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Estrogen-induced upregulation of Sftpb requires transcriptional control of neuregulin receptor ErbB4 in mouse lung type II epithelial cells ☆

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ABSTRACT

Estrogen is known for its positive stimulatory effects on surfactant proteins. ErbB4 receptor and its ligand neuregulin (NRG) positively stimulate lung development. ErbB receptors interact with nuclear receptors and ErbB4 co-regulates estrogen receptor (ER)α expression in breast cells. ERβ is highly expressed in pneumocytes and its deletion leads to fewer alveoli and reduced elastic recoil. A similar picture was seen in ErbB4-deleted lungs. We hypothesized that estrogen signals its effect on surfactant protein B (Sftpb) expression through interactions of ERβ and ErbB4. Estrogen and NRG treatment decreased cell numbers and stimulated Sftpb expression in type II cells. Estrogen and NRG both stimulated phosphorylation of ERβ and co-localization of both receptors. Overexpression of ERβ increased the cell number and Sftpb expression, which was further augmented by estrogen and NRG. Finally, estrogen and NRG stimulated ERβ and ErbB4 binding to the *Sftpb* promoter. Overexpression of these receptors stimulated *Sftpb* promoter activation, which was further enhanced by estrogen and NRG. The stimulatory effect of estrogen and NRG was abolished in ErbB4 deletion and reconstituted by re-expression of full-length ErbB4 in fetal ErbB4-deleted type II cells. Estrogen-induced nuclear translocation of ErbB4 required the intact γ-secretase cleavage site but not the nuclear localization sequence of the ErbB4 receptor, suggesting that ERβ might function as a nuclear chaperone for ErbB4. These studies demonstrate that estrogen effects on Sftpb expression require an interaction of ERβ and ErbB4. We speculate that the stimulatory effects of estrogen on Sftpb are under transcriptional control of ErbB4.

1. Introduction

Preterm infants are at high risk for developing "respiratory distress syndrome" (RDS) as a result of insufficient surfactant production [1,2]. The delayed onset of fetal surfactant synthesis [3] and the resulting increased risk for the development of RDS [4] in the male fetus are well known. Higher intrauterine androgen levels and interactions with transforming growth factor β receptor signaling pathways have been implicated in the mechanism of the sex-specific delay in lung maturation [5]. Female sex is associated with a lower incidence of RDS in premature infants [6]. The fetus is exposed to increased levels of estrogen and progesterone [7]. Despite this fact, replacement of these placental hormones does not decrease the incidence of bronchopulmonary dysplasia [8], the long-term pulmonary morbidity of preterm infants born with immature lungs. Estrogens are steroid hormones and there are two subtypes of estrogen receptors (ERs), ERα and ERβ. They belong to a large superfamily of nuclear receptors and act as ligand-activated transcription factors [9]. The development of multiple mammalian organ systems, including the reproductive system, skeletal, muscle, and brain, are dependent on estrogen [10,11]. ERβ is highly expressed in fetal pneumocytes (types I and II) [12] and deletion of fetal ERβ leads to fewer alveoli and reduced elastic recoil in adult mice [13]. This phenotypical picture is similar to the alveolar simplification and hyperreactive airway system seen in ErbB4-deleted lungs [14]. ErbB4 is the signaling receptor for the growth factor neuregulin (NRG). NRG is secreted by mature fetal lung fibroblasts and plays an important role in the initiation of fetal surfactant synthesis in type II epithelial cells [15]. It acts in a paracrine manner through mesenchymal–epithelial cell communication and signals its effects through formation of ErbB receptor dimer. ErbB4 is the preferred dimerization partner and functions as a ligand-induced co-factor for other transcription factors in fetal type II cells [16]. ErbB4 receptor is known for its involvement in differentiation processes [17–20] and is unique among transmembrane tyrosine kinase receptors. Its intracellular domain (4ICD) is proteolytic cleaved by TNFα-converting enzyme and translocated to the nucleus after being released into the cytosol [21] and processed by presenilin-dependent

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γ-secretase activity [22,23]. 4ICD harbors a PDZ domain recognition site [24] and an intrinsic nuclear localization sequence (NLS), which enables it to be translocated to the nucleus to activate gene expression leading to proliferation, cell cycle arrest, apoptosis, or differentiation. Shuttling of 4ICD between different subcellular compartments may regulate multiple cell functions including life or death decisions in a cell [18]. Since ErbB receptors interact with transcription factors [25] and nuclear receptors [26] and lung phenotypes of deletion of ERβ and ErbB4 are similar, we hypothesized that at least parts of the estrogen effects on lung development are signaled through interaction with ErbB4 signaling pathways. More detailed knowledge of the regulation of signaling pathways involved in lung development will help to create additional treatment options via enhancement of their biologic effects.

2. Materials and methods

2.1. Materials

Rabbit polyclonal ERβ (H-150), rabbit polyclonal IgG ErbB4 (C-18), and normal rabbit IgG were obtained from Santa Cruz Biotechnology (Heidelberg, Germany); mouse monoclonal c-ErbB4 antibody (clone HFR-1) was from Thermo Fisher scientific GmbH (Dreieich, Germany); goat anti-rabbit IgG (HRP-labeled, H + L), goat anti-mouse IgG (HRP-labeled, H + L) and rabbit anti-mouse IgG (H + L) were from Zymed Laboratories Inc (South San Francisco, CA); rabbit anti-sheep Sftpb was from Chemicon Europe (Schwalbach/Ts, Germany); purified mouse anti-phosphotyrosine antibody was from BD Biosciences (Heidelberg, Germany); mouse monoclonal anti-actin clone AC-40, 4,6-Diamidino-2-phenylindole, dilactate (DAPI) and 17-β-estradiol were obtained from Sigma (Hamburg, Germany); Alexa Fluor 488 goat anti-mouse IgG (H + L) and Alexa Fluor 568 anti-rabbit IgG (H + L) were from Molecular Probes (Karlsruhe, Germany). The Nanofectin™ transfection reagent was obtained from PAA-Laboratories (Pasching, A) and cDNA Kit was from Amersham Biotechnologies (Munich, Germany). Total RNA Isolation Reagent (TRIR) was obtained from ABgene (Darmstadt, Germany) and Plasmid Midi Kit from Qiagen (Hilden, Germany). The Dual-Glo Luciferase assay System was from Promega (Mannheim, Germany). TaqMan Universal PCR Master Mix and ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit were from Applied Biosystems (Darmstadt, Germany). ChIP Assay Kit was from USB Corporation (Staufen, Germany). The forward primers (FP), reverse primers (RP) and probes for *Actb*, *Sftpb*[14] and the *Sftpb* promoter were from Eurogentec (Cologne, Germany). Neuregulin 1β producer cells were kindly provided by Dr. Kermit Carraway III. (UC Davis, CA), and the growth factor was purified by the Phoenix Laboratory, Ann Kane (Tufts University, Boston, MA).

2.2. Cells

The mouse lung epithelial cell line (MLE-12) was obtained from American Type Culture Collection (Wesel, Germany). This line was established from pulmonary tumors in a mouse transgenic for the SV40 large T antigen under the control of the promoter region of the human surfactant protein C gene. The cells secrete phospholipids in response to phorbol esters and ATP, but not in response to Forskolin [27]. We used the MLE-12 cell line for all experiments since they express ErbB4, ERβ, and Sftpb similarly to primary fetal type II cells. Using primary cells would have required many time-dated pregnant animals since primary cells are less efficiently transfectable. MLE-12 cells, on the other hand, are easily transfectable and are known for maintaining functional characteristics of distal respiratory epithelial cells, including the expression of surfactant proteins and mRNAs and the ability to secrete phospholipids [27].

