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The receptor tyrosine kinase ERBB4 is expressed in skin keratinocytes and influences epidermal proliferation

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ABSTRACT

Background: The epidermal growth factor receptor (EGFR) and associated receptors ERBB2 and ERBB3 are important for skin development and homeostasis. To date, ERBB4 could not be unambiguously identified in the epidermis. The aim of this study was to analyze the ERBB-receptor family with a special focus on ERBB4 *in vitro* in human keratinocytes and *in vivo* in human and murine epidermis.

Methods: We compared the transcript levels of all ERBB-receptors and the seven EGFR-ligands in HaCaT and A431 cells. ERBB-receptor activity was analyzed after epidermal growth factor (EGF) stimulation by Western blot analysis. The location of the receptors was investigated by immunofluorescence in human keratinocytes and skin. Finally, we investigated the function of ERBB4 in the epidermis of skin-specific ERBB4-knockout mice.

Results: After EGF stimulation, all ligands were upregulated except for epigen. Expression levels of EGFR were unchanged, but all other ERBB-receptors were down-regulated after EGF stimulation, although all ERBB-receptors were phosphorylated. We detected ERBB4 at mRNA and protein levels in both human epidermal cell lines and in the basal layer of human and murine epidermis. Skin-specific ERBB4-knockout mice revealed a significantly reduced epidermal thickness with a decreased proliferation rate.

Conclusions: ERBB4 is expressed in the basal layer of human epidermis and cultured keratinocytes as well as in murine epidermis. Moreover, ERBB4 is phosphorylated in HaCaT cells due to EGF stimulation, and its deletion in murine epidermis affects skin thickness by decreasing proliferation.

General significance: ERBB4 is expressed in human keratinocytes and plays a role in murine skin homeostasis.

1. Introduction

The epidermal growth factor receptor (EGFR, ERBB1, HER1) and its ligands play an important role in skin homeostasis, and their deregulation promptly affects keratinocyte proliferation and differentiation, potentially resulting in inflammatory or hyperproliferative responses [1]. EGFR belongs to a family of receptor tyrosine kinase (RTK) receptors that also includes ERBB2 (NEU, HER2), ERBB3 (HER3), and ERBB4 (HER4) [2,3]. While evidence for a key role of EGFR in skin pathophysiology is ample, considerably less is known about the functions of the structurally related ERBB receptors in this tissue.

ERBB2, a ligand-less receptor, is co-expressed with EGFR in the epidermal basal layer and in proliferative cells of the pilosebaceous unit [4]. ERBB2 is activated by UV irradiation and increases UV induced skin tumorigenesis by suppressing S-phase arrest [5]. ERBB2 is overexpressed in several types of cancer, including human non-melanoma skin cancer [6,7], and transgenic overexpression of ERBB2 causes epidermal and follicular hyperplasia and spontaneous tumor formation [4,8–11]. Deletion of ERBB2 in HaCaT cells [12] and murine skin deteriorates wound healing and decreases tumor burden in a multistage chemical carcinogenesis protocol in ERBB2 knockout mice [13]. ERBB3 is expressed in all epidermal layers, with highest levels in the suprabasal and spinous layers [4,14]. ERBB3 has a potential involvement in wound repair [15,16], and it has an important function in chemically-induced skin tumorigenesis in mice [17]. So far, ERBB4 expression analysis in the skin resulted in contradictory findings. ERBB4 expression was not detectable in human [14,18] or murine [4] epidermis, and also not in human primary keratinocytes [19] and A431 [20,21]. Nevertheless, other groups detected ERBB4 in human epidermis [21], in A431 cells, although only a weak signal was observed [18], and not unambiguously identified in HaCaT cells [19]. Notably, Panchal and colleagues detected ERBB4 in murine epidermis and hair follicles [22]. This group overexpressed neuregulin 3 (NRG3), a highly specific ERBB4 ligand, under the control of the keratin 14 promoter in the skin, and observed a dramatically thickened epidermis.

