



OPEN

Epidermal growth factor strongly affects epithelial Na⁺ transport and barrier function in fetal alveolar cells, with minor sex-specific effects

Mandy Laube[✉], Diana Dornis, Fine Wenzel & Ulrich H. Thome

Male sex remains an independent risk factor for respiratory distress syndrome (RDS) in preterm infants. Insufficient Na⁺ transport-mediated alveolar fluid clearance contributes to RDS development and we previously demonstrated sex-specific differences in Na⁺ transport. The epidermal growth factor (EGF) is important during fetal lung development with possible influence on Na⁺ transport. Sex-specific effects of EGF during surfactant synthesis were shown. We thus determined whether EGF exerts sex-specific effects on Na⁺ transport in fetal alveolar cells. We analyzed sex-specific fetal distal lung epithelial (FDLE) cells exposed to EGF and related ligands with Ussing chambers, RT-qPCR and Western blots. EGF strongly reduced the epithelial Na⁺ channel (ENaC) mRNA levels in both male and female FDLE cells. This was corroborated by a markedly reduced ENaC activity, while amiloride-insensitive pathways as well as barrier function were raised by EGF. In contrast to chronic effects, acute effects of EGF were sex-specific, because Na⁺ transport was reduced only in males. AKT phosphorylation was elevated only in female cells, while pERK1/2 was increased in both male and female cells. EGF showed certain sex- and time-dependent effects in FDLE cells. Nevertheless, the results suggest that EGF is an unlikely cause for the sex-specific differences in Na⁺ transport.

During fetal development, lung epithelial cells actively secrete fluid, thereby filling the developing lung. Vectorial Cl⁻ transport-driven pulmonary fluid accumulation supports lung growth by establishing an intra-pulmonary pressure that promotes cellular proliferation. Animal studies with intrauterine tracheal drainage as well as tracheal occlusion demonstrated the pivotal relationship between fluid accumulation and fetal lung development¹. A contribution of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) to this process has been suggested²⁻⁴. More precisely, during fetal lung development CFTR expression exhibits a time- and tissue-dependent expression pattern. The highest CFTR expression level was observed in the 1st and 2nd gestational trimester, while its expression gradually declines during the 3rd trimester^{5,6}. Epithelial cell proliferation was accelerated by *Cftr* over-expression in the pseudoglandular stage resulting in an enhanced lung growth³. Prior to birth, the fetal lung fluid has to be removed to enable air breathing. Alveolar fluid clearance (AFC) is driven by epithelial Na⁺ transport accomplished by epithelial Na⁺ channels (ENaC) in the apical membrane compartment and the Na,K-ATPase in the basolateral membrane compartment of alveolar type II (ATII) cells. ENaC consist of three homologous subunits, α -, β -, and γ -ENaC⁷, and the Na-K-ATPase is composed of α_1 - and β_1 -subunits in ATII cells⁸. Vectorial Na⁺ transport establishes an osmotic driving force causing fluid absorption from the air spaces into the interstitium. In premature newborns a decreased AFC has been shown⁹, possibly due to a lower expression of epithelial Na⁺ channels¹⁰. In addition to surfactant deficiency, AFC insufficiency contributes to the development of the respiratory distress syndrome (RDS)¹⁰. Importantly, a sex ratio of 1:1.7^{11,12} was observed for the RDS incidence, with males developing RDS significantly more frequently compared with female infants of the same gestational age, raising male mortality¹³. Up until now, male sex remains an independent risk factor for RDS development^{11,12}. We have previously shown sex-specific differences in alveolar Na⁺ transport. Male sex was associated with lower Na⁺ transport and reduced levels of the ENaC and Na-K-ATPase subunits in fetal distal lung epithelial (FDLE) cells¹⁴. Na⁺ transport in female cells was more responsive to female sex steroids

Department of Pediatrics, Division of Neonatology, Center for Pediatric Research Leipzig (CPL), University of Leipzig, Liebigstrasse 19, 04103 Leipzig, Germany. ✉email: mandy.laube@medizin.uni-leipzig.de

possibly contributing to the observed female advantage¹⁵, while androgens and glucocorticoids were either lacking any effect or equally stimulated Na⁺ transport in both sexes^{15,16}. Another important growth factor during fetal lung development is the epidermal growth factor (EGF). EGF, via its receptor (EGFR), enhances terminal branch formation and stimulates proliferation and differentiation of epithelial and mesenchymal cells in culture¹⁷. EGFR deficient mice exhibit a neonatal lethal pulmonary phenotype and present a reduced branching as well as condensed lungs with collapsed alveoli, resembling a human neonatal RDS phenotype^{18,19}. EGFR is the prototypic member of the ErbB (erythroblastic oncogene B) family of receptor tyrosine kinases (reviewed in²⁰). To date, three other members of this family have been identified: ErbB2, ErbB3, and ErbB4, which are capable of forming homodimers, heterodimers, and possibly higher-order oligomers. Despite their large degree of structural homology, ErbB family members differ from each other in their patterns of expression, ligand specificity, and intracellular substrates. Complementing these receptors is a family of ligands: e.g. EGF and transforming growth factor- α (TGF- α) bind only to ErbB1 (EGFR), heparin-binding EGF (HB-EGF) binds to both ErbB1 and ErbB4, and neuregulin 1 (NRG1) which binds to ErbB3 and ErbB4. ErbB2 has no known ligand and acts through dimerization with one of the other three receptors, while ErbB3 is kinase impaired. Downstream ErbB signaling includes phosphoinositide 3-kinases (PI3-K)/ protein kinase B (AKT), mitogen-activated protein kinase (MAPK) signaling, and the phospholipase C (PLC) pathway²¹, all of which are known to affect ENaC functions.

Sex-specific responses to EGF have been previously noted regarding surfactant synthesis^{22–24} and an impact on epithelial Na⁺ transport has been described by others^{25,26}. However, a potential sex-specific effect of EGF on Na⁺ transport in fetal alveolar cells is currently unknown. We therefore aimed to determine the impact of EGF and its related ligands on Na⁺ transport in sex-specific FDLE cells and reveal contributing pathways in this context.

Methods

Cell isolation and culture. Sprague–Dawley rats were obtained from the Medical Experimental Center (MEZ) of Leipzig University. Animals were kept in rooms with a 12 h light–dark cycle, constant temperature (22 °C) and humidity (55%). Food and water were supplied ad libitum. At gestational day E20–21 (term E = 22) pregnant rats were anesthetized by CO₂ inhalation and euthanized by Pentobarbital injection. Separation of male and female fetuses was done through visual determination, as described before¹⁴. All experimental procedures were approved by the institutional review board (IRB: Landesdirektion Leipzig, permit number: T23/15) and complied with the ARRIVE guidelines. All methods were carried out in accordance with relevant guidelines and regulations.

FDLE cells, a model of fetal ATII cells, were isolated from fetal lungs as described previously^{27,28}. Male and female fetuses were derived from the same litter in equal numbers and prepared separately. Fetal lungs were mechanically dissociated with razor blades. The resulting cell suspension was enzymatically digested by incubation with 0.125% trypsin (Fisher Scientific, Schwerte, Germany) and 0.4 mg/ml DNase (CellSystems, Troisdorf, Germany) in HBSS (Fisher Scientific) for 10 min at 37 °C, followed by MEM containing 0.1% collagenase (CellSystems) and DNase for 15 min at 37 °C. To separate adjacent lung fibroblasts from FDLE cells, the cell mix was plated twice for one hour at 37 °C in cell culture flasks. FDLE cells were then seeded on permeable Snapwell inserts (Costar, # 3407, surface area 1.1 cm², Corning, NY) at a density of 10⁶ cells per insert. For RNA and protein isolation, cells were seeded on larger inserts (ThinCert, #657641, surface area 4.6 cm², Greiner Bio-One, Frickenhausen, Germany) at a density of 2 × 10⁶ cells per insert. To generate sex-specific epithelial–fibroblast co-cultures, FDLE cells were seeded on inserts as described above, while the adjacent lung fibroblasts obtained by differential adhesion were seeded in the compartment below the insert at the bottom of the well plate. FDLE cells were cultured in serum-supplemented medium consisting of MEM with 10% FBS (Biochrom, Berlin, Germany), glutamine (2 mM, Fisher Scientific) and antibiotic–antimycotic (Fisher Scientific). Cell culture medium was changed to serum-free medium (SF-Med, Cellgro, Mediatech, Herndon, VA, USA) supplemented with either EGF (10 ng/ml, # 354,052, BD-Bioscience, San Jose, CA, USA), HB-EGF (50 ng/ml, # 97560, Biomol, Hamburg, Germany), TGF- α (10 ng/ml, # T8250-05, Biomol) or NRG1 (50 μ g/ml, # SRP3055, Sigma-Aldrich, Taufkirchen, Germany) 24 h prior to analyses. The concentrations of estradiol (E2, 0.0037 μ M) and progesterone (P, 2.8 μ M) were chosen based on previous studies^{15,29}. The antagonist Erlotinib (10 μ M, # Cay10483-250, Biomol) was used to block EGFR and thereby the EGF-evoked response. Growth factors and the receptor antagonist were dissolved in dimethyl sulfoxide (DMSO). To exclude solvent influences on the evoked responses, control cells were treated with the same amount of the respective solvent.

Using chamber measurements. Ussing chamber measurements were performed five days after cell isolation, as previously reported¹⁴. Only monolayers with a transepithelial resistance (R_{te}) exceeding 300 Ω cm² were included in the analyses. Electrophysiological solutions consisted of: 145 mM Na⁺, 5 mM K⁺, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 125 mM Cl⁻, 25 mM HCO₃⁻, 3.3 mM H₂PO₄⁻ and 0.8 mM HPO₄²⁻ (pH 7.4). For the basolateral solution, 10 mM glucose was used, while 10 mM mannitol was used in the apical solution. During measurements, the solutions were continuously bubbled with carbogen (5% CO₂ and 95% O₂). Short-circuit currents (I_{sc}) were measured every 20 s with a transepithelial voltage clamp (Physiologic instruments, San Diego, CA, USA). After the I_{sc} reached a stable plateau (I_{base}), amiloride (10 μ M, # A7410, Sigma-Aldrich) was applied to the apical chamber to assess the amiloride-insensitive I_{sc} ($I_{amil-insens}$). The current reduction induced by amiloride (ΔI_{amil}) was used as a measure of ENaC activity. Forskolin (10 μ M, # F-6886, Sigma-Aldrich) was added to the apical compartment after amiloride application to increase the intracellular cyclic adenosine monophosphate (cAMP) concentration and thereby activate cAMP-sensitive ion channels like CFTR, contributing to the forskolin-induced I_{sc} (ΔI_{forsk}). Finally, CFTR_{inh172} (10 μ M, # 3430, Bio-Techne, Wiesbaden-Nordenstadt, Germany) was applied apically to determine the CFTR_{inh172}-sensitive I_{sc} (ΔI_{CFTR}), a measure of CFTR activity. Amiloride was dissolved in water; forskolin and CFTR_{inh172} were prepared in DMSO.