2.3. Plasmids

pEGFP N3 (control), pHER4 (full length ErbB4 receptor), pHER4-MuNLS (abrogated nuclear localization sequence), and pHER4V673IMu (abolished γ-secretase processing domain) were kindly provided by Dr. Frank Jones (Tulane University, New Orleans) [25]. YFPERβ was kindly provided by Dr. Elena Korenbaum (Hannover Medical School, Germany) [28]. *Sftpb* promoter luciferase reporter plasmid was kindly provided by Philip L. Ballard (University of California, San Francisco, CA) [29].

2.4. MTT-Assay

Cell number was examined using an MTT-assay. In this colorimetric assay, the yellow MTT is reduced to purple formazan in the mitochondria of living, metabolically active cells. The amount of formazan is directly proportional to the cell number [30]. MLE-12 cells were plated and grown to 50% confluence in a 96-well plate at a concentration of 2.5×10^3 cells per well. After a 3-hour serum starvation, cells were treated for 24 h with serum-free DMEM (controls), 17-β-estradiol (50, 100, 500, 1000, and 1500 pmol/l), or NRG (5 nM, 10 nM 20 nM, 33 nM, 40 nM and 100 nM). Different treatment concentrations were equally mixed from a stock solution to keep the vehicle media content similar. After incubating with MTT-solution for 2 h at 37 °C, the reaction was stopped by Isopropanol–HCl. Medium was removed and 3% SDS and Isopropanol–HCl was added. Absorption was measured at 570 nm.

2.5. RNA isolation and cDNA synthesis

Cells were plated on culture dishes until they reached 90% confluence. After being serum starved for 3 h, cells were treated with serum-free DMEM (controls), 17-β-estradiol (1000 pmol/l), or NRG (33 nM) for 24 h. Total RNA Isolation Reagent (TRIR) was used for cell lysis. RNA was isolated by guanidinium thiocyanate lysis followed by acid phenol/chloroform extraction [31]. After reversed transcription, 5 μg of total RNA was used in a 15 μl reaction volume containing 1× DTT, 0.2 μg Hexamer Primer and 5× Bulk Mix for 1 h. The resulting cDNA was used for real-time amplification reactions.

2.6. Real time polymerase chain reaction (RT-PCR)

The cDNA levels of the *Sftpb* gene and of the *Actb* gene were measured by real-time PCR. Actin was used as internal control to normalize the surfactant protein cDNA levels. We used actin as a housekeeping gene since stimulation with 17-β-estradiol (24.8 ± 0.5, $n = 6$, $p = 0.4$), NRG (24.7 ± 0.4, $n = 6$, $p = 0.3$), or overexpression with ERβ (23.7 ± 0.1, $n = 5$, $p = 0.2$) did not change the *Actb* mRNA expression when compared to control cells (25.5 ± 0.5, $n = 7$) or EGFP-transfected cells (23.4 ± 0.2, $n = 5$), respectively (data not shown). The 20 μl reaction mixture contained 1 μl of the cDNA template, 10 μl TaqMan universal master mix, 300 nm each of forward primer (FP) and reverse primer (RP), and 200 nm probe [14]. For amplification and detection of specific products the ABI PRISM 7900 sequence detection system was used. The amplification protocol consists of an initial denaturation and enzyme activation at 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s, and 60 °C for 1 min. The threshold cycle [32] was determined for each gene. Samples were run in quadruplicates. A semiquantitative method was used to estimate the relative expression level of the surfactant protein genes, which was done by calculating the DCt value, (difference in the Ct values of the target and the reference gene). The Ct (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold. The difference in the Ct values of the treated cells compared to the control cells was presented as –DDCt (DDCt values are inversely proportional to the levels of surfactant protein mRNA). For each assay, specificity was confirmed

by sequencing the PCR products on both strands using capillary electrophoresis with POP-6™-Polymer on an ABI 3100 Genetic Analyzer (Applied Biosystems).

2.7. Co-immunoprecipitation and Western blotting

MLE-12 cells were plated in culture dishes until 90% confluence. Cells were serum starved for 24 h and stimulated with serum-free DMEM (controls), 17-β-estradiol (1000 pmol/l), or NRG (33 nM) for 2 min and lysed in co-immunoprecipitation buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 1% TritonX-100, 10% Glycerol, 1 mM Na_3VO_4, 1 mM NaF, 1 mM $ZnCl_2$, 10 mM β-glycerol phosphate, 5 mM tetrasodium pyrophoshate, 1 mM phenylmethylsulfonylfluoride (PMSF) and 4 mg/ml each of aprotinin, leupeptin and pepstatin). Lysates were cleared by microcentrifugation at 4 °C for 15 min. A protein assay was used to determine total concentration of protein. 300 μg of total protein was incubated for 90 min with the specific receptor antibody (anti ErbB4 C-7, anti ERβ H-150) at 4 °C. Protein-A-sepharose was added and incubation was continued at 4 °C for another 90 min. The beads were collected by microcentrifugation at 14,000 rpm for 10 min, washed with co-immunoprecipitation washing buffer (20 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 1 mM Na_3VO_4, 1 mM NaF, 1 mM $ZnCl_2$, 10 mM β-glycerol phosphate, 5 mM tetrasodium pyrophoshate, 1 mM PMSF and 4 mg/ml each of aprotinin, leupeptin and pepstatin), and boiled in Laemmli buffer for 5 min at 100 °C. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked in 1% BSA and incubated with antibodies against individual proteins (purified mouse anti-phosphotyrosine antibody, anti-ERβ H-150, anti-ErbB4 C-18, anti-Sftpb, or anti-actin) overnight at 4 °C. Secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG, both HRP-labeled) was applied and the proteins were visualized by enhanced chemiluminescence [33]. Membranes were stripped in strip buffer (62.5 mM Tris (pH 6.8), 2% SDS, 0.8% β-mercaptoethanol) for 30 min at 50 °C up to three times. We performed an immunoprecipitation with a control IgG antibody to control for the antibody specificity.

2.8. Confocal microscopy

MLE-12 cells were grown on glass cover slips in 24-well plates for 24 h. After a serum starvation of 3 h, cells were treated with serum-free DMEM (controls) or stimulated for 2 min, 30 min, 1 h, 4 h, 8 h, 12 h, or 24 h with either 17-β-estradiol (1000 pmol/l) or NRG (33 nM). Cells were washed with PBS, fixed for 20 min in 3% paraformaldehyde, rinsed again with PBS, permeabilized in 0.5% TritonX-100/ PBS for 5 min and blocked for 1 h with 10% normal goat serum. After incubating with primary antibody (ErbB4 clone HFR-1, ERβ H-150) for 30 min, cells were washed with PBS and incubated with secondary antibody (Alexa Fluor 488, Alexa Fluor 568) for another 30 min. After staining with DAPI for 10 min, cells were mounted onto glass slides and examined using a Leica Inverted-2 DM IRB confocal laser scanning microscope connected to a TCS SP2 AOBS scanhead (Leica, Wetzlar, Germany).

2.9. Transfection experiments

To examine the effects of overexpression of ERβ and to study *Sftpb* promoter activation, MLE-12 cells were transfected with an ERβ construct (YFPERβ) [28], an ErbB4 construct (pHER4), or an empty control construct (pEGFP N3) [25]. The cells were transfected for 48 h using Nanofectin transfection reagent as described by the manufacturer. Briefly, cells were grown to 50% confluence in 96-well plates (for MTT assay and luciferase assay) or on 6-well plates (for real time PCR). 0.25 μg DNA (96-well plates) or 3 μg DNA (6-well plates) was diluted with the Nanofectin solution and incubated for 30 min before it was added drop-wise into the serum containing medium. To study the effect of estrogen on the ErbB4 nuclear translocation, cells were grown on glass cover slips and transfected with pHER4 (full-length ErbB4 receptor), pHER4MuNLS (defective nuclear localization sequence), or pHER4V673IMu (abolished γ-secreatase processing domain) as described above.