The aim of this work was to study the ERBB receptors and their

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ligands in the human keratinocyte cell line HaCaT, representing normal keratinocytes, and in the human skin epidermoid carcinoma cell line A431, which represents a squamous cell carcinoma. As EGFR, ERBB2, and ERBB3 are established receptors regulating skin homeostasis and tumorigenesis, we focused on the neglected ERBB4 receptor. Although in the past some studies investigated ERBB receptors and some of their ligands in these cell lines, to date no publication is available in which all ERBB receptors and all EGFR ligands are analyzed in both lines and directly compared to each other. We clearly detected ERBB4 expression in established keratinocyte cell lines, human and murine epidermis using three different antibodies. Generation of skin-specific ERBB4 knockout mice revealed that ERBB4 is dispensable for skin development, but it is involved in epidermal proliferation and homeostasis.

2. Materials and methods

2.1. Cell culture

HaCaT keratinocytes and A431 cells were cultured in DMEM® medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (FCS, Biochrom) and 1% penicillin/streptomycin (Biochrom). For starvation, cells were cultured in the medium indicated above without FCS for 12 h and were stimulated with 100 ng/ml EGF (R&D systems, Wiesbaden-Nordenstadt, Germany) for the indicated periods of time.

2.2. Mice

Mice carrying floxed *Erbb4* alleles [23] or expressing cre recombinase under the keratin 5 promoter have been described previously [24]. Genotyping of transgenic mice was done by PCR using the following primers *P20*: 5′-CAAATGCTCTCTCTGTTCTTTGTGTCTG-3′; *P22*: 5′-TTTTGCCAAGTTCTAATTCCATCAGAAGC-3′; *P23*: 5′-TATTGT GTTCATCTATCATTGCAACCCAG-3′; *K5Cre-Fw*: 5′-AATCGCCATCTTCC AGCAG-3′; *K5Cre-Rv*: 5′-GATCGCTGCCAGGATATACG-3′. Mouse strains were maintained in the C57BL/6N background under specific pathogen-free conditions and had access to water and standard rodent diet (V1534, Ssniff, Soest, Germany) *ad libitum*. All experiments were approved by the Committee on Animal Health and Care of the state of Upper Bavaria (Regierung von Oberbayern, Germany). Genotyping of mouse lines was performed according to the original publications. Eight-week-old *Erbb4*^{del} females and control littermates were dissected for further investigations. After euthanasia, skin samples were fixed in 4% paraformaldehyde (PFA, Sigma, Taufkirchen, Germany), dehydrated, and embedded in paraffin or snap-frozen and stored at −80 °C until use.

2.3. Quantitative RT-PCR

For RNA analysis the starved cells were stimulated with EGF for 2 h. Total RNA was isolated with TRIZOL reagent (Invitrogen, Darmstadt, Germany) and 1 μg of RNA samples were reverse-transcribed in a final volume of 20 μl using RevertAid Reverse Transcriptase (Thermo Scientific, Schwerte, Germany) according to the manufacturer's instructions. Quantitative RT-PCR was carried out in a LightCycler®480 (Roche, Mannheim, Germany) using the primers listed in Supplementary Table S1 (0.5 μM), 1 μl cDNA, 0.2 μM probe (Universal ProbeLibrary Set, Roche), and the LightCycler® 480 Probes Master Mix (Roche) in a final volume of 10 μl. Cycle conditions were 95 °C for 5 min for the first cycle, followed by 45 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 s. Transcript copy numbers were normalized to histone H3 (*H3F3A*) (Fig. 1) or peptidyl-prolyl cis-trans isomerase a (*PPIA*) (Fig. S3) mRNA copies. The ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the *H3F3A* gene. For each primer pair we performed no-template control and no-RT control assays, which produced negligible signals that were usually > 40 in Ct value. Experiments were performed in duplicates for each sample. All primers and probes are listed in Supplementary Table S1. For qualitative mRNA expression of human *ERBB4*, RT-PCR using reagents from Qiagen (Hilden, Germany) were performed. The final reaction volume was 20 μl, and cycle conditions were 94 °C for 5 min, followed by 35 cycles at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Following primers were employed: *ERBB4* forward primer 5′-GAGCAAGAATTGACTCGAATAGG-3′ and reverse primer 5′-TTCCTGACATGGGGGTGTAG-3′; *JM* forward primer 5′-TCC AGATGGCTTACAGGG-3′ and reverse primer 5′-TCTCATTAAGAATCTT AATAGC-3′; *CYT* forward primer 5′-ATCTCTTGGATGAAGAGGATT-3′ and reverse primer 5′-TTGTCTCGCATAGGAGTCAT-3′; *GAPDH* forward primer 5′-TCATCAACGGGAAGCCCATCAC-3′ and reverse primer 5′-AGACTCCACGACATACTCAGCACCG-3′.