Gene	Primer (forward, 5'-3')	Primer (reverse, 3'-5')
<i>α-ENaC</i> NM_031548.2	TTCTGGGCGGTGCTGTGGCT	GCGTCTGCTCCGTGATGCGG
<i>β-ENaC</i> NM_012648.1	TGCAGGCCCAATGCCGAGGT	GGGCTCTGTGCCCTGGCTCT
<i>γ-ENaC</i> NM_017046.1	CACGCCAGCCGTGACCCTTC	CTCGGGACACCACGATGCGG
<i>Na,K-ATPases-α₁</i> NM_012504.1	GGACGAGACAAGTATGAGCCCGC	CATGGAGAAGCCACCGAACAGC
<i>Na,K-ATPases-β₁</i> NM_012505.2	GCGCAGCACTCGCTTTCCT	GGGCCACACGGTCTTGGTACG
<i>CFTR</i> NM_031506.1	GCCTTCGCTGGTTGCACAGTAGTC	GCTTCTCCAGCACCCAGCACTAGA
<i>Sftpa</i> NM_001270645.1	CCTCTTCTTGACTGTTGTCGCTGG	GCTGAGGACTCCCATTGTTGTCAG
<i>Sftpb</i> NM_138842.1	GGAGCTAATGACCTGTGCCAAGAG	CTGGCCCTGGAAGTAGTCGATAAC
<i>Sftpc</i> NM_017342.2	GATGGAGAGCCACCGGATTACTC	GAACGATGCCAGTGGAGCCAATAG
<i>EGFR</i> NM_031507.1	GGGATCGGCCTTTCATGCG	GTGCCAAATGCTCCTGAACCCAG
<i>Rpl13a</i> NM_173340.2	GGGCCATCTTCTGGGCCGC	CATGCCTCGCACAGTGCGCC

Table 1. Primer sequences.

mRNA expression analyses. RNA isolation, reverse transcription and real-time quantitative PCR (RT-qPCR) were done as described before¹⁶. Gene-specific primers are listed in Table 1.

Western blot analyses. FDLE cell inserts were placed on ice, and protein lysates prepared and analyzed as described elsewhere³⁰. Phosphorylation of AKT was analyzed using antibodies against phospho-AKT at Ser473 (# 9271, Cell Signaling Technology, Frankfurt am Main, Germany), and AKT (# 9272, Cell Signaling Technology, both kindly provided by A. Garten). Phosphorylation of ERK1/2 (extracellular-signal regulated kinases) was analyzed using antibodies against phospho-ERK1/2 at Tyr202/Tyr204 (# 9101, Cell Signaling Technology), and ERK1/2 (# 9102, Cell Signaling Technology, both kindly provided by A. Garten). EGFR (C74B9, # 2646, Cell Signaling Technology) expression was measured and α -Tubulin (11H10, # 2125, Cell Signaling Technology) expression was used as a reference.

MTT. Metabolic activity in male and female FDLE cells stimulated with EGF was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as described before³¹.

Cell permeability assay. Epithelial permeability was assessed with FDLE cells cultured on Snapwell inserts. After two days in culture, the medium was changed to EGF-containing or control medium. After 24 h the medium was replaced by phenol red-free MEM (Fisher Scientific) in the lower compartment and phenol red-free MEM containing FITC-dextran (0.25 mg/ml, 3–5 kDa, # 46944, Sigma-Aldrich) in the upper insert compartment. Cells were incubated for another 24 h, followed by analysis of FITC-dextran fluorescence intensity in the lower compartment.

Statistical analysis. Differences between two groups were analyzed with the unpaired *t*-test. Otherwise, significant differences were determined by ANOVA with Dunnett's post hoc test. A probability of $p < 0.05$ was considered significant for all statistical analyses. Statistical analysis was performed with GraphPad Prism software (GraphPad Prism 9.1.1, <https://www.graphpad.com>, GraphPad Software, La Jolla, CA, USA).

Results

Effect of EGF on Na⁺ and Cl⁻ transporters in sex-specific FDLE cells. We addressed the effect of EGF on epithelial Na⁺ channels in male and female FDLE cells separately. Cells were cultured in SF-Med to exclude potential serum effects. In male and female FDLE cells, application of EGF significantly decreased *α-ENaC*, *β-ENaC* and *γ-ENaC* mRNA expression after 24 h compared with control cells (Fig. 1a, b). In detail, EGF reduced *α-ENaC* mRNA expression by approximately 30%, and *β-ENaC* and *γ-ENaC* mRNA expression by approximately 60% ($p < 0.01$; $p < 0.001$). EGF further reduced *Na,K-ATPase-α₁* in male FDLE cells by 10%, and *Na,K-ATPase-β₁* mRNA expression by 25% in both, male and female cells ($p < 0.05$; $p < 0.001$, Fig. 1c). In contrast to the reduction of Na⁺ transporter mRNA levels, EGF significantly elevated the mRNA expression of surfactant protein A (*Sftpa*), B (*Sftpb*) and C (*Sftpc*) in both sexes (Fig. 1d). Moreover, EGF increased *Sftpa* mRNA levels by 50%, *Sftpb* by more than 400%, and *Sftpc* by approximately 20–30% ($p < 0.05$; $p < 0.01$; $p < 0.001$), without any pronounced sex difference.

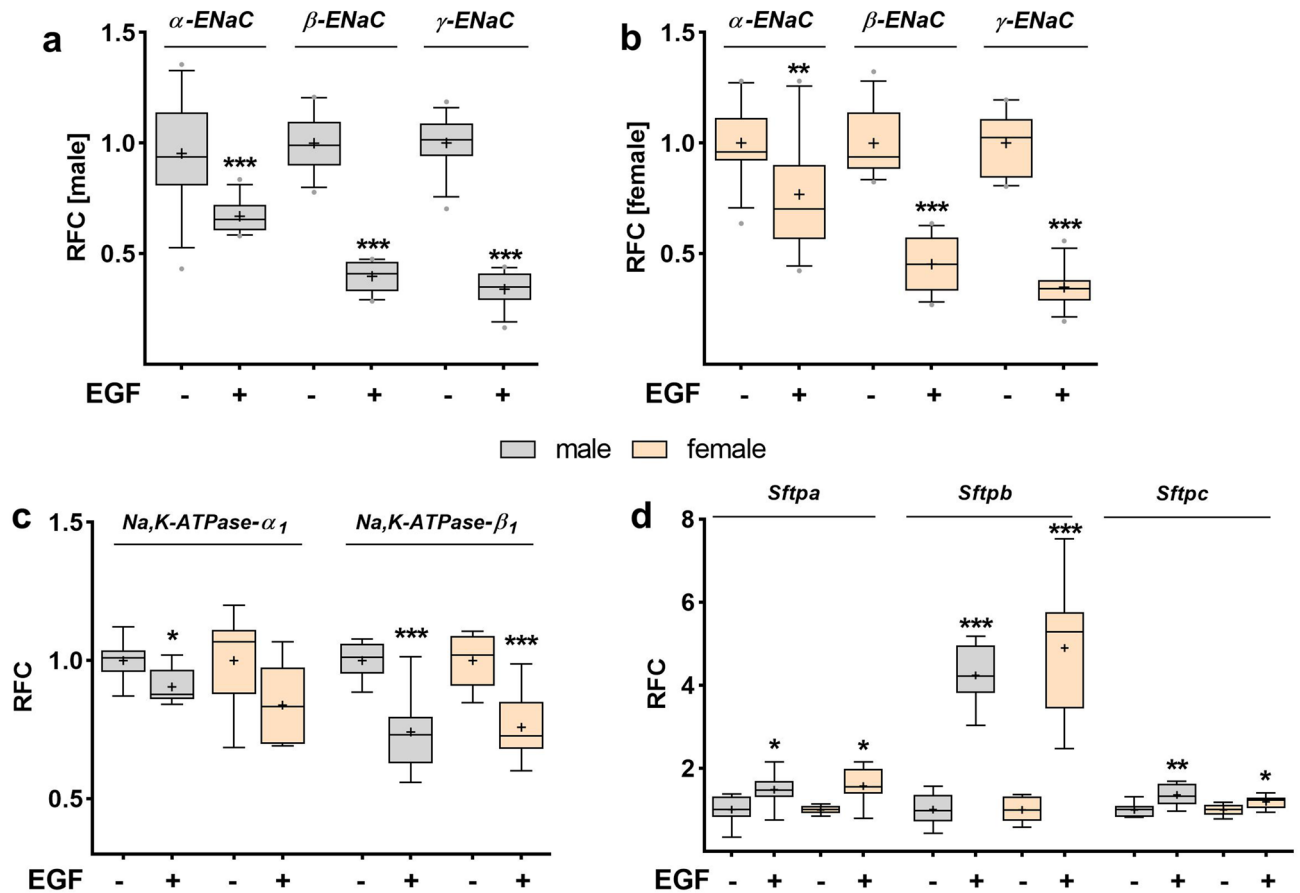


Figure 1. EGF reduced mRNA expression of Na^+ transporters in both sexes. Sex-specific FDLE cells were isolated from fetal rats, cultured in SF-Med and stimulated with EGF for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line) of relative fold change (RFC). In male (a) and female (b) FDLE cells, EGF significantly decreased α -ENaC, β -ENaC and γ -ENaC mRNA expression ($n = 11$ –12; ** $p < 0.01$; *** $p < 0.001$, t -test). (c) EGF also reduced Na,K -ATPase- α_1 in male FDLE cells, and Na,K -ATPase- β_1 mRNA expression in both, male and female cells ($n = 8$; * $p < 0.05$; *** $p < 0.001$, t -test). (d) Surfactant protein ($Sftpa$, $Sftpb$ and $Sftpc$) mRNA expression was elevated by EGF in male and female FDLE cells ($n = 8$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, t -test). (Grey boxes) male; (yellow boxes) female.

Ussing chamber analyses demonstrated that baseline I_{SC} (I_{base}) of male cells was significantly decreased by EGF after 24 h compared with untreated male controls ($p < 0.01$; Fig. 2a). The amiloride-sensitive I_{SC} (ΔI_{amil}) was also significantly decreased by EGF in male FDLE cells ($p < 0.001$). Similarly, EGF significantly decreased I_{base} and ΔI_{amil} in female FDLE cells ($p < 0.01$; $p < 0.001$; Fig. 2a). The results suggest that epithelial Na^+ transport of male and female FDLE cells is equally reduced by EGF after 24 h. In contrast, epithelial barrier function with regard to R_{te} was highly increased by EGF in both sexes ($p < 0.001$; Fig. 2b). Furthermore, the amiloride-insensitive I_{SC} ($I_{amil-insens}$), most likely Cl^- transport, was strongly increased by EGF in male and female cells ($p < 0.001$; Fig. 2c). This is further supported by the significantly elevated forskolin-induced I_{SC} (ΔI_{forsk}) and the increased CFTR_{inh172}-sensitive I_{SC} (ΔI_{CFTR}) induced by EGF in female FDLE cells ($p < 0.01$; $p < 0.001$; Fig. 2d). Surprisingly, male FDLE cells did not show a significant increase of CFTR activity, since ΔI_{forsk} and ΔI_{CFTR} were not affected by EGF addition. Finally, CFTR mRNA expression was strongly increased by EGF in both, male and female FDLE cells ($p < 0.001$; Fig. 2e). Taken together, EGF negatively regulated mRNA level and activity of Na^+ transporters in both sexes after 24 h. On the other hand, EGF strongly elevated Cl^- transport in females and CFTR mRNA expression in both, male and female FDLE cells.

Persistence of the EGF response was tested by stimulating FDLE cells with EGF for 24 h, followed by another 24 h culture in SF-Med without EGF. These experiments were done in mixed FDLE cells of both sexes. Culture with SF-Med restored I_{base} and ΔI_{amil} of initially EGF-treated cells to control levels (Fig. 3a). In contrast, EGF's effect on $I_{amil-insens}$ persisted even after 24 h culture in SF-Med, shown by the significantly higher $I_{amil-insens}$ in formerly EGF-treated cells ($p < 0.001$). The elevated barrier function shown above was abolished by 24 h culture in SF-Med (Fig. 3b), suggesting that the effect of EGF on Na^+ transport and R_{te} requires its continuous presence.