2.10. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was done using a ChIP assay kit as described by the manufacturer. Briefly, MLE-12 cells were cultured until confluence in culture dishes. Cells were serum starved for 3 h and treated with DMEM (control), 17-β-estradiol (1000 pmol/l), or NRG (33 nM) for 24 h. Cells were cross-linked with 1% formaldehyde and chromatin was sonicated to an average size of 200 to 1000 bp. For immunoprecipitation the sonicated chromatin was incubated over night with 5 μg antibody (ErbB4 C-18 or ERβ H-150). The input DNA was used as a positive control (Input) and a no antibody containing negative control (mock) was included. To capture the immune-complexes 50 μl pre-blocked protein A agarose beads were added for 1 h. Immune complexes were washed and eluted. The protein-DNA cross-linking was reversed at 65 °C overnight. The primers for PCR amplification of the surfactant protein B promoter are: *Sftpb*1 FP 5′-CACTTACCCTGCGTCAAGAG-3′, RP 5′-GCCTGACTTTGTTCACGTC-3′ and *Sftpb*2 FP 5′-AAGGACTAGGAACCGA-CATC-3′, RP 5′-ACTGCAGTAGGTGCGACTTG-3′. The PCR products were sequenced using BigDye chemistry and an Avant 3100 Genetic Analyzer (Applied Biosystems) to show product specificity.

2.11. Luciferase reporter gene assay

Cells were transfected with the *Sftpb* promoter luciferase reporter plasmid [29] and the pRL-TK control Vector (renilla luciferase) alone or together with the EGFP control plasmid, pHER4, or YFPERβ, as described above. 48 h after transfection cells were starved for 3 h and treated with DMEM (control), 17-β-estradiol (1000 pmol/l), or NRG (33 nM) for 24 h. Luciferase assay was done using the Dual-Glo Luciferase assay system. Briefly, luciferase reagent was added to cells in growth medium. After 10 minute incubation the firefly luminescence was measured. Stop and Glo reagent was added to the cells and renilla luciferase was measured after 10 minute incubation. For data evaluation the ratio of luminescence from the experimental reporter to the luminescence from the control reporter was calculated and normalized to a control well.

2.12. Preparation of primary fetal mouse ErbB4 deleted type II epithelial cell cultures

Primary type II cells were freshly isolated as previously described [34,35]. Time dated pregnant transgenic ErbB4 mice, rescued from their lethal cardiac defects by expressing a human ErbB4 ($HER4^{heart}$) cDNA under the cardiac-specific α-myosin heavy chain promoter [36] (kindly provided by Carmen Birchmeier in agreement with Martin Gassmann) were used. All animals were housed in an animal facility at Hannover Medical School, and the protocols were approved by the appropriate governmental and institutional authorities at Hannover Medical School. Time-dated pregnant $HER4^{heart}$ mice were sacrificed at E17.5 of gestation by CO_2 inhalation. The fetal lungs were removed, washed in sterile Hank's buffered salt solution, and minced with a razorblade. The minced lungs were incubated with Collagenase Type II for 2 h at 37 °C. The reaction was stopped on ice for 30 min. The tissue was centrifuged, the pellet was resuspended in DMEM, and incubated for another 30 min on ice. After a second centrifugation the pellet was resuspended in DNase and trypsin and incubated for 12 min at 37 °C. The reaction was stopped by DMEM containing 10% fetal calf serum (FCS). The cells were filtered, centrifuged, resuspended in DMEM containing 10% FCS, and

Table 1
Dose response of estrogen effect on cell viability.

17-β-Estradiol concentration	0 pmol/l	60 pmol/l	100 pmol/l	500 pmol/l	1000 pmol/l	1500 pmol/l
Cell viability	100 ± 2%	85 ± 2%	74 ± 3%	82 ± 2%	76 ± 2%	78 ± 2%
P value		P = 0.0002	P < 0.0001	P < 0.0001	P < 0.0001	P < 0.0001
Experimental repeats	7	3	6	3	7	3

plated in culture dishes for 60 min at 37 °C (21%0_2/5%$C0_2$) to allow differential adherence of lung fibroblasts. For type II cell isolation the supernatants from the first differential adherence were centrifuged again. The cell pellet was resuspended in DMEM containing 10% FCS, and plated in culture flasks for 60 min at 37 °C for a second differential adherence. Supernatants were removed and centrifuged. Cell pellets were resuspended, plated in DMEM containing 20% FCS, and grown until further use.

2.13. Data analysis

All treatment values were mean ± SEM of experimental specific controls unless otherwise stated. The effects of ERβ and ErbB4 overexpression on cell viability, surfactant protein expression, and *Sftpb* promoter activity were expressed as percentages of EGFP-treated controls. To evaluate the results for their statistical significance a two-way ANOVA or two tailed t-test with post hoc Bonferroni correction for multiple comparisons was used when appropriate.

3. Results

3.1. Estrogen and NRG reduces cell number

We first examined the effect of estrogen and NRG on the cell number in a dose dependent manner. 17-β-estradiol (Table 1) as well as NRG (Table 2) reduced the cell number at all tested concentrations. We used 1000 pmol/l 17-β-estradiol or 33 nM NRG for all of the following experiments since these doses are known to stimulate ERβ and ErbB4 receptor phosphorylation, respectively [37].

3.2. Estrogen and NRG stimulate Sftpb mRNA and Sftpb protein expression

1000 pmol/l 17-β-estradiol and 33 nM NRG significantly stimulated *Sftpb* mRNA expression to 0.61 ± 0.2 (n = 6, P = 0.02) and 0.56 ± 0.2 (n = 5, P = 0.03), respectively (Fig. 1A), when compared to untreated control cells. This stimulatory effect on *Sftpb* mRNA was less intense on the protein level, implying the presence of post-transcriptional modification. Sftpb protein expression was significantly increased after 17-β-estradiol (114 ± 5%, n = 5, P = 0.02) or NRG (119 ± 3%, n = 5, P = 0.004) treatment compared to untreated control cells (100 ± 4%, n = 5) (Fig. 1B).

3.3. ERβ and ErbB4 co-immunoprecipitate each other

Interactions of ERβ and ErbB4 were studied using a co-immunoprecipitation (co-IP) protocol. ERβ and ErbB4 co-precipitated each other (Fig. 2), independently of treatment with 17-β-estradiol or NRG. In the ERβ co-IP, 17-β-estradiol (116 ± 10%, n = 4, P = 0.1) and NRG (106 ± 15%, n = 3, P = 0.6) did not significantly change the baseline endogenous ErbB4 tyrosine phosphorylation, but significantly stimulated the phosphorylation of ERβ (135 ± 5%, n = 4, P = 0.0002; 109 ± 4%, n = 3, P = 0.03, respectively) above the endogenous phosphorylation of this receptors (100 ± 0.3%, n = 4; 100 ± 0.3%, n = 4 respectively) (Fig. 2A). In the ErbB4 co-IP, treatment with 17-β-estradiol or NRG decreased ErbB4 (88 ± 5%, n = 4, P = 0.05; 91 ± 10%, n = 3, P = 0.3 respectively) and the co-precipitated ERβ (86 ± 6%, n = 4, P = 0.05; 74 ± 13%, n = 3, P = 0.05 respectively) phosphorylation compared to the endogenous phosphorylation of this receptors (100 ± 3%, n = 4; 100 ± 3%, n = 4 respectively) (Fig. 2B). Immunoprecipitations with a control IgG antibody did not show any non-specific antibody binding (Fig. S1).

