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ErbB4 is an upstream regulator of TTF-1 fetal mouse lung type II cell development *in vitro*

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ABSTRACT

TTF-1 is an important transcription factor in lung development and lung disease and is essential for lung cell differentiation, specifically surfactant protein (Sftp) expression. The molecular mechanisms that drive the expression and transcriptional control of TTF-1 are not fully understood. In the fetal lung, ErbB4 functions as a transcriptional co-factor and regulates the timely onset of fetal Sftp expression. We speculate that ErbB4 is an upstream regulator of TTF-1 and regulates Sftpb expression via this pathway in alveolar type II cells. Neuregulin-induced ErbB4 and TTF-1 signaling interactions were studied by co-immunoprecipitation and confocal microscopy. Overexpression of ErbB4 and TTF-1 was analyzed in its effect on cell viability, Sftpb expression, TTF-1 expression, and *Sftpb* and *TTF-1* promoter activity. The effect of ErbB4 deletion and ErbB4 nuclear translocation on TTF-1 expression was studied in primary fetal type II epithelial cells, isolated from transgenic HER4$^{heart(-/-)}$ mice. ErbB4 ligand neuregulin induces ErbB4 and TTF-1 co-precipitation and nuclear colocalization. Combined ErbB4 and TTF-1 overexpression inhibits cell viability, while promoting Sftpb expression more than single overexpression of each protein. NRG stimulates TTF-1 expression in ErbB4-overexpressing epithelial cells, while this effect is absent in ErbB4-depleted cells. In primary fetal type II cells, ErbB4 nuclear translocation is critical for its regulation of TTF-1-induced Sftpb upregulation. TTF-1 overexpression did not overcome this important requirement. We conclude that ErbB4 is a critical upstream regulator of TTF-1 in type II epithelial cells and that this interaction is important for Sftpb regulation.

1. Introduction

Thyroid transcription factor (TTF)-1, also known as Nkx2-1, is a homeodomain containing transcription factor and a member of the NK2 homeobox 1 (Nkx2) family, which activates the expression of genes in the thyroid, lung, and brain [1]. TTF-1 is a transcriptional regulator that plays a key role in embryonic lung development and lung cell differentiation [2,3]. In the lung, TTF-1 regulates the expression of genes coding for the surfactant proteins Sftpa1, Sftpb, and Sftpc, and clara cell secretory protein (*CCSP*). All these proteins are critical for lung function as surface tension reducing, anti-inflammatory, and immunomodulatory factors [1]. Suppression of TTF-1 translation in a cultured lung explant model results in inhibition of branching morphogenesis [4], while its deletion leads to extensive defects in the ventral region of the forebrain [5] and non-viable pups with a complete lack of lung parenchyma and thyroid tissue [5]. Beside the effects in the developing lung, TTF-1 has a prominent role in epithelial cell regeneration [6,7] and lung cancer [7]. In lung cancer biology, TTF-1 seems to have a zwitter function, driving oncogenic aspects in some [8–11] and a more favorable prognosis in other lung adenocarcinoma cases [12]. This is paralleled by the observation that ErbB4, a member of the receptor tyrosine kinase family can be either oncogenic or tumor suppressive [13,14]. Myosin Binding Protein H (MYBPH) has recently been discovered as a TTF-1 downstream target in its inhibition of invasion and metastasis development [15]. The upstream molecular mechanism that controls the expression of TTF-1 and its transcriptional activity is still not fully understood.

ErbB4 is known for its involvement in differentiation processes in the breast, the central nervous system, and the lung [16–21]. ErbB4, similar to TTF-1, is critical for the progression of morphologic and functional late fetal lung development [19,22]. ErbB4 deletion leads to delayed fetal Sftpb expression [19] and alveolar simplification and a hyperreactive airway system in the adult lung [23]. Recently it has been shown that neuregulin (NRG)-induced ErbB4 activation leads to proteolytic cleavage [24,25] of the receptor and the 80 kDa intracellular domain (4ICD) [26] translocates to the nucleus [27] to interact with other nuclear receptors and transcription factors. In its promoting effect on Sftpb expression, ErbB4 interacts with Estrogen

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Receptor (ER)β [28] and the transcription factor Signal Transducer and Activator of Transcription (Stat)5A [29]. There is limited information on the regulation of TTF-1 gene expression by hormones, cytokines and other biological agents [2,30]. Since both, ErbB4 and TTF-1 are critical in terminal fetal lung development and function we here mechanistically analyze their interactions by hypothesizing that ErbB4 is an upstream regulator of TTF-1.