Sex-specific expression of EGFR. In the presence of fetal serum, female FDLE cells displayed a higher EGFR protein expression ($p < 0.001$; Fig. 4a). In FDLE cells cultured in SF-Med no sex-specific protein expression of EGFR was observed, and EGF application significantly downregulated EGFR protein expression after 24 h in both sexes ($p < 0.001$; Fig. 4b). In accordance, EGF also reduced EGFR mRNA expression in male and

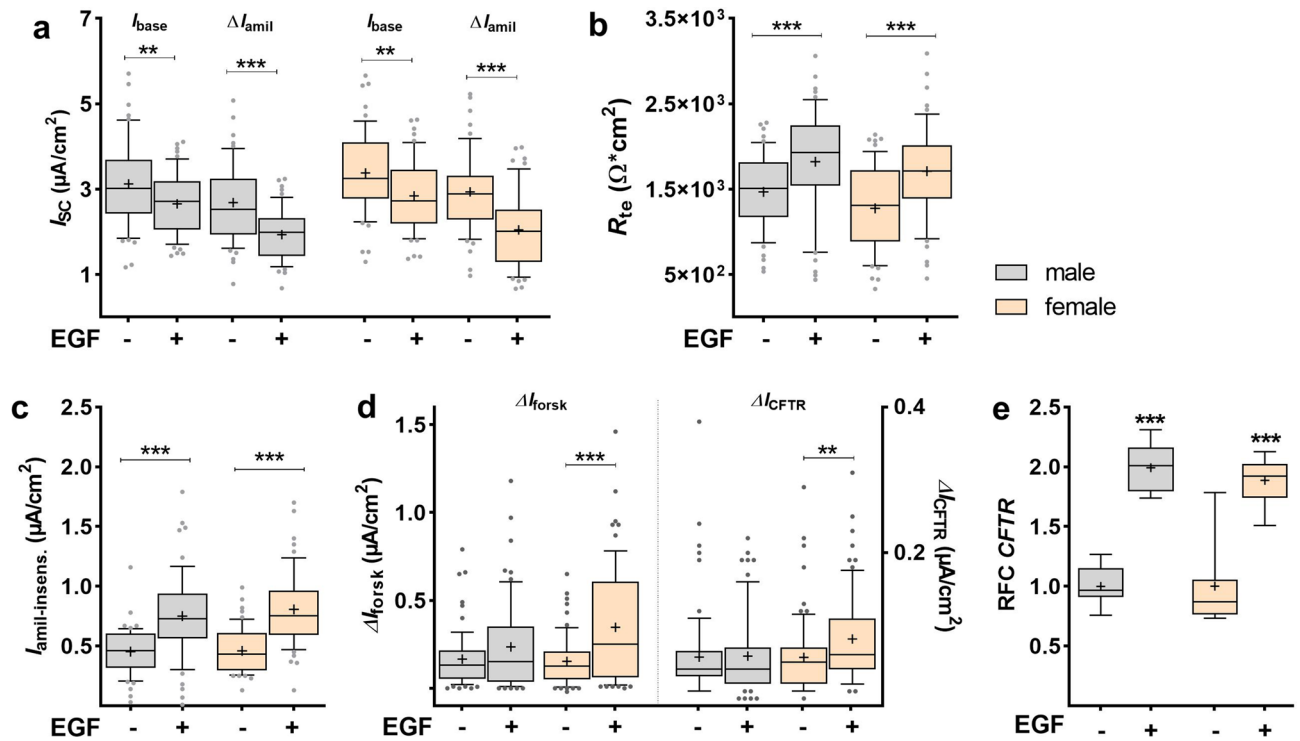


Figure 2. EGF decreased Na^+ transport, but increased Cl^- transport and CFTR mRNA expression. FDLE cells were cultured in SF-Med and stimulated with EGF for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). (a) In male and female FDLE cells, EGF significantly decreased I_{base} and ΔI_{amil} ($n = 52-59$; $**p < 0.01$; $***p < 0.001$ by t -test). EGF further significantly increased R_{te} (b) and $I_{amil-insens.}$ (c) in both sexes ($***p < 0.001$). (d) EGF strongly increased ΔI_{forsk} and ΔI_{CFTR} , but only in female FDLE cells ($n = 71-76$; $**p < 0.01$; $***p < 0.001$ by t -test). (e) EGF significantly increased CFTR mRNA expression in both sexes ($n = 8$; $***p < 0.001$; t -test). (Grey boxes) male; (yellow boxes) female.

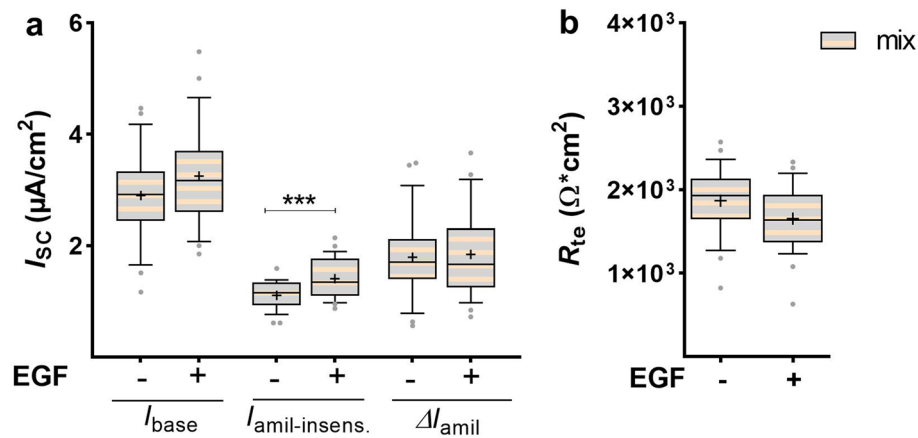


Figure 3. Persistence of EGFs' effect. FDLE cells (mixed sexes) were cultured in SF-Med and stimulated with or without EGF for 24 h, followed by a medium exchange to SF-Med lacking EGF in both groups and culture for another 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). (a) The reducing effect of EGF on I_{base} and ΔI_{amil} did not persist after 24 h culture in SF-Med, while the increasing effect on $I_{amil-insens.}$ was retained in mixed FDLE cells ($n = 26/27$; $***p < 0.001$ by t -test). (b) Furthermore, the stimulating effect of EGF on R_{te} was lost after 24 h in SF-Med.

female cells by approximately 80% ($p < 0.001$; Fig. 4c), and EGFR mRNA expression was not sex-specific. The results thus suggest that EGFR expression is sex-specific only in the presence of fetal serum, while EGF equally reduces the expression of its own receptor in both sexes.

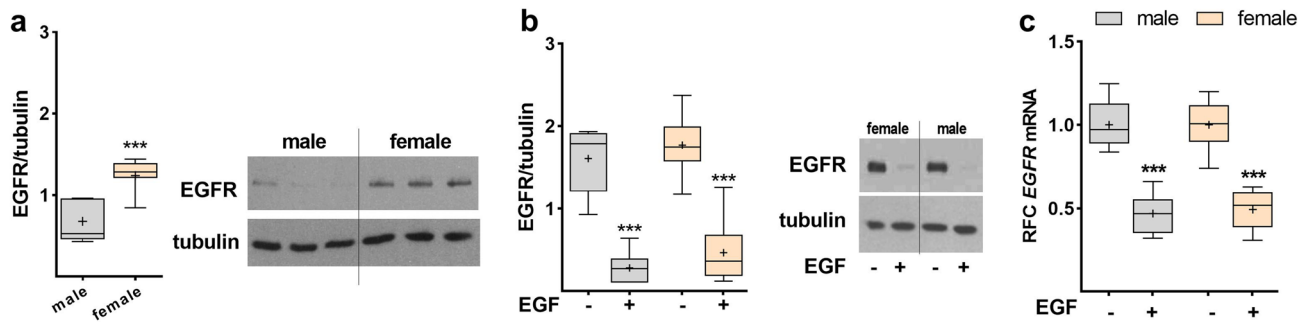


Figure 4. Sex-specific and EGF-dependent expression of EGFR. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). (a) EGFR protein expression was significantly higher in female FDLE cells compared to male cells in the presence of serum ($n = 7$; $***p < 0.001$ by t -test). EGF application to FDLE cells cultured in SF-Med reduced EGFR protein (b) and mRNA expression (c) after 24 h in FDLE cells of both sexes ($n = 6/8$; $***p < 0.001$ by t -test). (Grey boxes) male; (yellow boxes) female.

Acute effects of EGF in sex-specific FDLE cells. Previous studies suggested a biphasic response with acute EGF effects differing from chronic effects. Sex-specific FDLE cells were thus cultured in SF-Med for 24 h prior to the addition of EGF for 45 min. In accordance to the chronic EGF treatment, I_{base} and ΔI_{amil} were significantly decreased by EGF after 45 min ($p < 0.01$; $p < 0.001$; Fig. 5a). However, this effect was only observed in male FDLE cells, while female FDLE cells were not affected by acute EGF addition. Moreover, $I_{amil-insens}$ was significantly increased by EGF only in male cells ($p < 0.05$; Fig. 5b). Barrier function was not affected by short-term EGF treatment since the R_{te} was not different compared to controls or between sexes (Fig. 5c). Western blot demonstrated a significantly higher AKT phosphorylation in female FDLE cells after acute EGF treatment that was not observed in males ($p < 0.05$; Fig. 5d). ERK phosphorylation was significantly elevated by acute EGF application in both male and female cells, although the response to EGF with regard to ERK1/2 phosphorylation was stronger in male cells ($p < 0.001$; Fig. 5e). Since AKT phosphorylation is suggested to increase ENaC activity, and ERK1/2 phosphorylation downregulates ENaC, elevated pAKT in combination to a lower increase of pERK1/2 in females possibly prevents the decrease in ENaC activity that is observed in males after acute EGF treatment. These results show that at least for the acute effects of EGF sex-specific differences were observed with regard to Na^+ and Cl^- transport. EGFR protein expression was also decreased by short-term EGF exposure by approximately 30% in both male and female FDLE cells ($p < 0.05$; $p < 0.001$; Fig. 5f).

In contrast to the acute effects of EGF, AKT phosphorylation was reduced in male FDLE cells after chronic (24 h) EGF treatment ($p < 0.05$; Fig. 6a), while AKT phosphorylation in females was not different between EGF-treated and untreated cells. Furthermore, ERK phosphorylation was not different in chronically EGF-treated and controls in both male and female FDLE cells (Fig. 6b). Thus, the acute effects of EGF on AKT/ERK phosphorylation differ from its chronic effects.

EGF affects barrier permeability and metabolic activity in sex-specific FDLE cells. As shown above, chronic EGF treatment strongly elevated R_{te} suggesting an improved barrier permeability. To confirm this observation a FITC-dextran assay was conducted showing a significantly lower epithelial permeability induced by EGF in both male and female FDLE cells ($p < 0.001$; Fig. 7a). The elevated R_{te} and the reduced permeability support an improved barrier function induced by EGF. On the other hand, metabolic activity determined by an MTT assay was elevated only in male cells, while female cells were not affected by EGF ($p < 0.01$; Fig. 7b). FDLE cell numbers after 24 and 48 h of EGF treatment were unchanged in both male and female cells, suggesting that EGF did not stimulate proliferation of epithelial monolayers (Fig. 7c).

Response to EGF in epithelial-mesenchymal co-cultures. EGF is known to stimulate the epithelial-mesenchymal interaction during fetal lung development. We therefore treated co-cultures of sex-specific FDLE cells and sex-specific fetal lung fibroblasts with EGF for 24 h. EGF significantly reduced I_{base} and ΔI_{amil} in both sexes ($p < 0.001$; Fig. 8a, b). Moreover, $I_{amil-insens}$ was increased by EGF in male and female FDLE cells as shown above, lacking sex-specific effects ($p < 0.01$; $p < 0.001$; Fig. 8a, b). Epithelial barrier function with regard to R_{te} was highly increased by EGF only in males ($p < 0.001$; Fig. 8c), although the same trend was observed in females. EGFR protein expression in sex-specific fetal lung fibroblasts cultured in the presence (Fig. 8d) or absence of fetal serum (Fig. 8e) was not different between male and female fibroblasts. EGF application again significantly reduced EGFR protein levels in both male and female fibroblasts ($p < 0.01$; Fig. 8e).