3.4. Estrogen and NRG stimulate cellular co-localization of ERβ and ErbB4

The intracellular receptor localization was studied using confocal microscopy. In non-stimulated control cells, ERβ showed a diffuse intracellular localization pattern, mainly in the nucleus and the perinuclear region. ErbB4 is localized in the cell membrane and the cytoplasm (Fig. 3A). A short 2-minute stimulation with 17-β-estradiol or NRG led to nuclear localization of ERβ and perinuclear localization of ErbB4 with some co-localization with estrogen stimulation only (Fig. 3B, F). Estrogen or NRG treatment for 30 min (data not shown), 1 h (Fig. 3C, G), 4 h (data not shown), and 8 h (data not shown) led to a cytoplasmatic localization of ErbB4 and no co-localization with the nuclear localized ERβ. After 12 h of 17-β-estradiol or NRG treatment co-localization of both receptors was found in the nucleus and the perinuclear region (Fig. 3D, H). 24 h of NRG treatment further increased the co-localization of both receptors mostly in the perinuclear region (Fig. 3E, I), whereas estrogen induced some co-localization at the cell membrane. Overall, NRG induced a more efficient and faster localization of ERβ in the cytoplasm with an eventual pronounced localization in the perinuclear region and a strong co-localization with ErbB4.

3.5. ERβ, but not ErbB4 overexpression increases cell number

To study the effect of ERβ overexpression on the cell number, MLE-12 cells were transfected with ERβ (YFPERβ) resulting in a significant increase to 152 ± 5% (n = 16, P < 0.001) in comparison to EGFP-transfected control cells (100 ± 2%, n = 12). 17-β-estradiol and NRG treatment further enhanced this stimulatory effect to 155 ± 8% (n = 10, P < 0.001) and 167 ± 5% (n = 7, P < 0.001), respectively, while 17-β-estradiol and NRG treatment had no effect on EGFP-transfected cells (84 ± 6%, n = 12, P > 0.05; 94 ± 5%, n = 6, P > 0.05, respectively). Overexpression of ErbB4 using a human HER4 construct

Table 2
Dose response of NRG effect on cell viability.

NRG concentration	0 nM	5 nM	10 nM	20 nM	33 nM	40 nM	100 nM
Cell viability	100 ± 2%	79 ± 3%	83 ± 3%	84 ± 2%	86 ± 2%	90 ± 2%	91 ± 4%
P value		P < 0.0001	P < 0.0001	P = 0.0002	P < 0.0001	P = 0.015	P = 0.063
Experimental repeats	7	3	3	3	4	3	3

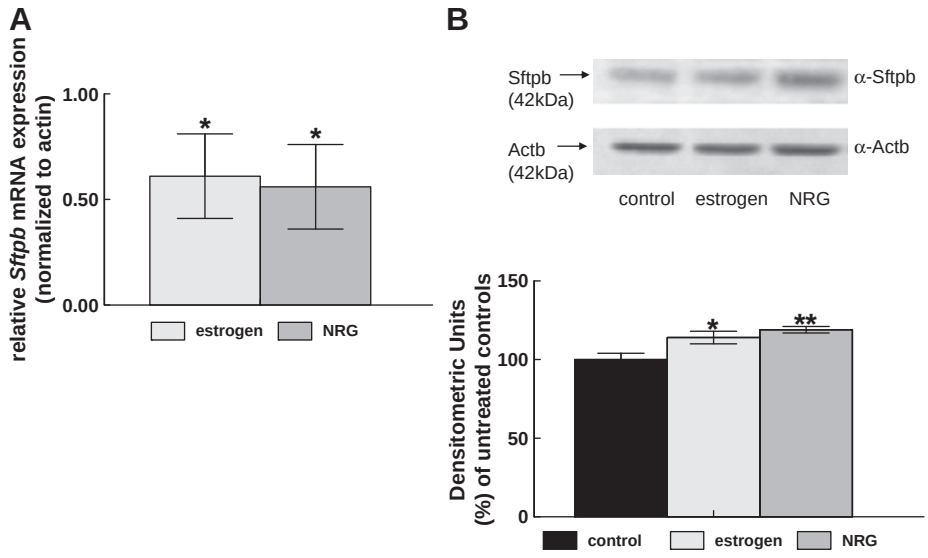

Fig. 1. Estrogen and NRG stimulate *Sftpb* mRNA and Sftpb protein expression. *Sftpb* mRNA expression was measured by real time PCR. 17-β-estradiol (light gray bar) and NRG (dark gray bar) significantly stimulated *Sftpb* mRNA expression in MLE-12 cells ($n=5–6$, $^{*}P\leq0.05$) (A). Sftpb protein expression was studied by Western blotting (representative Western blot is shown on the upper part of B) and analyzed by densitometry. 17-β-estradiol (light gray bar) and NRG (dark gray bar) stimulated Sftpb protein expression, when compared to untreated control cells ($n=5$, $^{*}P\leq0.05$, $^{**}P\leq0.005$), (black bar) (B).

did not change the cell number neither under control conditions ($99\pm6\%$, $n=6$, $P>0.05$), nor after 17-β-estradiol ($92\pm9\%$, $n=6$, $P>0.05$), or NRG ($98\pm4\%$, $n=6$, $P>0.05$) treatment in comparison to EGFP-transfected control cells ($100\pm2\%$, $n=12$) (Fig. 4).

3.6. ERβ overexpression stimulates Sftpb mRNA and protein expression

Overexpression of ERβ stimulated *Sftpb* mRNA expression (0.8 ± 0.1, $n=5$, $P>0.05$), but only the combination of ERβ and 17-β-estradiol (1.4 ± 0.3, $n=5$, $P<0.01$) or NRG (1.2 ± 0.1, $n=4$, $P<0.05$) treatment significantly increased *Sftpb* mRNA expression when compared to cells transfected with the EGFP control construct (Fig. 5A).

Sftpb protein expression was on the other hand significantly increased after ERβ overexpression alone to $115\pm2\%$ ($n=3$, $P<0.01$). 17-β-estradiol treatment further increased Sftpb protein expression to $120\pm5\%$ ($n=3$, $P<0.001$) while NRG treatment did not add further stimulation ($116\pm4\%$, $n=3$, $P<0.01$). Results were compared to

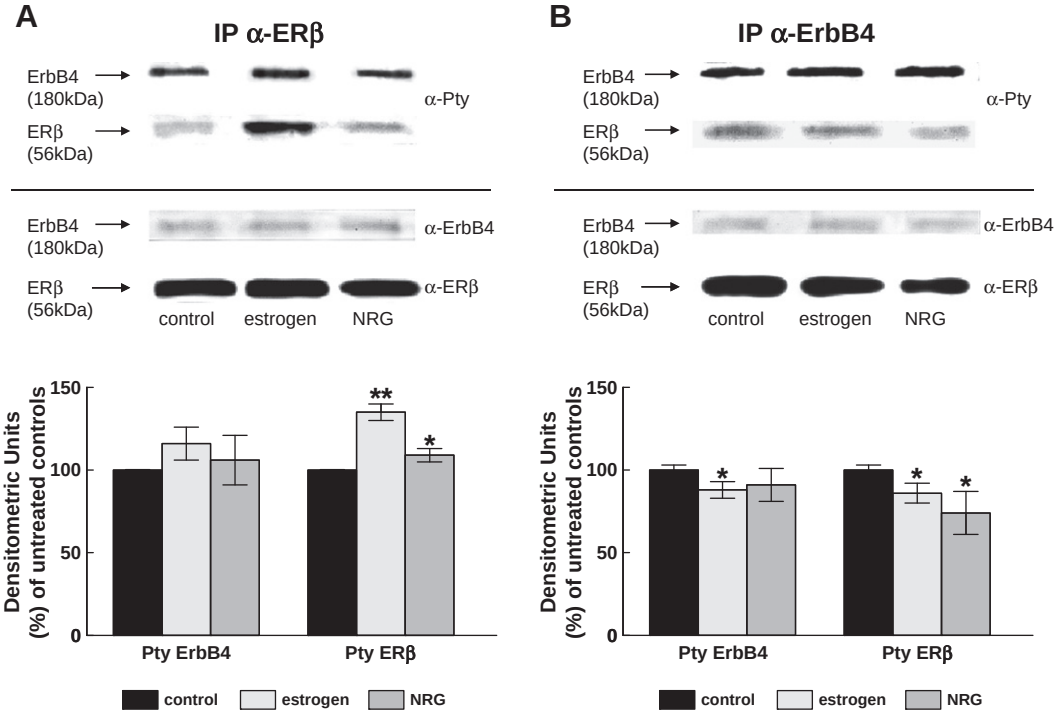

Fig. 2. ERβ and ErbB4 co-immunoprecipitated each other. Co-immunoprecipitation (Co-IP) protocols were used to co-precipitate ERβ (A) and ErbB4 (B) under control conditions (left panel), after 17-β-estradiol (middle panel) and after NRG (right panel) stimulation. Blots were probed with anti-phosphotyrosine, anti-ErbB4, and anti-ERβ antibody as stated on the right next to the blots. Both receptors co-precipitated each other under all conditions. Blots were analyzed by densitometric measurements. Phosphorylation of 17-β-estradiol (light gray bar) or NRG (dark gray bar) stimulated cells ($n=3–4$, $^{*}P\leq0.05$, $^{**}P\leq0.005$) was compared to non-stimulated control cells (black bar).