2. Materials and methods

The mouse lung epithelial cell line (MLE-12) was obtained from American Type Culture Collection (Wesel, Germany); rabbit polyclonal TTF-1 (H-190), rabbit polyclonal ErbB4 (C-18) (suitable for Western Blot), and normal rabbit IgG were obtained from Santa Cruz Biotechnology (Heidelberg, Germany); mouse monoclonal c-ErbB4 antibody (clone HFR-1) (suitable for immunofluorescence) 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 10% non-immune goat serum were from Zymed Laboratories Inc. (South San Francisco, CA); Alexa Fluor 488 goat anti-mouse IgG (H + L) and Alexa Fluor 568 anti-rabbit IgG (H + L) were from Molecular Probes (Karlsruhe, Germany); rabbit polyclonal anti-sheep Sftpb was from Chemicon Europe (Schwalbach/Ts, Germany); purified mouse monoclonal anti-phosphotyrosine antibody was from BD Biosciences (Heidelberg, Germany); rabbit polyclonal anti-phosphoserine antibody was from Millipore (Schwalbach/Ts, Germany); mouse monoclonal anti-actin clone AC-40, 4,6-Diamidino-2-phenylindole, dilactate (DAPI), and bovine serum albumin (BSA) were obtained from Sigma (Hamburg, Germany); protein A Sepharose™ CL-4B, Western Blotting Detection Reagents (enhanced chemiluminescence, ECL), and cDNA Kit were from Amersham Biotechnologies (Munich, Germany); Dulbecco's Modified Eagles Medium containing glutamine (DMEM) was obtained from PAA-Laboratories (Coelbe, Germany). Fetal calf serum was from PAN Biotech GmbH (Aidenbach, Germany); TaqMan Universal PCR Master Mix and ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit was from Applied Biosystems (Darmstadt, Germany); Total RNA Isolation Reagent (TRIR) was obtained from ABgene (Darmstadt, Germany) and Plasmid Midi Kit from Qiagen (Hilden, Germany). FuGene® HD Transfection Reagent was from Roche Applied Science (Mannheim, Germany). The Dual-Glo Luciferase Assay System was from Promega (Mannheim, Germany). The forward primers (FP), reverse primers (RP) and probes for *Actb*, *Sftpb and TTF-1* were from Eurogentec (Cologne, Germany). Neuregulin 1β was produced using an expression vector kindly provided by Kermit Carraway III (UC Davis, CA) and purified by Dr. Ann Kane, Phoenix Laboratory (Tufts Medical Center, Boston, MA). NRG was diluted in glutamine containing DMEM and used it in a final concentration of 33 nM.

2.1. Overall experimental approach

The first aspect of our approach was focused on the individual and combined effects of ErbB4 and TTF-1 on cell viability (Section 3.1), Sftpb mRNA and protein expression (Section 3.2) and promoter activity (Section 3.3). This was followed by studies elucidating the interactions of these two proteins by studying their cellular colocalization (Section 3.4), and confirming it in subcellular fractionation (Section 3.5) and co-immunoprecipiation studies (Section 3.6). Next we studied the effect of neuregulin on TTF1 expression (Section 3.7) and promoter activity (Section 3.8). In order to exclude endogenous ErbB4 effects on this interaction we repeated the experiments in primary fetal ErbB4-deleted type II cells. Finally we analyzed whether ErbB4 cleavage and nuclear trafficking were critical for TTF-1-induced Sftpb regulation (Section 3.9) and TTF-1 expression (Section 3.10).

2.2. Plasmids

pEGFP N3 (control), pHER4 (full-length human ErbB4 receptor), pHER4muNLS (human ErbB4 receptor with defective nuclear localization signal), and *Sftpb* promoter luciferase reporter plasmid were used as previously published [27,29,31]. HER4 refers to the human ErbB4 receptor plasmid in the rest of the manuscript, pRC/CMV/Nkx2.1 (TTF-1 expression plasmid) and *TTF-1* promoter luciferase reporter plasmid was kindly provided by Dr. Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) [32].

2.3. Transfection experiments

A mouse lung epithelial cell line (MLE-12) was used for most of the transfection experiments. This cell 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 *Sftpc* gene. The cells secrete phospholipids in response to phorbol esters and ATP, but not in response to forskolin [33]. MLE-12 cells are easily transfectable and express Sftpb, TTF-1, and ErbB4. Also, their response to NRG is comparable to fetal type II cells [22]. Transfection protocols were used as previously published [29]. To examine the effects of overexpression of ErbB4 and TTF-1 and to study its effect on *Sftpb* promoter and *TTF-1* promoter activity, MLE-12 cells were transfected with a human ErbB4 construct [28], a TTF-1 construct, an empty Enhanced Green Fluorescent Protein (EGFP) control construct, and a *Sftpb* promoter luciferase reporter plasmid [28] or a *TTF-1* promoter luciferase reporter plasmid. The amount of DNA was adjusted as suggested by the manufacturer and previously published [28,29] to keep the amount of transfected DNA and transfection reagent similar in each transfection experiment. To study the importance of the ErbB4 nuclear translocation, cells were transfected with an HER4 mutant, defective in the nuclear localization signal (HER4muNLS) [28]. The cells were transfected for 48 h using FuGene transfection reagent as previously described [28] in accordance to the manufacturer suggestions.

2.4. MTT-assay

Cell viability was examined using a MTT-assay. In this colorimetric assay the yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) is reduced to purple formazan in the mitochondria of living, metabolically active cells. The amount of formazan is directly proportional to the cell number [34]. MLE-12 cells were grown 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) or NRG (33 nM). After incubating with MTT-solution for 2 h at 37 °C, the reaction was stopped by isopropanol-HCl. Media was removed and 3% SDS and isopropanol-HCl were added. Absorption was measured at 570 nm.

2.5. RNA isolation and cDNA synthesis

Cells were plated in 6 well plates until they reached 90% confluence. After being serum-starved for 3 h, cells were treated for 24 h with serum-free DMEM (controls) or NRG (33 nM). Total RNA isolation reagent was used for cell lysis. RNA was isolated by guanidinium thiocyanate lysis followed by acid phenol/chloroform extraction [35]. After reversed transcription, 5 μg of total RNA was used for cDNA synthesis 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.