Effects of EGF after inhibition of EGFR with Erlotinib. Inhibition of EGFR with Erlotinib did not prevent the decrease of Na^+ transport induced by EGF, but even further decreased I_{base} and ΔI_{amil} in both sexes ($p < 0.05$; $p < 0.01$; $p < 0.001$; Fig. 9a, b). On the other hand, Erlotinib prevented the increase of $I_{amil-insens}$ induced by EGF ($p < 0.01$; $p < 0.001$; Fig. 9a, b). Furthermore, the significant increase of R_{te} induced by EGF was completely abolished by inhibition with Erlotinib ($p < 0.001$; Fig. 9c). Thus, Erlotinib prevents only some of the EGF-induced effects on epithelial ion transport, whereas other effects were even amplified.

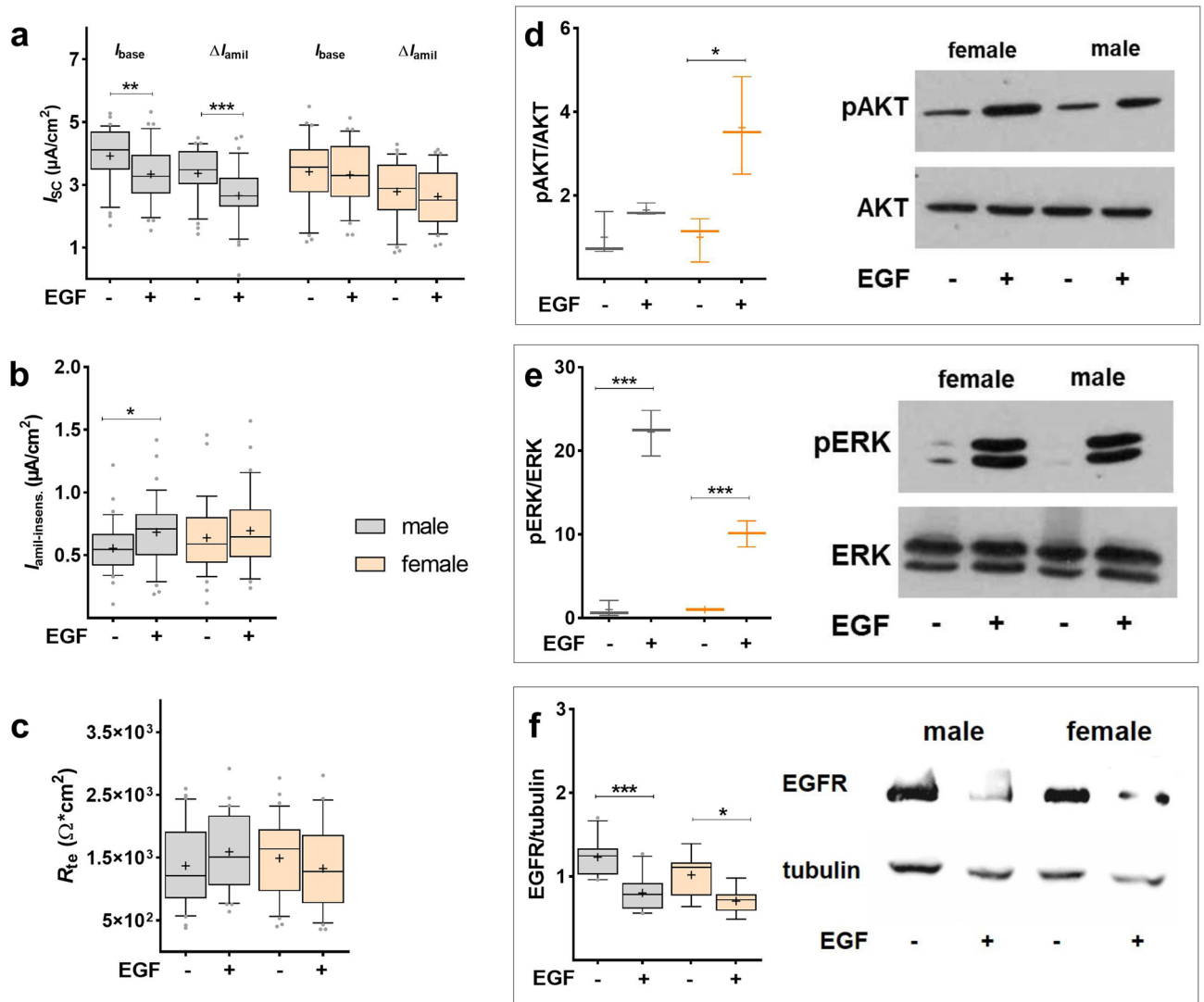


Figure 5. Acute sex-specific effects of EGF via AKT-ERK1/2 signaling. FDLE cells were cultured in SF-Med and stimulated with EGF for 45 min. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). **(a)** In male FDLE cells, EGF significantly decreased I_{base} and ΔI_{amil} after 45 min ($n = 32-37$; $**p < 0.01$; $***p < 0.001$ by t -test), while female FDLE cells were not significantly affected by acute EGF application. **(b)** EGF acutely increased $I_{amil-insens}$ in male FDLE cells ($*p < 0.05$), while R_{te} **(c)** was not affected within the experimental time frame. **(d)** EGF significantly increased AKT phosphorylation within 15 min only in female FDLE cells ($n = 3$; $*p < 0.05$ by t -test). **(e)** In contrast, EGF significantly increased ERK1/2 phosphorylation within 15 min in both, male and female FDLE cells ($n = 3$; $***p < 0.001$ by t -test). **(f)** Short-term EGF exposure decreased EGFR protein expression ($n = 10/9$; $*p < 0.05$; $***p < 0.001$ by t -test). (Grey boxes) male; (yellow boxes) female.

Effects of EGF in combination with female sex steroids. Sex steroid, especially female sex steroids, have been previously shown by us to stimulate Na^+ transport in FDLE cells^{15,29}. Whether this stimulation is affected by the presence of EGF was analyzed in male and female FDLE cells treated with E2/P and EGF, alone or in combination. Female sex steroids prevented the reducing effect of EGF on Na^+ transport. EGF significantly decreased ΔI_{amil} in male FDLE cells, which was reversed by the addition of E2/P ($p < 0.05$; $p < 0.01$; Fig. 10a). Moreover E2/P stimulated ΔI_{amil} in females and prevented the reduction induced by EGF ($p < 0.05$; $p < 0.01$). EGF significantly increased $I_{amil-insens}$ in both male and female FDLE cells, which persisted in the presence of E2/P ($p < 0.05$; $p < 0.001$; Fig. 10b). EGF significantly increased R_{te} as shown above, while E2/P reduced R_{te} in male and female FDLE cells. EGF prevented this decrease of R_{te} induced by E2/P in both male and female FDLE cells ($p < 0.01$; $p < 0.001$; Fig. 10c). In conclusion, the presence of female sex steroids alters the response to EGF, independent of the cells' sex. Notably, the concentration of female sex steroids in utero is equal for both sexes, as they are produced by the maternal placenta. Therefore, a sex specific effect of EGF in combination with female sex steroids is unlikely, at least for the fetal situation.

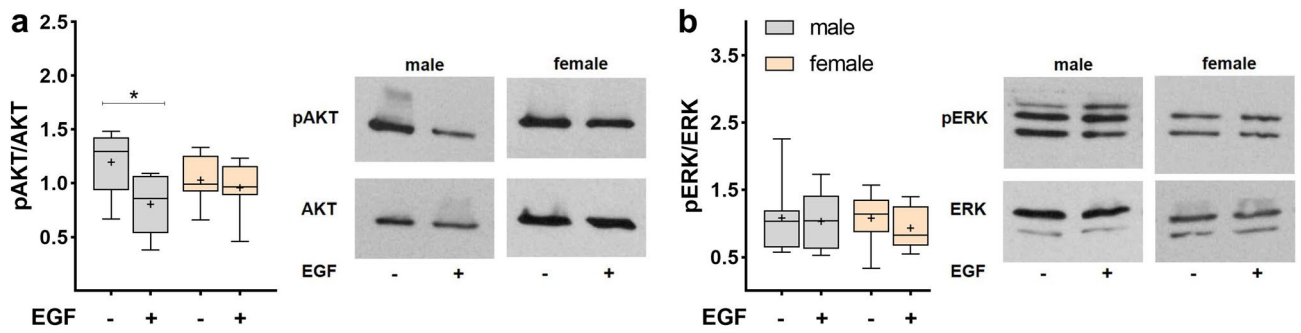


Figure 6. Effect of chronic EGF exposure on AKT-ERK1/2 phosphorylation. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). FDLE cells were cultured in SF-Med and stimulated with EGF for 24 h. (a) EGF reduced AKT phosphorylation in male FDLE cells ($n=8$; $*p<0.05$ by t -test). (b) In contrast, chronic EGF exposure had no significant effect on ERK1/2 phosphorylation ($n=8$).

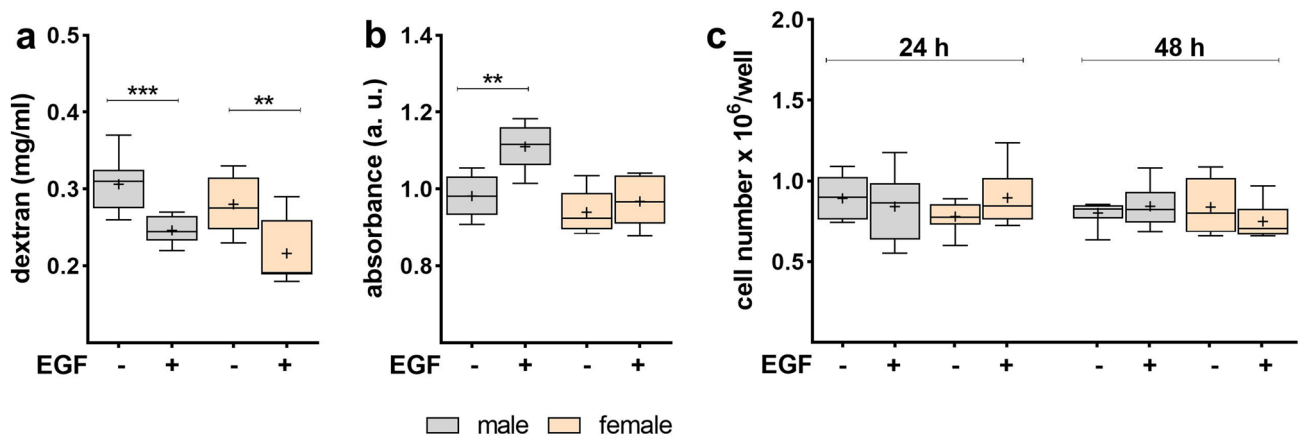


Figure 7. EGF decreased permeability in both sexes, but increased metabolic activity only in males. FDLE cells were cultured in SF-Med and stimulated with EGF for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). (a) FITC-dextran assay demonstrated a significantly reduced permeability induced by EGF in male and female FDLE cells ($n=8$; $**p<0.01$; $***p<0.001$ by t -test). (b) EGF further increased the metabolic activity in male FDLE cells, in contrast to female cells, as shown with a MTT assay ($n=6$; $**p<0.01$ by t -test). (c) The cell number was not altered by EGF addition in male and female FDLE cells after 24 and 48 h ($n=6-8$). (Grey boxes) male; (yellow boxes) female.