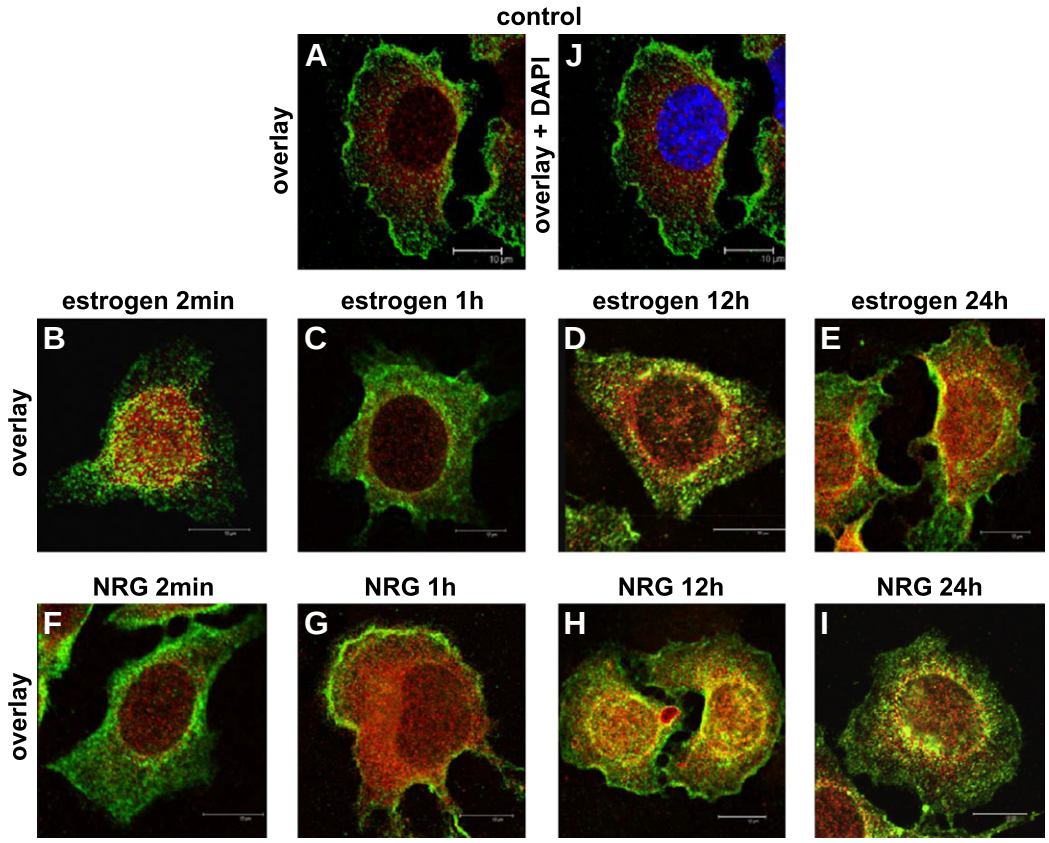

Fig. 3. Estrogen and NRG stimulate cellular co-localization of ERβ and ErbB4. Cellular co-localization of ERβ (red dye) and ErbB4 (green dye) was examined in non-stimulated control cells (A) and after 2 min (B, F), 1 h (C, G), 12 h (D, H) or 24 h (E, I) 17-β-estradiol (estrogen) or NRG stimulation. DAPI staining was used to distinguish the nuclear region from the cytoplasm (J). Nuclear receptor co-localization was most pronounced after 2 min 17-β-estradiol, and perinuclear co-localization after 12 h (D, H) and 24 h (E, I) 17-β-estradiol or NRG treatment.

EGFP-transfected control cells (100 ± 1%, n = 4). In EGFP-transfected cells, 17-β-estradiol (102 ± 1%, n = 4, P > 0.05) and NRG (106 ± 2%, n = 4, P > 0.05) alone had only a slight stimulatory effect on Sftpb protein expression compared to EGFP-transfected control cells (100 ± 1%, n = 4) (Fig. 5B).

3.7. ERβ and ErbB4 bind to the Sftpb promoter

In order to study if ERβ and ErbB4 are binding to the *Sftpb* promoter region, MLE-12 cells were treated with 17-β-estradiol or NRG and chromatin immunoprecipitation was performed. Primers were designed for the *Sftpb* promoter region encompassing approximately 200 bp intervals (Fig. 6A). Under control conditions (C), ErbB4 and ERβ antibody precipitated two *Sftpb* promoter regions (nt −379 to −199 and nt −175 to +39). 17-β-estradiol treatment also recruited both receptors to these regions whereas NRG recruited only ErbB4 to these two regions and ERβ only to the nt −379 to −199 region (Fig. 6B). The PCR products were sequenced using BigDye chemistry and an Avant 3100 Genetic Analyzer (Applied Biosystems) to confirm product specificity (data not shown).

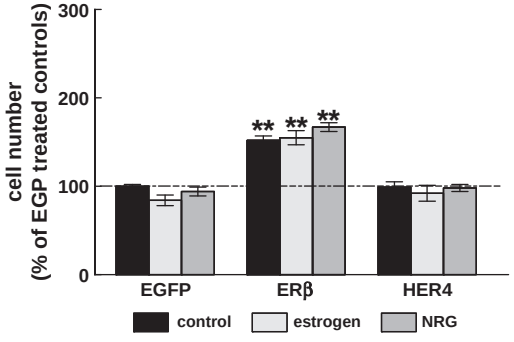

Fig. 4. ERβ, but not ErbB4 overexpression increased cell number. Cell number was measured by MTT assay in MLE 12 cells transfected with ERβ or HER4 under control conditions (black bar), and after treatment with 17-β-estradiol (light gray bar) or NRG (dark gray bar). Results were compared to EGFP-transfected non treated control cells (n = 6–16, **P ≤ 0.005).