2.4. Western blot analysis

For Western blot experiments the starved cells were stimulated with EGF for 10 min. Protein of cells and murine tissue was extracted using Laemmli-extraction-buffer, and the protein concentration was estimated *via* bicinchoninic acid protein assay. 25 μg of total protein were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Schwalbach, Germany) by semidry blotting. Membranes were blocked in 5% w/v fat-free milk powder (Roth, Karlsruhe, Germany) for 1 h at room temperature (RT). After washing in Tris-buffered saline solution (TBS) with 0.1% Tween20 (Sigma) (TBS-T), membranes were incubated over night at 4 °C in 5% w/v BSA (Sigma) in TBS-T with the appropriated primary antibody. All primary and secondary antibodies and their dilutions are listed in Supplementary Table S2. After washing, membranes were incubated in 5% w/v fat-free milk powder with a horseradish peroxidase-labeled secondary antibody. Signals were detected using an enhanced chemiluminescence detection reagent (GE Healthcare, Munich, Germany) and appropriated X-ray films (GE Healthcare).

2.5. Immunofluorescence

For *in vitro* immunofluorescence stainings, cells were grown on cover slips and fixed for 15 min in 4% PFA in phosphate buffered saline (PBS). After fixation, the cells were permeabilized for 10 min with 0.5% Triton X-100 (Roth) in PBS and then washed with PBS. Cover slips were incubated for 2 h at RT with primary antibodies listed in Supplementary Table S3. Sections were washed and incubated with secondary antibody together with Alexa Fluor 594 conjugated Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA, #A-12381). All sections were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) with DAPI and stored at 4 °C.

Biopsy sample (normal femoral skin from a female, Caucasian, 52 years old patient and healthy frontal skin) obtained after informed consent were kindly provided by R. Wolf, M.D. (Klinik für Dermatologie und Allergologie, Universitätsklinikum Giessen-Marburg, Marburg, Germany). Human and murine samples were embedded in Tissue-Tek O.C.T. (VWR, Darmstadt, Germany), frozen on dry ice and cryosectioned (10 μm). After fixation for 15 min at RT in 4% PFA in PBS sections were washed with TBS-T and blocked in blocking buffer (5% donkey serum (Millipore), 0.1% Tween20 in TBS) for 2 h at RT. For immunolabeling the primary and secondary antibodies listed in Supplementary Table S3 were used. Sections were incubated with primary antibody in staining buffer (2.5% donkey serum, 0.1% Tween20 in TBS) at 4 °C over night. After washing with 0.1% TBS-T tissue sections were incubated with appropriate fluorescent secondary antibodies in staining buffer for 1–2 h at RT and nuclei were additionally stained with 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at RT. At last, sections were mounted with Vectashield with DAPI after washing with 0.1% TBS-T. Images were acquired using Zeiss LSM710 laser-scanning confocal microscope (Carl Zeiss MicroImaging) with a 40× water