Response of sex-specific FDLE cells to other ErbB ligands. Finally, other ErbB receptor ligands were analyzed in sex-specific FDLE cells. TGF- α exerted no effect on I_{base} in male and female cells (Fig. 11a, b), which is in contrast to EGF as detailed above. However, ΔI_{amil} was significantly decreased and $I_{amil-insens}$ strongly increased by TGF- α in both sexes ($p<0.05$; $p<0.001$; Fig. 11a, b). We assume that the increased $I_{amil-insens}$ together with the small decrease of ΔI_{amil} counterbalanced total I_{SC} and thus resulted in no net difference of I_{base} . In accordance to EGF, TGF- α significantly increased R_{te} ($p<0.05$; Fig. 11c). Thus, both EGFR (ErbB1) ligands, EGF and TGF- α , equally affect Na^+ and Cl^- transport, and barrier function in a sex-independent manner, even though not to the same degree. HB-EGF increased $I_{amil-insens}$ and R_{te} in males, with a similar trend in females ($p<0.05$; Fig. 11a, c), while I_{base} and ΔI_{amil} were not affected, neither in male nor in female FDLE cells (Fig. 11a–c). ErbB2 ligand NRG1 further increased $I_{amil-insens}$ in male and female cells, while ΔI_{amil} was reduced only in females ($p<0.05$; $p<0.001$; Fig. 11d). Barrier function with regard to R_{te} was not affected by NRG1 (Fig. 11e). In mixed FDLE cells, NRG1 further significantly increased ΔI_{forsk} and ΔI_{CFTR} ($p<0.01$; Fig. 11f). NRG1 therefore shares the Cl^- transport stimulating properties of EGF, which were sex-independent.

Discussion

We aimed to determine whether EGF, a prominent mitogen during fetal lung development, exerts sex-specific effects on epithelial Na^+ transport in fetal alveolar cells. EGF strongly reduced the ENaC subunit mRNA levels in both male and female FDLE cells. This was corroborated by a markedly reduced Na^+ transport and ENaC activity, while amiloride-insensitive pathways as well as the barrier function (R_{te}) were raised by EGF. Notably, CFTR-mediated Cl^- transport was elevated only in female cells, while CFTR mRNA levels were increased in male and female cells. The effects of EGF were reproduced with other ligands of the EGFR (TGF- α), ErbB3 (NRG1)

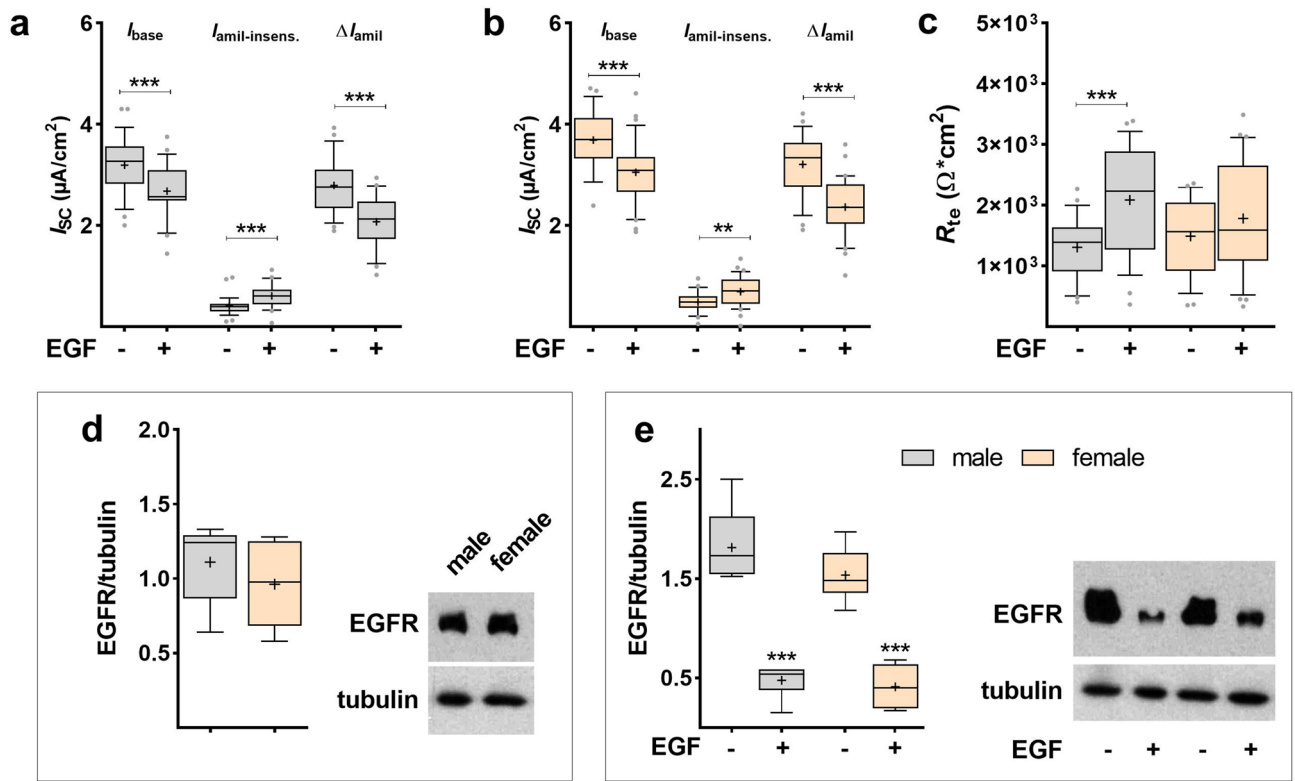


Figure 8. EGF decreased Na^+ transport in epithelial-fibroblast co-cultures of both sexes. FDLE cells were co-cultured with fetal lung fibroblasts in SF-Med and stimulated with EGF for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). In male (a) and female (b) FDLE cells, EGF significantly decreased I_{base} and ΔI_{amil} and increased $I_{amil-insens.}$ ($n = 26-32$; $**p < 0.01$; $***p < 0.001$ by t -test). (c) EGF significantly increased R_{te} only in male cells ($***p < 0.001$). (d) EGFR protein expression was not different between male and female fetal lung fibroblasts cultured in serum-supplemented medium ($n = 6$). (e) EGF application to fetal lung fibroblasts cultured in SF-Med strongly decreased EGFR protein levels after 24 h in both, male and female fibroblasts ($n = 4$; $***p < 0.001$ by t -test). (Grey boxes) male; (yellow boxes) female.

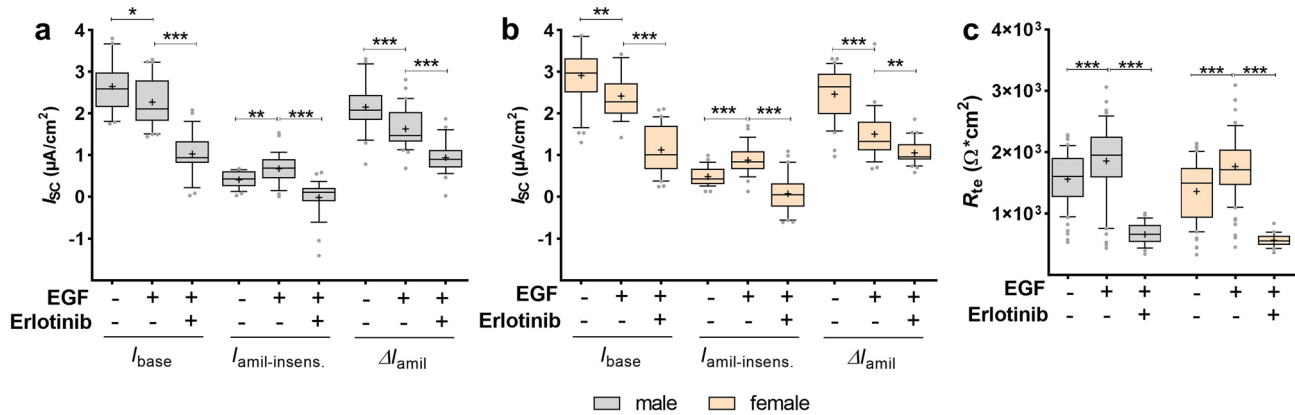


Figure 9. EGFR inhibitor prevented the effect of EGF on $I_{amil-insens.}$ and R_{te} , but not on Na^+ transport. FDLE cells were cultured in SF-Med and stimulated with EGF with or without erlotinib for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). EGF significantly decreased I_{base} and ΔI_{amil} in both, male (a) and female (b) FDLE cells, which was further decreased by erlotinib ($n = 26-31$; $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by t -test). In contrast, erlotinib prevented the significantly increased $I_{amil-insens.}$ induced by EGF in both sexes. (c) Erlotinib further prevented the significant increase of R_{te} induced by EGF in both sexes ($***p < 0.001$). (Grey boxes) male; (yellow boxes) female.

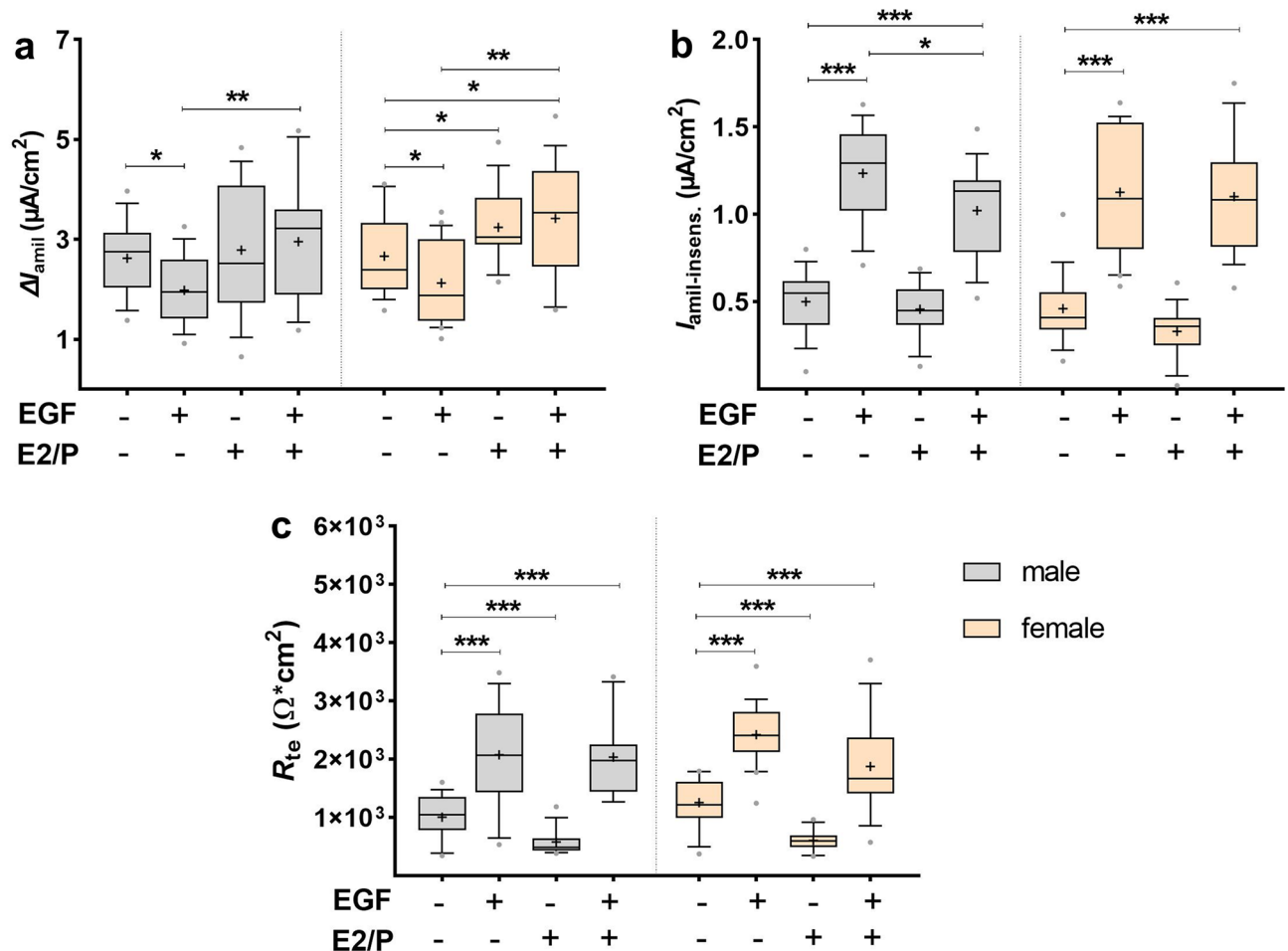


Figure 10. Female sex steroids prevented the reducing effect of EGF on Na⁺ transport. FDLE cells were cultured in SF-Med and stimulated with EGF with or without female sex steroids (E2/P). Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). (a) EGF significantly decreased ΔI_{amil} in male FDLE cells, which was reversed by the addition of E2/P (n = 16–18; * $p < 0.05$; ** $p < 0.01$ by *t*-test). Moreover E2/P stimulated ΔI_{amil} in females and prevented the reduction induced by EGF (n = 15–18; * $p < 0.05$; ** $p < 0.01$). (b) EGF significantly increased $I_{amil-insens}$ in both male and female FDLE cells, which persisted in the presence of E2/P (* $p < 0.05$; *** $p < 0.001$). (c) EGF significantly increased R_{te} , while E2/P reduced R_{te} in male and female FDLE cells. In addition, EGF prevented the decrease of R_{te} induced by E2/P (** $p < 0.01$; *** $p < 0.001$). (Grey boxes) male; (yellow boxes) female.