3.8. ERβ and ErbB4 overexpression induce Sftpb promoter activity

To determine if estrogen, ERβ, and/or ErbB4 overexpression would stimulate the *Sftpb* promoter activity we transfected MLE-12 cells with an *Sftpb* promoter luciferase reporter plasmid. 1000 pmol/l 17-β-estradiol and 33 nM NRG significantly stimulated luciferase activity to 121 ± 7% (n = 7, P = 0.002) and 120 ± 4% (n = 10, P < 0.0001), respectively. The treated cells were compared to untreated control cells (100 ± 2%, n = 11) (Fig. 7A). 17-β-estradiol treatment increased luciferase activity to 134 ± 18% (n = 7, P > 0.05) and NRG treatment to 111 ± 14% (n = 10, P > 0.05) in EGFP-transfected cells. We have previously shown that co-transfection of the *Sftpb* promoter luciferase reporter and full-length ErbB4 receptor significantly increased luciferase activity under control conditions and after NRG treatment [38]. 17-β-estradiol treatment increased luciferase activity to 165 ± 23% (n = 5, P > 0.05) in ErbB4 overexpressing cells (data not shown). Co-transfection of the *Sftpb* promoter luciferase reporter and ERβ increased luciferase activity to 157 ± 45% (n = 5, P > 0.05) under control conditions, to 229 ± 81% (n = 5, P > 0.05) after 17-β-estradiol treatment, and to 173 ± 61% (n = 5, P > 0.05) after NRG treatment. A combined overexpression of HER4 and ERβ further potentiated this

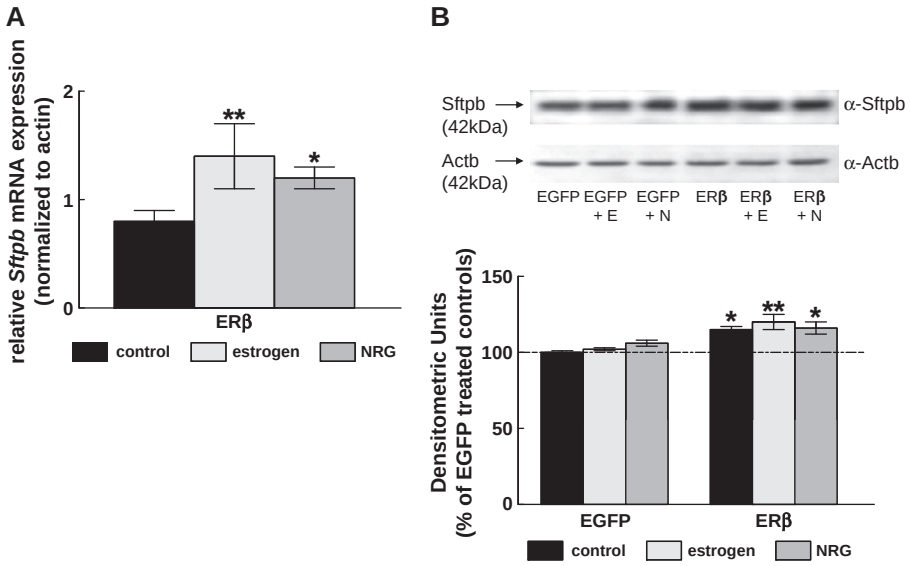

Fig. 5. ERβ overexpression stimulated *Sftpb* mRNA and protein expression. *Sftpb* mRNA expression was measured by real time PCR (5A). *Sftpb* mRNA was significantly increased in cells transfected with ERβ under control conditions (black bar) and after 17-β-estradiol (light gray bar) or NRG (dark gray bar) treatment. Results were compared to EGFP transfected control cells (n = 4–5, *P ≤ 0.05, **P ≤ 0.005). Sftpb protein expression was studied by Western blotting and analyzed by densitometry (5B) (representative Western blot was shown on the upper part of B). Again the ERβ overexpression significantly stimulated Sftpb protein expression when compared to EGFP-transfected cells under control conditions (black bar) and after 17-β-estradiol (light gray bar) or NRG (dark gray bar) treatment (n = 3–4, *P ≤ 0.05, **P ≤ 0.005).

effect showing an increase in luciferase activity to 229 ± 24% (n = 5, P < 0.05) under control conditions, to 371 ± 53% (n = 4, P < 0.001) after 17-β-estradiol treatment, and to 278 ± 78% (n = 4, P < 0.05) after NRG treatment. Transfected cells were compared to EGFP-transfected cells under control conditions (100 ± 4%, n = 12) (Fig. 7B).

3.9. Estrogen induces ErbB4 nuclear translocation

To further analyze the importance of membrane cleavage and nuclear entry of ErbB4 in the effects of estrogen induced co-regulation of ERβ and ErbB4, we transfected MLE-12 cells with ErbB4 mutants with an abrogated nuclear localization sequence (HER4muNLS) or with an abolished γ-secretase processing domain (HER4V673IMu) [25]. Full-length ErbB4 receptor (HER4) and both ErbB4 mutants (HER4muNLS or HER4V673IMu) localized to the cell membrane and the cytoplasm in the control condition. After 17-ß-estradiol treatment the full-length ErbB4 receptor (HER4) and the ErbB4 mutant with an abrogated nuclear localization sequence (HER4muNLS) localized to

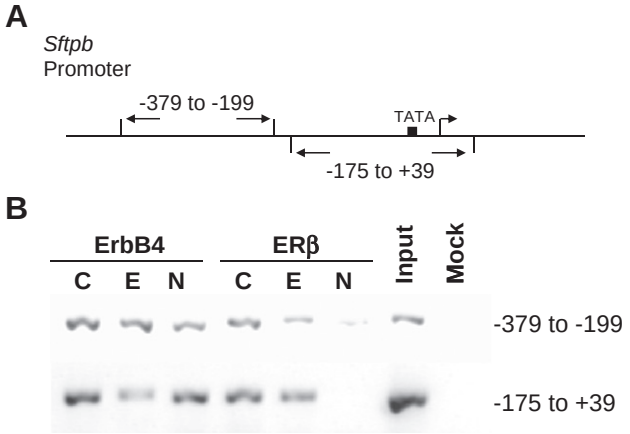

Fig. 6. Binding of ErbB4 and ERβ to the *Sftpb* promoter. Primers were designed for the *Sftpb* promoter region encompassing approximately 200 bp intervals (A). Cells were mock treated (C) or treated with 17-β-estradiol (E) or NRG (N). ChIP assay was performed and DNA bound to ErbB4 or ERβ was analyzed by semi-quantitative PCR amplification. The chromatin was immunoprecipitated using antibodies directed against ErbB4 and ERβ. The input DNA was used as a positive control (Input) and a no antibody negative control (Mock) was included. Under control conditions and after 17-β-estradiol or NRG treatment, ErbB4 and ERβ precipitated with two *Sftpb* promoter regions (B).

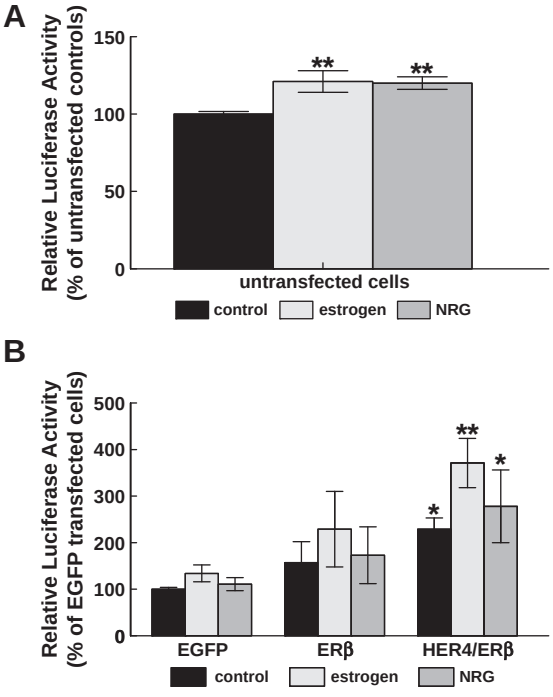

Fig. 7. ERβ and ErbB4 overexpression induced *Sftpb* promoter activity. MLE-12 cells were transfected with a *Sftpb* promoter luciferase reporter. Luciferase activity was measured after 17-β-estradiol (light gray bar) or NRG (dark gray bar) treatment. Results were compared to non-stimulated control cells (black bar), (n = 4–11, **P ≤ 0.005) (A). Cells were co-transfected with EGFP, ERβ or a combination of HER4 and ERβ and luciferase activity was measured under control conditions (black bar) and after 17-β-estradiol (light gray bar) or NRG (dark gray bar) treatment. Results were compared to cells transfected with a control plasmid (EGFP) (n = 4–12, *P ≤ 0.05, **P ≤ 0.005) (B).

