Online Supplemental information

Neuregulin receptor ErbB4 functions as a transcriptional cofactor for Sftpb expression in the fetal lung

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Materials and methods

The mouse lung epithelial cell line (MLE-12) was obtained from American Type Culture Collection (Wesel, Germany). Mouse monoclonal anti beta-actin clone AC-40 and 4,6-Diamidino-2-phenylindole, dilactate (DAPI) were obtained from Sigma (Hamburg, Germany); goat anti-rabbit IgG (HRP-labeled), goat anti-mouse IgG (HRP-labeled) and 10% non-immune goat serum were from Zymed Laboratories Inc (South San Francisco, CA); rabbit polyclonal anti IgG antibody, rabbit polyclonal anti Stat5a antibody, rabbit polyclonal anti ErbB4 antibody C-18, rabbit polyclonal TTF-1 (H-190), and mouse monoclonal anti ErbB4 antibody C-7 were from Santa Cruz (Heidelberg, Germany); Alexa Fluor 568 goat anti-rabbit IgG (H+L) was from Molecular Probes (Karlsruhe, Germany). Rabbit anti-sheep Sftpc was from Chemicon Europe (Schwalbach/Ts, Germany). First-Strand cDNA Synthesis Kit was from GE Healthcare (Munich, Germany); Nanofectin™ and Dulbecco's modified eagle's medium (DMEM) were from PAA-Laboratories (Pasching, A). Fetal calf serum was from PAN Biotech GmbH (Aidenbach, Germany). Total RNA Isolation Reagent was from ABgene (Darmstadt, Germany); the forward primers (FP), reverse primers (RP) and probes specific for Actb, Sftpa1, Sftpb, Sftpc, Sftpd and the *Sftpb* promoter were from Eurogentec (Cologne, Germany); TaqMan Universal PCR Master Mix and ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit were from Applied Biosystems (Darmstadt, Germany); Collagenase Type II was from Worthington Biochemical Corp (St. Katharinen, Germany); Qproteome Nuclear Subfractionation Kit and Plasmid Midi Kit were from Quiagen (Hilden, Germany). ChIP Assay Kit was from USB (Staufen, Germany).

Electron microscopy

Primary fetal type II cells were cultured in transwell plates with 0.4 µm pore size and fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.2M HEPES. Cells were postfixed for 2 hours in 1% OsO_4 in 0.1M cacodylate buffer and stained overnight in uranyl acetate. Aceton was used to dehydrate the cells and they were embedded in epon. Analyses were done using an EM 10 electron microscope (Zeiss, Göttingen, Germany).

RNA isolation and cDNA synthesis

After transfection cells were lysed in RNA isolation reagent. RNA was isolated by guanidinium thiocyanate lysis followed by acid phenol/chloroform extraction. 5 µg of total RNA was used for reverse transcription using a cDNA synthesis kit in a 15µl reaction volume containing 1x DTT, 0.2 µg Hexamer Primer, and 5x Bulk Mix for 1 hour. The resulting cDNA was used for real-time amplification reactions.

Quantitative real-time PCR

The cDNA levels from *Sftpb* and *Actb* gene transcripts were measured by real-time PCR as previously described (10). Actin was used as an internal control to normalize the surfactant

protein cDNA levels. The 20µl reaction mixture contained 1µl of the cDNA template, 10µl TaqMan universal master mix, 300nM each of forward and reverse primer and 200nM probe. For amplification and detection of specific products the ABI PRISM 7500 sequence detection system was used (Applied Biosystems). The threshold cycle was determined for each gene. Samples were run in quadruplicates. A semiquantitative method was used to estimate the relative expression level of the *Sftp* genes by calculating the DCt value, representing the difference in the Ct values of the target and the reference gene. The differences in the Ct values of the ErbB4 transfected cells compared to cells transfected with the EGFP control plasmid were presented as DDCt (DDCt values are inversely proportional to the levels of *Sftp* mRNA). The values for differences between cells transfected with the full-length HER4 and the HER4 mutant were presented as DDDCt. For each assay, specificity was confirmed by sequencing the PCR products on both strands using capillary electrophoresis with POP-6^{TM}-Polymer on an ABI 3100 Genetic Analyzer (Applied Biosystems).