and ErbB4 (HB-EGF, NRG1), diminishing Na⁺ transport in FDLE cells. In contrast to chronic effects of EGF after 24 h, acute effects of EGF were sex-specific, because Na⁺ transport was reduced only in males. Furthermore, pAKT was elevated only in female cells in response to acute EGF addition, while pERK1/2 was increased in both male and female cells. The study thus showed certain sex- and time-dependent effects on epithelial ion transport induced by EGF, while other analyzed aspects were similar between male and female FDLE cells.

Prior studies reported contradictory results about the effect of EGF on epithelial Na⁺ transport. In alveolar epithelial cells derived from adult male rats EGF was shown to increase net Na⁺ flux and the activity of the Na₂K-ATPase via basolaterally located EGFR³². The elevated Na⁺ transport was attributed to an increased Na₂K-ATPase- α_1 and - β_1 mRNA and protein expression³³. Furthermore, in adult rat ATII cells EGF treatment increased the whole-cell conductance and density of ENaC channels, whereas mRNA expression of all three ENaC subunits was decreased³⁴. In contrast, the majority of studies observed a negative effect of EGF on Na⁺ transport. EGF markedly and quickly decreased ENaC activity by lowering its open probability, paralleled by a reduction of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP₂) levels in ENaC-reconstituted CHO cells³⁵. Anionic phospholipids such as PIP₂ and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) were shown to regulate ENaC and the stimulation of PLC by EGFR activation decreased PIP₂ and consequently ENaC activity^{35–37}. EGF rapidly diminished amiloride-sensitive Na⁺ currents in perfused rabbit and mouse collecting tubules^{38,39}, which was attributed to a rapid phosphorylation of ERK1/2. EGF further raised basal intracellular Ca²⁺ levels after 24 h and reduced ENaC function by a Ca²⁺-mediated process that affected trafficking and surface expression of ENaC in a human nasal airway epithelial cell line derived from a CF patient²⁵. These contrasting results may suggest cell and/or tissue specific EGF-dependent regulation of ENaC activity. It is proposed that EGF increases Na⁺ absorption in the airways and the intestine, whereas it supposedly reduces Na⁺ transport in CCD cells, CF cells,

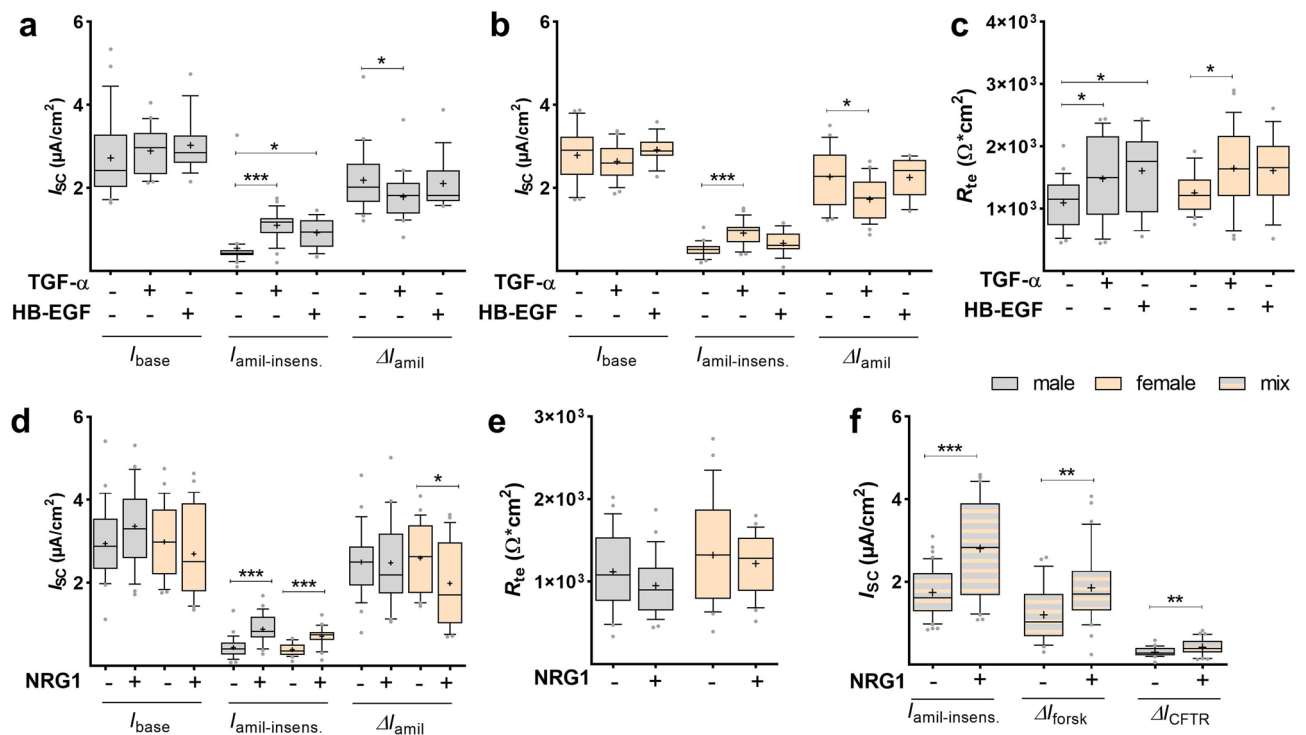


Figure 11. ErbB ligands equally increased amiloride-insensitive I_{SC} in both sexes. FDLE cells were cultured in SF-Med and stimulated with TGF- α , HB-EGF or NRG1 for 24 h. Data are displayed as box and whiskers with the 10–90 percentile, mean (+) and median (horizontal line). TGF- α increased $I_{amil-insens.}$ and decreased ΔI_{amil} in male (a) and female (b) FDLE cells ($n=23-38$; $***p < 0.001$ by t -test). HB-EGF increased $I_{amil-insens.}$ in male cells ($n=15-23$; $*p < 0.05$). (c) TGF- α increased R_{te} in male and female cells, and HB-EGF increased R_{te} in male cells, with the same trend observed in females ($*p < 0.05$). (d) NRG1 increased $I_{amil-insens.}$ in male and female cells, while NRG1 decreased ΔI_{amil} in female FDLE cells ($n=25-27$; $*p < 0.05$; $***p < 0.001$). (e) R_{te} was not affected by NRG1 in male and female cells. (f) NRG1 increased $I_{amil-insens.}$, ΔI_{forsk} and ΔI_{CFTR} in mixed FDLE cells ($n=39-43$; $**p < 0.01$; $***p < 0.001$). (Grey boxes) male; (yellow boxes) female; (white square) mix.

CHO cells overexpressing ENaC and EGFR, and other kidney-derived cells⁴⁰. In contrast to this, we observed an antagonizing effect of EGF on Na^+ transport in alveolar epithelial cells of fetal origin, which suggests that EGF effects also depend on the developmental stage of the cells. Furthermore, chronic incubation with EGF reduced Na^+ transport in both male and female cells, while short-term incubation reduced ENaC activity only in males. To our knowledge, this is the first study demonstrating a sex-specific effect of EGF on Na^+ transport. Different modes of EGF-dependent modulation of ENaC-mediated Na^+ absorption have been proposed. EGF caused a transient increase in Na^+ transport in A6 cells, which was PI3-K dependent, followed by a chronic reduction of ENaC membrane abundance mediated through MAPK1/2^{40,41}. Inhibition of MEK1/2, thereby preventing ERK1/2 phosphorylation, stimulated the acute increase and abolished the chronic decrease of Na^+ transport in response to EGF⁴⁰. An increased pERK1/2 was demonstrated in male and female cells after short-term EGF exposure, whereas pAKT was significantly increased only in females. While activation of PI3-K/AKT signaling has been associated with enhanced ENaC activity, ERK1/2 signaling usually results in a decreased ENaC function. We suggest that the acutely increased pAKT counterbalances the diminishing effect of induced ERK1/2 signaling, resulting in no net changes of Na^+ transport in females, whereas this compensatory effect is lacking in males leading to a decreased Na^+ transport. Unlike the acute effects, chronic EGF application resulted in a reduction of pAKT in males, while no difference was observed for pERK1/2 in male and female FDLE cells after 24 h.

Importantly, EGF strongly stimulated the amiloride-insensitive fraction of epithelial ion transport. It has been previously shown that FDLE cells exhibit different amiloride-insensitive pathways, including secretion of Cl^- and HCO_3^- ^{28,42}. Therein the presence of cAMP-activated Cl^- and HCO_3^- secretion across rat FDLE cells was demonstrated, which was mediated via CFTR⁴². Raising intracellular cAMP levels by forskolin after inhibition of ENaC activity with amiloride increased I_{SC} in female FDLE cells. Furthermore, CFTR activity was strongly increased by EGF, but only in females. Therefore, EGF increased amiloride-insensitive pathways in both sexes, but this increase was attributable to an enhanced CFTR activity only in female FDLE cells. In contrast to this, CFTR mRNA expression was elevated by EGF in both male and female FDLE cells. Short-term exposure to EGF elevated amiloride-insensitive currents only in males. The effects of EGF on Na^+ transport and barrier function were normalized by removing EGF from the culture medium for 24 h, while the effect on amiloride-insensitive pathways persisted. In conclusion, EGF affects Na^+ transport and amiloride-insensitive pathways in both sexes, but with varying magnitude and time specifics. Only few studies dealt with a relationship between EGF and epithelial Cl^- secretion. In T84 cells acute EGF chronically increased I_{SC} responses to a broad range of

secretagogues, and bumetanide, an inhibitor of the Na,K,2Cl-cotransporter, abolished the effect of EGF, suggesting an enhanced Cl⁻ secretion⁴³. In addition, Cl⁻ secretion was enhanced by EGF through upregulation of the Ca²⁺-dependent Cl⁻ channel, transmembrane protein 16A (TMEM16A), in the apical membrane of colonic cells⁴⁴. Another group demonstrated that EGF inhibited Ca²⁺-dependent Cl⁻ secretion via stimulation of the PI3-K through recruitment of the regulatory PI3-K subunit p85 to ErbB2 in colonic epithelial cells⁴⁵. Thus, regulation of epithelial Na⁺ transport and Cl⁻ secretion by EGF is not consistent between different studies and cell-, sex- and developmental-specific effects have to be considered.