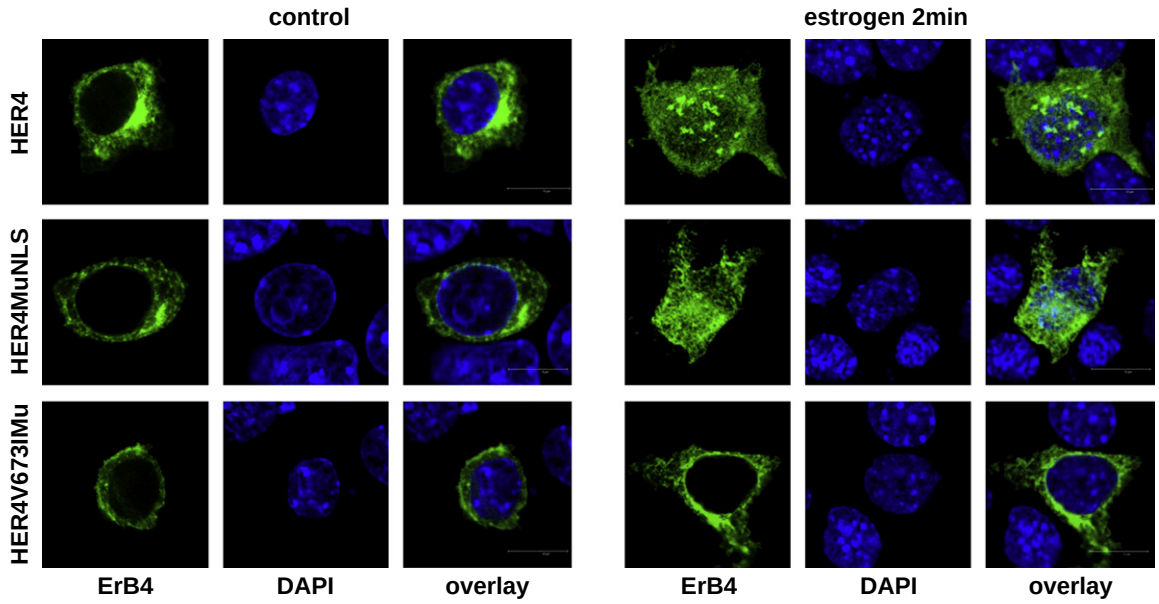

Fig. 8. Estrogen induced ErbB4 nuclear translocation. MLE-12 cells were transfected with HER4 (full length ErbB4 receptor, upper panel), HER4MuNLS (abrogated nuclear localization sequence, middle panel) or HER4V673IMu (abolished γ-secreatase processing domain, lower panel). Cellular localization of HER4 (green dye) was determined under control conditions (left panel) and after 2 min of 17-β-estradiol stimulation (right panel). DAPI staining was used to distinguish the nuclear region from the cytoplasm (blue dye).

the nucleus whereas the ErbB4 mutant with an abolished γ-secretase processing domain (HER4V673IMu) remained in the cell membrane and the cytoplasm. This underlines that γ-secretase processing of ErbB4 seems to be required, while the nuclear localization sequence seems not to be required for estrogen-induced nuclear localization of ErbB4 (Fig. 8), suggesting that ER might function as a NRG-independent nuclear shuttle for ErbB4.

3.10. ErbB4 deletion abolishes estrogen-induced increase in Sftpb mRNA expression

In primary fetal E17.5 ErbB4-deleted type II cells freshly isolated from $HER4^{heart}-/-$ mouse lungs, 17-β-estradiol (-0.04 ± 0.2, $n=3$, $P=0.3$) had no stimulatory effect on *Sftpb* mRNA expression, while NRG significantly decreased *Sftpb* mRNA (-0.4 ± 0.3, $n=3$, $P<0.0001$). Expression of full-length human ErbB4 receptor (HER4) restored the estrogen- (0.6 ± 0.1, $n=3$, $P=0.01$) and the NRG-induced (1.5 ± 0.3, $n=3$, $P<0.0001$) effects on *Sftpb* mRNA expression in ErbB4-depleted type II cells (Fig. 9).

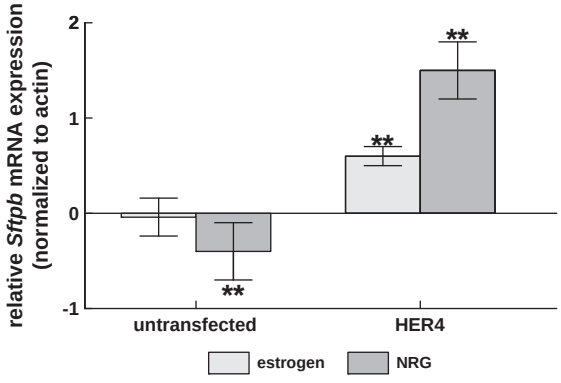

Fig. 9. ErbB4 deletion abolished estrogen-induced increase in *Sftpb* mRNA expression. $HER4^{heart}-/-$ cells were treated with 17-β-estradiol. Real time PCR measurements showed that this treatment had an inhibitory effect on *Sftpb* mRNA expression ($n=3$; $^{**}P \leq 0.005$). Expression of HER4 increased *Sftpb* mRNA expression in 17-β-estradiol treated cells ($n=3$, $^{**}P \leq 0.005$).

4. Discussion

Hormones and growth factors play important roles in the developing lung [39]. Glucocorticoids are widely used in the clinical setting to reduce the risk for RDS [40]. Progesterone, which is known to prevent preterm birth [41] is being studied for its positive effect on the surfactant system [42]. Sex differences in lung development are well studied in the clinical and animal setting and are due to the inhibitory influence of androgens [3] and the positive effect of estrogen [8]. Even the effects of the antenatal glucocorticoid therapy are more prominent in female newborns [43] suggesting a positive interaction with estrogens. NRG [14,15], an important differentiation factor, also exerts stimulatory effects on fetal lung maturation and surfactant synthesis and is known to positively interact with ER in other organ systems [26], but its signaling interaction with estrogen in the fetal lung has to be elucidated. Since type II cells highly express ERβ and ErbB4 and both receptors are known to be very interactive with other signaling pathways or proteins, we hypothesized that estrogen stimulates Sftpb expression and that its stimulatory effect is signaled through an interaction of ERβ and ErbB4 signaling pathways.

NRG-induced ErbB receptor signaling is known to be critical for normal female sex development [20,44] and ErbB4 co-regulates ERα in breast tumor cells [26]. The nuclear ERα/4ICD complex is selectively recruited to estrogen-inducible gene promoters. Consistent with 4ICD-selective promoter binding, suppression of ErbB4 expression shows that 4ICD co-activates ERα transcription. Noteworthy, ErbB4 itself is an estrogen-inducible gene and the ErbB4 promoter harbors a consensus estrogen response element (ERE) half-site with overlapping activator protein-1 elements that bind ERα and 4ICD in response to estrogen. ERs are highly expressed in the fetal lung. In fetal mouse lungs, ERα expression is highest earlier in gestation at E15, while ERβ expression peaks at E17 [12] when fetal surfactant synthesis is initiated. This underlines the importance of ER function in fetal lung development, while the exact intracellular signaling and interaction with other known important promoters of fetal lung development still need to be elucidated. ERα was only weakly expressed in mouse lung epithelial cells (MLE-12 cells) and had no effect on *Sftpb* promoter activation (data not shown). Therefore we here focused on the interaction of estrogen-induced ERβ signaling

interactions with the ErbB4 receptor in a mouse lung epithelial cell line. We are well aware that it might be difficult to draw direct conclusions from an epithelial cell line, established from a mouse lung tumor and further elucidation in primary fetal type II cells is required. We used this cell line here, since transfection efficiency is less potent in primary freshly isolated type II cells and on the other hand these cells express characteristics of type II cells, including the expression of Sftpb [27].