MTT assay

Cell viability 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. Primary fetal HER4^{heart} (+/+) type II cells were plated in a 96-well plate at a concentration of 2.5 x 10^3 cells per well. After transfection cells were incubated with MTT-solution, the reaction was stopped by Isopropanol-HCl. Medium was removed and 3% SDS and Isopropanol-HCl were added. Absorption was measured at 570nm.

Co-Immunoprecipitation and Western Blotting

Primary fetal type II cells of HER4^{heart} (+/+) animals were cultured in culture dishes, serum starved for 4 hours and stimulated with DMEM (control) or DMEM:FCM (1:1) for 24 hours. They were washed with ice cold PBS and lysed in co-immunoprecipitation (Co-IP) buffer as previously published (30). 300 µg of total protein was incubated for 90 minutes with the specific antibody (anti-Stat5a, anti-IgG) at 4^oC. Protein-A-Sepharose was added and the incubation was continued at 4^oC for another 90 minutes. The beads were collected by microcentrifugation, washed three times with Co-IP washing buffer. The proteins were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The blots were blocked using 1% bovine serum albumin (BSA), and then incubated with antibodies against individual proteins (ErbB4, Stat5a, or actin) overnight at 4^oC. Secondary antibody was applied and protein bands were visualized by enhanced chemiluminescence.

Confocal Microscopy

Primary fetal type II cells of HER4^{heart} (-/-) animals were cultured on glass cover slips. After transfection, cells were serum starved for 4 hours and stimulated with DMEM (control) or DMEM:FCM (1:1) for another 24 hours. Immunofluorescence was done as described before (30). Briefly, cells were fixed for 20 minutes in 3% paraformaldehyde and permeabilized for 2 minutes with 0.2% Triton X-100 in PBS. After blocking in 10% normal goat serum, cells were incubated with primary antibody (Stat5a, TTF-1 Sftpc) for 30 minutes at room temperature, washed with PBS and incubated with secondary antibody (Alexa Fluor 568) for 30 minutes at room temperature followed by a 10 minutes DAPI

incubation for a nuclear staining. Cells were mounted in Gelvatol/DABCO and analyzed using a Leica DM IRB confocal laser scanning microscope connected to a TCS SP2 AOBS scanhead (Leica, Wetzlar, Germany).

Nuclear Subfractionation

Nuclear subfractionation of proteins was done in primary fetal $HER4^{heart}$ (+/+) type II cells using the Qproteome Nuclear Subfractionation Kit as described by the manufacturer. Briefly, cells were cultured in 6 well plates, serum starved for 4 hours, and stimulated with DMEM (control) or g.

DMEM:FCM (1:1) for another 24 hours. Cells were lysed in lysis buffer and centrifuged. The supernatant, containing the cytosolic fraction, was stored at -80°C and the pellet, containing the nuclear fraction, was resuspended in nuclear protein lysis buffer. After centrifugation the nuclear pellet was resuspended in extraction buffer and incubated with gentle agitation. The supernatant, containing the nucleic acid binding proteins, was stored at -80°C until further use. Proteins of the different cell fractions were analyzed by Western Blottin

Figure S1: *Sftpc* mRNA expression in fetal type II cells

Sftpc mRNA expression was measured in wild-type fetal type II cells (HER4^{heart} +/+) at different time points. The *Sftpc* expression remains stable for the first 5 days and slowly decreases afterwards. The mRNA expression was lowest after 9 days of cell culture (n=3).

Figure S2: Homogeneity of isolated fetal type II cells

Cell homogeneity of isolated fetal type II cells was evaluated by electron micrograph (Figure S2A) and immunofluorescence staining for TTF-1 (red) (Figure S2B, left panel) and Sftpc (red) (Figure S2B, right panel). Nuclear staining was done by DAPI (blue).

Figure S3: ErbB4 does not change cell proliferation

Cell viability, measured by MTT assay, was significantly stimulated in cells transfected with full-length ErbB4 (HER4) (black bar) and with the ErbB4 mutant (HER4muNLS) (grey bar) when compared to EGFP transfected cells (white bar).

Figure S4: ErbB4 is a nucleic acid binding protein

Nuclear cell fractionation was performed to analyze cellular ErbB4 intra cellular domain (4ICD) distribution in wild-type fetal type II cells (HER4^{heart} +/+). The blots were reprobed with a specific ErbB4 antibody (upper blot) and an actin antibody (lower blot). The left panel shows the cytoplasmic proteins and the right panel the nucleic acid binding proteins under control conditions (C) and after stimulation with fibroblast conditioned medium (FCM).