Notably, prolonged EGF application strongly stimulated R_{te} in both male and female cells, suggesting an improved barrier integrity that was confirmed by a permeability assay. Neither metabolic activity nor cell number was affected by EGF, suggesting that the enhanced barrier function is not due to an increased cellular proliferation. Other studies demonstrated that EGF increased R_{te} across adult alveolar epithelial cell monolayers and reduced the tight junction permeability³², possibly by regulating the expression of certain claudins⁴⁶. In addition, EGF has been shown to be an effective intestinal regulator helping to protect intestinal barrier integrity via binding to EGFR and subsequent activation of MAPK/ERK, PI3-K/AKT, PLC- γ /PKC (protein kinase C), and STATS (signal transducers and activators of transcription substrates) signal pathways⁴⁷.

In general, EGFR signaling is associated with lung growth and maturation, and a reduced EGFR expression was observed in hypoplastic fetal lungs^{48,49}. In addition, lower EGF levels in the bronchoalveolar lavage fluid at birth were detected in infants with RDS or bronchopulmonary dysplasia compared with control infants⁵⁰. On the other side, EGFR overactivation has been associated with acute respiratory distress syndrome (ARDS) and fibrosis development^{51,52}. Protein expression of EGFR was higher in female FDLE cells. It has been shown previously that estrogen increases the uterine levels of EGFR⁵³ and that EGFR density is higher in the female fetal rabbit lung, possibly due to a negative impact of androgens⁵⁴. Chronic androgen treatment has been shown to reduce EGFR activity inhibiting surfactant protein gene expression in ATII cells and fetal lungs^{55-57,57}. Serum-free cultivation of FDLE cells abolished the sex difference in EGFR protein expression. Thus, sex differences in EGFR protein expression were affected by the presence of fetal serum in the culture and possibly the fetal circulation. Both, the mRNA and protein expression of EGFR was strongly decreased by EGF in both sexes. This was observed after acute (45 min) and chronic (24 h) EGF treatment, although the extent was more pronounced after prolonged application. Similarly, EGFR protein expression was reduced by EGF in fetal lung fibroblasts, while no sex-specific expression was observed in the latter cell type. It can be assumed that this negative feedback regulation prevents hyperproliferation as shown in preterm baboon lungs with hyaline membrane disease possibly induced by elevated levels of the EGFR ligand TGF- α ⁵⁸.

Studies showed that EGF increased thymidine incorporation and DNA accumulation in fetal lung fibroblasts and ATII cells, and elevated choline incorporation into surfactant phosphatidylcholine²³. EGF positively influenced the epithelial-mesenchymal interaction, and EGFR binding in fetal rat lung fibroblasts peaks on embryonic day 18–19, prior to surfactant surge⁵⁶. EGFR inactivation or EGFR deficiency resulted in ATII cell immaturity apparent from an increased glycogen content and a reduced number of lamellar bodies⁵⁹. EGF further stimulated *Sftpc* expression in normal but not in EGFR-deficient lungs⁶⁰. It is suggested that EGF may elaborate a fibroblast-derived factor that stimulates surfactant synthesis in ATII cells⁶¹. We also demonstrated an enhanced mRNA expression of the surfactant proteins in response to EGF that was observed in male and female FDLE cells, lacking any pronounced sex difference. Previous studies observed sex differences in surfactant synthesis with males lagging behind, although both sexes achieved comparable responses in late gestation²⁴. Our results could thus be explicable by the fact that FDLE cells are isolated from late gestation lungs during the saccular stage, while the surge in surfactant synthesis occurs during the canalicular stage. Furthermore, the prior studies addressed surfactant synthesis and not surfactant protein mRNA expression possibly explaining the differential outcomes.

As noted above, EGF supposedly exerts its effect on lung epithelial cells by affecting the epithelial-mesenchymal relationship. EGF has been shown to increase the proliferation of epithelial and mesenchymal cells in monoculture, and within the embryonic lung, the mesenchyme represents the major source of EGF as a paracrine factor acting on epithelial cells expressing EGFR¹⁷. Thereby epithelial proliferation and differentiation is stimulated. Due to this, we co-cultured FDLE cells with fetal lung fibroblasts in a sex-specific manner and repeated the preceding experiments. Despite the presence of fibroblasts, the effects of EGF on Na⁺ transport were essentially the same as we observed before. The presence of fibroblasts did not affect the response of epithelial Na⁺ transport to EGF, ruling out any indirect stimulating effects in this context.

Inhibition of EGFR with erlotinib prevented only some effects of EGF. Erlotinib inhibited the increase of amiloride-insensitive pathways and barrier function, while the reduction of Na⁺ transport was not affected. It is unclear why erlotinib is unable to prevent the reduced Na⁺ transport. However, the effects of erlotinib were not sex-specific. Erlotinib is a reversible inhibitor of EGFR that also exerts inhibitory activity on purified ErbB2⁶². The inhibitory effect of erlotinib in ErbB2-expressing cells is suggested to arise at least in part through direct interaction with ErbB2 rather than indirectly through a process, that requires the presence of EGFR. Since ligandless ErbB2 also dimerizes with ErbB3 or ErbB4 and thereby exerts diverging biological actions, it might be conceivable that some EGF effects were affected by erlotinib while others differed.

Finally, female sex steroids prevented the reducing effect of EGF on Na⁺ transport in both male and female FDLE cells, while the effects of EGF on barrier function and Cl⁻ transport persisted in the presence of female sex steroids. Since the fetal concentration of female sex steroids is equal for both sexes, as they are provided by the maternal placenta, no sex-specific effect of EGF in combination with female sex steroids is assumed. Concerning male sex steroids, no androgen receptor-specific mRNA expression was detected in male or female FDLE cells¹⁵. Thus, FDLE do not express androgen receptors during the saccular stage at which cell isolation was performed. Furthermore, flutamide, an androgen receptor antagonist showed no effect on Na⁺ transport in FDLE cells¹⁵. Together, the inability of flutamide to affect Na⁺ transport and the undetectable androgen receptor expression suggest a negligible impact of androgens on Na⁺ transport in FDLE cells.

Conclusions

EGF strongly reduced the ENaC mRNA expression and channel activity in both male and female FDLE cells. In contrast, amiloride-insensitive pathways as well as the barrier function were raised by EGF. While chronic effects were not sex-specific, short-term effects were limited to males. In conclusion, we showed certain sex- and time-dependent differences in epithelial ion transport induced by EGF, while other analyzed aspects were similar between male and female FDLE cells. From the results obtained in this study it cannot be assumed that EGF is responsible for the sex specific differences in Na⁺ transport observed in FDLE cells.

Data availability

All data generated or analyzed during this study are either included in this published article or can be obtained from the corresponding author on reasonable request.

Received: 16 December 2020; Accepted: 18 July 2021

Published online: 05 August 2021

References

1. Alcorn, D. *et al.* Morphological effects of chronic tracheal ligation and drainage in the fetal lamb lung. *J. Anat.* **123**, 649–660 (1977).
2. Larson, J. E., Delcarpio, J. B., Farberman, M. M., Morrow, S. L. & Cohen, J. C. CFTR modulates lung secretory cell proliferation and differentiation. *Am. J. Physiol. Lung Cell Mol. Physiol.* **279**, L333–L341 (2000).
3. Larson, J. E. & Cohen, J. C. Improvement of pulmonary hypoplasia associated with congenital diaphragmatic hernia by in utero CFTR gene therapy. *Am. J. Physiol. Lung Cell Mol. Physiol.* <https://doi.org/10.1152/ajplung.00372.2005> (2006).
4. Hudak, J. J., Killeen, E., Chandran, A., Cohen, J. C. & Larson, J. E. Adult onset lung disease following transient disruption of fetal stretch-induced differentiation. *Respir. Res.* **10**, 34. <https://doi.org/10.1186/1465-9921-10-34> (2009).
5. Trezise, A. E., Chambers, J. A., Wardle, C. J., Gould, S. & Harris, A. Expression of the cystic fibrosis gene in human foetal tissues. *Hum. Mol. Genet.* **2**, 213–218 (1993).
6. Tizzano, E. F., Chitayat, D. & Buchwald, M. Cell-specific localization of CFTR mRNA shows developmentally regulated expression in human fetal tissues. *Hum. Mol. Genet.* **2**, 219–224 (1993).
7. Canessa, C. M. *et al.* Amiloride-sensitive epithelial Na⁺ channel is made of three homologous subunits. *Nature* **367**, 463–467. <https://doi.org/10.1038/367463a0> (1994).
8. Ridge, K. M. *et al.* Differential expression of Na⁺-ATPase isoforms in rat alveolar epithelial cells. *Am. J. Physiol.* **273**, L246–L255 (1997).
9. Barker, P. M., Gowen, C. W., Lawson, E. E. & Knowles, M. R. Decreased sodium ion absorption across nasal epithelium of very premature infants with respiratory distress syndrome. *J. Pediatr.* **130**, 373–377 (1997).
10. Helve, O., Pitkänen, O., Janér, C. & Andersson, S. Pulmonary fluid balance in the human newborn infant. *Neonatology* **95**, 347–352. <https://doi.org/10.1159/000209300> (2009).
11. Altman, M., Vanpee, M., Cnattingius, S. & Norman, M. Risk factors for acute respiratory morbidity in moderately preterm infants. *Paediatr. Perinat. Epidemiol.* **27**, 172–181. <https://doi.org/10.1111/ppe.12035> (2013).
12. Anadkat, J. S., Kuzniewicz, M. W., Chaudhari, B. P., Cole, F. S. & Hamvas, A. Increased risk for respiratory distress among white, male, late preterm and term infants. *J. Perinatol.* **32**, 780–785. <https://doi.org/10.1038/jp.2011.191> (2012).
13. Stevenson, D. K. *et al.* Sex differences in outcomes of very low birthweight infants: The newborn male disadvantage. *Arch. Dis. Child Fetal Neonatal. Ed.* **83**, F182–F185 (2000).
14. Kaltofen, T., Haase, M., Thome, U. H. & Laube, M. Male sex is associated with a reduced alveolar epithelial sodium transport. *PLoS One* **10**, e0136178. <https://doi.org/10.1371/journal.pone.0136178> (2015).
15. Haase, M., Laube, M. & Thome, U. H. Sex-specific effects of sex steroids on alveolar epithelial Na⁺ transport. *Am. J. Physiol. Lung Cell Mol. Physiol.* **312**, L405–L414. <https://doi.org/10.1152/ajplung.00275.2016> (2017).
16. Laube, M., Riedel, D., Ackermann, B., Haase, M. & Thome, U. H. Glucocorticoids Equally Stimulate Epithelial Na⁺ Transport in Male and Female Fetal Alveolar Cells. *Int. J. Mol. Sci.* **21**; <https://doi.org/10.3390/ijms21010057> (2019).
17. Schuger, L., Varani, J., Mitra, R. & Gilbride, K. Retinoic acid stimulates mouse lung development by a mechanism involving epithelial–mesenchymal interaction and regulation of epidermal growth factor receptors. *Dev. Biol.* **159**, 462–473. <https://doi.org/10.1006/dbio.1993.1256> (1993).
18. Kaplan, F. Molecular determinants of fetal lung organogenesis. *Mol. Genet. Metab.* **71**, 321–341. <https://doi.org/10.1006/mgme.2000.3040> (2000).
19. Bourbon, J. R. Gene expression in alveolar development. In *Gaultier C., Bourbon J.R., Post M. (eds) Lung Development. Clinical Physiology Series.* (Springer, New York, NY, 1999).
20. Yarden, Y. & Sliwkowski, M. X. Untangling the ErbB signalling network. *Nat. Rev. Mol. Cell. Biol.* **2**, 127–137. <https://doi.org/10.1038/35052073> (2001).
21. Roskoski, R. The ErbB/HER family of protein-tyrosine kinases and cancer. *Pharm. Res.* **79**, 34–74. <https://doi.org/10.1016/j.phrs.2013.11.002> (2014).
22. Nielsen, H. C. Epidermal growth factor influences the developmental clock regulating maturation of the fetal lung fibroblast. *Biochim. Biophys. Acta* **1012**, 201–206 (1989).
23. Fraslon, C. & Bourbon, J. R. Comparison of effects of epidermal and insulin-like growth factors, gastrin releasing peptide and retinoic acid on fetal lung cell growth and maturation in vitro. *Biochim. Biophys. Acta* **1123**, 65–75 (1992).
24. Klein, J. M. & Nielsen, H. C. Sex-specific differences in rabbit fetal lung maturation in response to epidermal growth factor. *Biochim. Biophys. Acta* **1133**, 121–126 (1992).
25. Cao, L., Owsianik, G., Becq, F. & Nilius, B. Chronic exposure to EGF affects trafficking and function of ENaC channel in cystic fibrosis cells. *Biochem. Biophys. Res. Commun.* **331**, 503–511. <https://doi.org/10.1016/j.bbrc.2005.03.201> (2005).
26. Falin, R. A. & Cotton, C. U. Acute downregulation of ENaC by EGF involves the PY motif and putative ERK phosphorylation site. *J. Gen. Physiol.* **130**, 313–328. <https://doi.org/10.1085/jgp.200709775> (2007).
27. Jassal, D., Han, R. N., Caniggia, I., Post, M. & Tanswell, A. K. Growth of distal fetal rat lung epithelial cells in a defined serum-free medium. *Vitro Cell Dev. Biol.* **27**, 625–632 (1991).
28. Thome, U. H., Davis, I. C., Nguyen, S. V., Shelton, B. J. & Matalon, S. Modulation of sodium transport in fetal alveolar epithelial cells by oxygen and corticosterone. *Am. J. Physiol. Lung Cell Mol. Physiol.* **284**, L376–L385. <https://doi.org/10.1152/ajplung.00218.2002> (2003).
29. Laube, M., Küppers, E. & Thome, U. H. Modulation of sodium transport in alveolar epithelial cells by estradiol and progesterone. *Pediatr. Res.* **69**, 200–205. <https://doi.org/10.1203/PDR.0b013e3182070ec8> (2011).
30. Bossmann, M., Ackermann, B. W., Thome, U. H. & Laube, M. Signaling cascade involved in rapid stimulation of cystic fibrosis transmembrane conductance regulator (CFTR) by dexamethasone. *Int. J. Mol. Sci.* <https://doi.org/10.3390/ijms18081807> (2017).