First, we studied the effects of estrogen and NRG on Sftpb expression, the most critical surfactant protein in reducing surface tension in the lung, supporting the adequate neonatal transition, and their effects on cell number. Estrogen as well as NRG significantly stimulated the expression of Sftpb. The overall effects on *Sftpb* gene expression were more pronounced than on Sftpb protein, suggesting the presence of either a latency of protein production or posttranslational modifications. To increase the estrogen-induced effect on Sftpb expression we overexpressed ERβ, because the expression intensity and the amount of ER might have an influence on multiple cell functions.

Despite the fact that estrogen is known to be involved in cell proliferation, it is also found to be present in more differentiated cells with low proliferative activity [45,46]. It is known for multiple growth factors that they are able to switch from a proliferative to a more differentiation promoting activity. Both, estrogen as well as NRG decreased the cell number in our type II epithelial cells. The growth factor NRG is required for the initiation of fetal surfactant synthesis [15] and its signaling receptor ErbB4 is known to be preferably involved in differentiation processes than in cell proliferation [17–20]. Since different concentrations of estrogen and NRG did not reveal significant differences on the cell number and 1000 pmol/l 17-β-estradiol and 33 nM NRG had a stimulatory effect on Sftpb, these concentrations were used for all experiments. The physiological 17-β-estradiol concentration in amniotic fluid increases with gestational age [47]. The concentration we used correlates well with the concentration found in fetal plasma of guinea pigs [48], in fetal fluids and cord blood of pigs [49], and in fetal mouse lungs [50] at an early time point in gestation. This estrogen dose is also known for its stimulatory effect on ER and ErbB4 interactions in breast cancer cells [37]. The NRG dose we used is known to stimulate ErbB receptor phosphorylation in these cells [15].

Secondly, we used co-immunoprecipitation experiments to study the intracellular and nuclear interactions of ERβ and ErbB4 in these type II cells. Interactions found with this technique were endogenous and independent of further estrogen and NRG stimulation. Estrogen significantly stimulated tyrosine phosphorylation of ERβ in type II cells, but had an inhibitory effect on ErbB4 phosphorylation. Despite this observation, interactions of both receptors were critical for the effect of estrogen on Sftpb as seen with the following experiments. Intracellular localization studies confirmed cellular interactions. Both estrogen and NRG stimulated nuclear co-localization of both receptors in a time-dependent manner, underlining the importance of their interactions. ErbB4 is unique in the ErbB receptor family, since the intracellular domain of ErbB4 (4ICD) is able to translocate to the nucleus through a proteolytic cleavage mechanism [22]. Nuclear localization sequences (NLSs) of EGFR family members are required for the nuclear function of these receptors [51]. Estrogen receptors, on the other hand, are nuclear receptors and the classic estrogen signaling is initiated by binding of estrogen to the ER [52]. The non-classical signaling of estrogen through a membrane-associated ER, results in the formation of complexes with receptor tyrosine kinases, G-protein coupled receptors, or secondary messengers like cyclic AMP [53,54]. In breast cancer cells, estrogen promotes direct coupling of the transmembrane ErbB4 and ERα, leading to subsequent complex formation of ERα and the proteolytic processed ErbB4 intracellular domain (4ICD). This complex translocates to the nucleus and binds to gene promoters containing EREs [26]. Our present experiments show for the first time that estrogen effects on *Sftpb* require ErbB4 signaling interactions in lung epithelial type II cells. Estrogen-induced co-localization of both receptors occurred in the nucleus and in the perinuclear region of type II cells. NRG induced co-localization in a more timely fashion in the membrane first, followed by a more intense co-localization in the perinuclear region. The membrane co-localization is in alignment with the membrane coupling of ErbB4 and ERα described in mammary cells [26]. It might be speculated that such a response is also present in lung epithelial cells and that the estrogen receptor serves as a nuclear shuttle for ErbB4.

Thirdly to further study the effects of estrogen- and NRG-induced ERβ signaling on the cell number and surfactant protein expression we overexpressed this receptor in the MLE-12 cells, resulting in stimulation of both, cell number and *Sftpb* expression. The stimulatory effect on the cell number, which was further increased by estrogen and NRG treatment, is in keeping with the known stimulatory effects of estrogen in ER overexpressing tumor cells [45,46]. This also confirms that NRG-induced ErbB4 activation is required for the growth promoting action of estrogen [26]. It is somewhat surprising that estrogen treatment alone decreased the cell number in these cells. In many developing organ systems, including the fetal lung, cell differentiation is accompanied by reduced cell proliferation [55,56]. It is known that estrogen has a stimulatory effect on the cell number in ER overexpressing tumor cells, but in more differentiated cells estrogen leads to a lower proliferative activity [45,46]. The expression intensity, the amount, and the cellular localization of ER might also be involved in the diversity of effects on multiple cell functions including cell proliferation and cell differentiation. For example the estrogen-induced proliferation of neural stem/progenitor cells is mediated via nuclear ER while estrogen-induced differentiation of these cells is mediated by membrane-associated ER [57].

The effects of ERβ overexpression on cell proliferation were distinct from the effects of ErbB4 overexpression, suggesting that estrogen effects on the cell number might be independent of ErbB4 signaling. This was not the case for their effect on the Sftpb expression, requiring an interaction of both receptors. This is in agreement with the known fact that ErbB4 is a member of the ErbB receptor family that is mostly involved in differentiation of organ function [58]. These differences in effect might also be involved in the known “tension” between maturation and proliferation processes happening in many areas of organ development [58,59] where cell differentiation is accompanied by reduced cell proliferation [55,56].

Finally, co-localization of ERß and ErbB4 and the stimulatory effect of estrogen and NRG on *Sftpb* expression, raise the possibility that both receptor proteins form a functionally important complex to stimulate *Sftpb* expression by binding to the promoter region of this gene. There are highly conserved regions located 100 and 300 bp downstream of the transcription start region in the *Sftpb* promoter, which contain DNase hypersensitivity and footprinting sites, suggesting the presence of transcription factor binding sites [60]. Our results presented here suggest that both proteins bind to the promoter region, but ErbB4 is needed for estrogen-induced effects on *Sftpb* promoter activity. Since the estrogen-induced nuclear entry did not require a competent nuclear localization sequence in the ErbB4 receptor, we speculate that ERβ functions as a nuclear chaperone for ErbB4 through a different nuclear entry mechanism. We conclude that estrogen-induced nuclear translocation of the proteolytic processed 4ICD is required for the stimulatory effect of estrogen on *Sftpb* expression. ErbB4 might function as a transcriptional co-factor in the regulation of estrogen-induced *Sftpb* gene upregulation in type II epithelial cells. There is growing evidence from clinical and basic research that NRG receptor ErbB4 regulates estrogen-related molecular biology of disease processes, mostly through interaction of ErbB4 and ERα [26,61]. This might hold true for the development of RDS in the setting of preterm labor. Since NRG might be a potential protector in preterm infants [62–64], further elucidation of this interaction in

the primary fetal setting seems warranted, since most of our studies were done in a cell line.

5. Conclusion

In summary, our data show that ErbB4 regulates estrogen effects on Sftpb expression in this mouse cell line of epithelial type II cell origin. We speculate that the interactions of ERß and ErbB4 signaling pathways might play an important role in lung development by positively influencing the fetal surfactant system. Estrogen is well-known for its positive postnatal effects, improving pulmonary function and decreasing ventilatory support requirement in preterm primates [65], but evidence is missing that it leads to a significant reduction in the incidence or severity of BPD [66]. Activating ErbB4 signaling might be a possibility to positively enhance morphologic lung development in addition to the known positive effects of estrogen treatment on the surfactant system. Further studies in the developing lung are warranted to confirm the role of ErbB4 as a transcriptional regulator of estrogen signaling.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbamcr.2011.06.020.

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