratou C, Milonas A, Perrea D, Donta I, Papadopoulos S: Early intestinal morphological changes following benazkolinium chloride treatment in a rat model of short bowel syndrome. *Ann Ital Chir* 2009;80:135-139.
- 50 Pedemonte N, Lukacs GL, Du K, Caci E, Zegar-Moran O, Galletta LJV, Verkman AS: Small-molecule correctors of defective deltaF508 cellular processing identified by high throughput screening. *J Clin Invest* 2005;115:2564-2571.
- 51 Caputo A, Hinzpeter A, Caci E, Pedemonte N, Arous N, Di Duca M, Zegar-Moran O, Fanen P, Galletta LJV: Mutation-specific potency and efficacy of cystic fibrosis transmembrane conductance regulator chloride channel potentiators. *J Pharmacol Exp Ther* 2009;330:783-791.
- 52 Van Goor F, Hadida S, Grootenhuys PD, Burton B, Cao D, Neuberger T, Turnbull A, Singh A, Joubert J, Hazlewood A, Zhou J, McCartney J, Arumugam V, Decker C, Yang J, Young C, Olson ER, Wine JJ, Frizzell RA, Ashlock M, Negulescu P: Rescue of CF airway epithelial cell function in vitro by a CFTR potentiator, VX-770. *PNAS USA* 2009;106:18825-18830.
- 53 Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, Sagel SD, Hornick DB, Konstan MW, Donaldson SH, Moss RB, Pilewski JM, Rubenstein RC, Uller AZ, Aitken ML, Freedman SD, Rose LM, Mayer-Hamblett N, Dong Q, Zha J, Stone AJ, Olson ER, Ordoñez CL, Campbell PW, Ashlock MA, Ramsey BW: Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. *N Engl J Med* 2010;363:1991-2003.
- 54 Marini H, Minutoli L, Polito F, Bitto A, Altavilla D, Atteritano M, Gaudio A, Mazzaferro S, Frisina A, Frisina N, Lubrano C, Bonaiuto M, D'Anna R, Cannata ML, Corrado F, Adamo EB, Wilson S, Squadrito F: Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women. *Ann Intern Med* 2007;146:839-847.
- 55 Abebe W, Agrawal DK: Role of tyrosine kinases in norepinephrine contraction of vascular smooth muscle. *J Cardiovasc Pharmacol* 1995;26:153-159.