31. Obendorf, J., Fabian, C., Thome, U. H. & Laube, M. Paracrine stimulation of perinatal lung functional and structural maturation by mesenchymal stem cells. *Stem Cell Res. Ther.* <https://doi.org/10.1186/s13287-020-02028-4> (2020).
32. Borok, Z. *et al.* Effects of EGF on alveolar epithelial junctional permeability and active sodium transport. *Am. J. Physiol.* **270**, L559–L565 (1996).
33. Danto, S. I. *et al.* Mechanisms of EGF-induced stimulation of sodium reabsorption by alveolar epithelial cells. *Am. J. Physiol.* **275**, C82–92 (1998).
34. Kemp, P. J. *et al.* Epidermal growth factor regulation in adult rat alveolar type II cells of amiloride-sensitive cation channels. *Am. J. Physiol. Cell Physiol.* **277**, C1058–C1065. <https://doi.org/10.1152/ajpcell.1999.277.6.C1058> (1999).
35. Tong, Q. & Stockand, J. D. Receptor tyrosine kinases mediate epithelial Na⁺ channel inhibition by epidermal growth factor. *Am. J. Physiol. Renal Physiol.* **288**, F150–F161. <https://doi.org/10.1152/ajprenal.00261.2004> (2005).
36. Ma, H.-P. & Eaton, D. C. Acute regulation of epithelial sodium channel by anionic phospholipids. *J. Am. Soc. Nephrol.* **16**, 3182–3187. <https://doi.org/10.1681/ASN.2005040434> (2005).
37. Ma, H.-P., Saxena, S. & Warnock, D. G. Anionic phospholipids regulate native and expressed epithelial sodium channel (ENaC). *J. Biol. Chem.* **277**, 7641–7644. <https://doi.org/10.1074/jbc.C100737200> (2002).
38. Vehaskari, V. M., Hering-Smith, K. S., Moskowitz, D. W., Weiner, I. D. & Hamm, L. L. Effect of epidermal growth factor on sodium transport in the cortical collecting tubule. *Am. J. Physiol. Renal Physiol.* **256**, F803–F809 (1989).
39. Shen, J.-P. & Cotton, C. U. Epidermal growth factor inhibits amiloride-sensitive sodium absorption in renal collecting duct cells. *Am. J. Physiol. Renal Physiol.* **284**, F57–F64. <https://doi.org/10.1152/ajprenal.00028.2002> (2003).
40. Zheleznova, N. N., Wilson, P. D. & Staruschenko, A. Epidermal growth factor-mediated proliferation and sodium transport in normal and PKD epithelial cells. *Biochim. Biophys. Acta* **1812**, 1301–1313. <https://doi.org/10.1016/j.bbadis.2010.10.004> (2011).
41. Liu, L., Duke, B. J., Malik, B., Yue, Q. & Eaton, D. C. Biphasic regulation of ENaC by TGF- α and EGF in renal epithelial cells. *Am. J. Physiol. Renal Physiol.* **296**, F1417–F1427. <https://doi.org/10.1152/ajprenal.90337.2008> (2009).
42. Lazrak, A. *et al.* cAMP regulation of Cl⁻ and HCO₃⁻(3) secretion across rat fetal distal lung epithelial cells. *Am. J. Physiol. Lung Cell Mol. Physiol.* **282**, L650–L658. <https://doi.org/10.1152/ajplung.00370.2001> (2002).
43. O'Mahony, F., Toumi, F., Mroz, M. S., Ferguson, G. & Keely, S. J. Induction of Na⁺/K⁺/2Cl⁻ cotransporter expression mediates chronic potentiation of intestinal epithelial Cl⁻ secretion by EGF. *Am. J. Physiol. Cell Physiol.* **294**, C1362–C1370. <https://doi.org/10.1152/ajpcell.00256.2007> (2008).
44. Mroz, M. S. & Keely, S. J. Epidermal growth factor chronically upregulates Ca(2+)-dependent Cl⁻ conductance and TMEM16A expression in intestinal epithelial cells. *J. Physiol.* **590**, 1907–1920. <https://doi.org/10.1113/jphysiol.2011.226126> (2012).
45. Keely, S. J. & Barrett, K. E. ErbB2 and ErbB3 receptors mediate inhibition of calcium-dependent chloride secretion in colonic epithelial cells. *J. Biol. Chem.* **274**, 33449–33454 (1999).
46. Chen, S. P. *et al.* Effects of transdifferentiation and EGF on claudin isoform expression in alveolar epithelial cells. *J. Appl. Physiol.* **98**, 322–328. <https://doi.org/10.1152/jappphysiol.00681.2004> (2005).
47. Tang, X. *et al.* Epidermal growth factor and intestinal barrier function. *Mediators Inflamm.* **2016**, 1927348. <https://doi.org/10.1155/2016/1927348> (2016).
48. Chinoy, M. R., Chi, X. & Cilley, R. E. Down-regulation of regulatory proteins for differentiation and proliferation in murine fetal hypoplastic lungs: Altered mesenchymal-epithelial interactions. *Pediatr. Pulmonol.* **32**, 129–141 (2001).
49. Yoshimura, S. *et al.* Effect of epidermal growth factor on lung growth in experimental fetal pulmonary hypoplasia. *Early Hum. Dev.* **57**, 61–69 (2000).
50. Currie, A. E., Vyas, J. R., MacDonald, J., Field, D. & Kotecha, S. Epidermal growth factor in the lungs of infants developing chronic lung disease. *Eur. Respir. J.* **18**, 796–800. <https://doi.org/10.1183/09031936.01.00088201> (2001).
51. Nakayama, I. *et al.* Regulation of epidermal growth factor receptor expression and morphology of lung epithelial cells by interleukin-1 β . *J. Biochem.* <https://doi.org/10.1093/jb/mvaa015> (2020).
52. Plantier, L. *et al.* Ectopic respiratory epithelial cell differentiation in bronchiolised distal airspaces in idiopathic pulmonary fibrosis. *Thorax* **66**, 651–657. <https://doi.org/10.1136/thx.2010.151555> (2011).
53. Couse, J. F. & Korach, K. S. Estrogen receptor null mice: What have we learned and where will they lead us?. *Endocr. Rev.* **20**, 358–417. <https://doi.org/10.1210/edrv.20.3.0370> (1999).
54. Klein, J. M. & Nielsen, H. C. Androgen regulation of epidermal growth factor receptor binding activity during fetal rabbit lung development. *J. Clin. Investig.* **91**, 425–431. <https://doi.org/10.1172/JCI116218> (1993).
55. Carey, M. A. *et al.* The impact of sex and sex hormones on lung physiology and disease: Lessons from animal studies. *Am. J. Physiol. Lung Cell Mol. Physiol.* **293**, L272–L278. <https://doi.org/10.1152/ajplung.00174.2007> (2007).
56. Dammann, C. E. & Nielsen, H. C. Regulation of the epidermal growth factor receptor in fetal rat lung fibroblasts during late gestation. *Endocrinology* **139**, 1671–1677. <https://doi.org/10.1210/endo.139.4.5934> (1998).
57. Dammann, C. E., Ramadurai, S. M., McCants, D. D., Pham, L. D. & Nielsen, H. C. Androgen regulation of signaling pathways in late fetal mouse lung development. *Endocrinology* **141**, 2923–2929 (2000).
58. Mino, P. & King, R. J. Epithelial–mesenchymal interactions in lung development. *Annu. Rev. Physiol.* **56**, 13–45. <https://doi.org/10.1146/annurev.ph.56.030194.000305> (1994).
59. Kumar, V. H., Lakshminrusimha, S., El Abiad, M. T., Chess, P. R. & Ryan, R. M. Growth factors in lung development. *Adv. Clin. Chem.* **40**, 261–316 (2005).
60. Miettinen, P. J. *et al.* Impaired lung branching morphogenesis in the absence of functional EGF receptor. *Dev. Biol.* **186**, 224–236. <https://doi.org/10.1006/dbio.1997.8593> (1997).
61. Sen, N. & Cake, M. H. Enhancement of disaturated phosphatidylcholine synthesis by epidermal growth factor in cultured fetal lung cells involves a fibroblast-epithelial cell interaction. *Am. J. Respir. Cell Mol. Biol.* **5**, 337–343. <https://doi.org/10.1165/ajrcmb/5.4.337> (1991).
62. Schaefer, G., Shao, L., Totpal, K. & Akita, R. W. Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. *Cancer Res.* **67**, 1228–1238. <https://doi.org/10.1158/0008-5472.CAN-06-3493> (2007).

Acknowledgements

We wish to thank Jessica Schneider for excellent technical assistance. We acknowledge support from Leipzig University for Open Access Publishing. The study was funded by a research grant from the Roland Ernst Stiftung für Gesundheitswesen to ML and UHT. The foundation had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Author contributions

Conceptualization, M.L.; Methodology, M.L.; Validation, M.L., D.D., F.W.; Formal Analysis, M.L., D.D., F.W.; Investigation, M.L., D.D., F.W.; Resources, M.L.; Writing—Original Draft Preparation, M.L.; Writing—Review and Editing, M.L., D.D., F.W. and U.H.T.; Visualization, M.L.; Supervision, M.L.; Project Administration, M.L. and U.H.T. The authors read and approved the final manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-021-95410-y>.

Correspondence and requests for materials should be addressed to M.L.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2021