Developmental Biology 320 (2008) 60–71

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Identification of dkk4 as a target of Eda-A1/Edar pathway reveals an unexpected role of ectodysplasin as inhibitor of Wnt signalling in ectodermal placodes

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ARTICLE INFO

Article history:
Received for publication 10 August 2007
Revised 26 March 2008
Accepted 14 April 2008
Available online 26 April 2008

Keywords:
Tabby
NF-κB
Mouse
lrp4
Tooth
Hair
Mammary gland
Placode

ABSTRACT

The development of epithelial appendages, including hairs, glands and teeth starts from ectodermal placodes, and is regulated by interplay of stimulatory and inhibitory signals. Ectodysplasin-A1 (Eda-A1) and Wnts are high in hierarchy of placode activators. To identify direct targets of ectodysplasin pathway, we performed microarray profiling of genes differentially regulated by short exposure to recombinant Eda-A1 in embryonic *eda*$^{-/-}$ skin explants. Surprisingly, there were only two genes with obvious involvement in Wnt pathway: *dkk4* (most highly induced gene in the screen), and *lrp4*. Both genes colocalized with Eda-A1 receptor *Edar* in placodes of ectodermal organs. They were upregulated upon Edar activation while several other Wnt associated genes previously suggested as Edar targets were unaffected. However, low *dkk4* and *lrp4* expression was retained in the absence of NF-κB signalling in *eda*$^{-/-}$ hair placodes. We provide evidence that this expression was dependent on Wnt activity present prior to Eda-A1/Edar signalling. Dkk4 was recently suggested as a key Wnt antagonist regulating lateral inhibition essential for correct patterning of hair follicles. Several pieces of evidence suggest Lrp4 as a Wnt inhibitor, as well. The finding that Eda-A1 induces placode inhibitors was unexpected, and underlines the importance of delicate fine-tuning of signalling during placode formation.

Introduction

Ectodermal organ development has been extensively studied using several models such as hairs, glands, teeth, and feathers (for reviews see Pispa and Thesleff, 2003; Veltmaat et al., 2003; Mikkola and Millar, 2006; Lin et al., 2006). Despite their diversity in shape and function, these organs share common morphological and molecular features during the early steps of morphogenesis. They all develop as a result of interactions between ectoderm and underlying mesenchymal cells (Hardy, 1992). The first sign of ectodermal organ formation is a local thickening of the epithelium, the placode, which is accompanied by condensation of the underlying mesenchyme. The communication between and within the two tissues is mediated by several families of signalling molecules including Wnts, fibroblast growth factors (FGFs), transforming growth factors-β (TGF-βs), bone morphogenetic proteins (BMPs), and sonic hedgehog (Shh) which are produced in the placode or the associated mesenchymal condensate during early morphogenesis, and are conserved between species (for review see Pispa and Thesleff, 2003; Mikkola and Millar, 2006). Numerous studies have shown that a balance between stimulating and inhibiting signals governs the patterning of ectodermal appendages.

Ectodysplasin is a signalling molecule in the tumor necrosis factor (TNF) family which triggers a pathway required for the establishment of the placode in a number of ectodermal organs, and it has been shown to operate rather early in the hierarchy of signalling molecules regulating placode formation (Mikkola and Thesleff, 2003; Mustonen et al., 2004). This pathway is composed of the ligand ectodysplasin (Eda), Edar, the receptor of the Eda-A1 isoform of ectodysplasin, and the cytoplasmic Edar-associated death domain adapter protein (Edaradd). Mutations in any of these three genes cause a syndrome called hypohidrotic (anhidrotic) ectodermal dysplasia characterized by defective development of multiple ectodermal organs (Kere et al., 1996; Monreal et al., 1999). In mice, the mutations in the corresponding genes are responsible for the mouse mutants *tabby*, *downless*, and *crinkled* respectively (Srivastava et al., 1997; Headon and Overbeek, 1999; Headon et al., 2001). Mice carrying these mutations lack specifically the long guard hairs and show defects in several exocrine glands and the number and shapes of teeth. Guard hair follicles which develop as the first wave of mouse hairs at embryonic day (E) 14, are missing in the mutant mice, whereas placodes of the next wave giving rise to awl hairs form correctly at E16 (Vielkind and Hardy, 1996; Laurikkala et al., 2002). Several studies have shown that the binding of the Eda-A1 isoform to Edar leads to activation of transcription factor NF-κB (Yan et al., 2000; Koppinen et al., 2001; Kumar et al., 2001). Mice and humans carrying mutations perturbing NF-κB activation show similar phenotypes in ectodermal organs as those with deficiency in Eda-A1/Edar pathway

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doi:10.1016/j.ydbio.2008.04.023

components (Schmidt-Ullrich et al., 2001; Mikkola and Thesleff, 2003; Orange et al., 2005), suggesting that NF-κB activation is required for proper Eda-A1/Edar signal transduction. Targeted epithelial overexpression of Eda-A1 in transgenic mice under the control of Keratin 14 promoter (K14) results in supernumerary tooth and mammary placodes, and enlarged or fused hair placodes (Mustonen et al., 2003, 2004). Moreover, recombinant Eda-A1 protein fused to the C-terminus of an IgG1 Fc domain (Fc-EdaA1) permanently restores a majority of the defects of *eda*$^{-/-}$ litters when injected to *eda*$^{-/-}$ pregnant mice (Gaide and Schneider, 2003), and rescues the first wave of hair placode formation when administered to E13 *eda*$^{-/-}$ skin in culture (Mustonen et al., 2004).

Wnt signalling plays a crucial role in ectodermal organ placode formation, most probably via the canonical β-catenin/LEF1/TCF pathway (Pispa and Thesleff, 2003; Mikkola and Millar, 2006). Canonical Wnt signalling has been extensively characterized during the last decades (Clevers, 2006). Wnt ligands activate Frizzled transmembrane receptors, which leads to the stabilization of cytoplasmic β-catenin through inhibition of the GSK3β kinase. β-catenin then translocates to the nucleus where it binds to LEF/TCF transcription factors to promote transcription of target genes. TOPGAL mice reporting LEF/TCF and β-catenin activity via β-galactosidase expression show canonical Wnt signal activity in the basal epithelial cell layer of hair pregerms and subsequently in cells of dermal condensates suggesting its involvement in the first steps of hair morphogenesis (DasGupta and Fuchs, 1999; Mikkola and Millar, 2006). Transgenic overexpression of the Wnt signal inhibitor Dkk1 in ectoderm inhibits hair and mammary placode formation, and blocks tooth morphogenesis before the bud stage (Andl et al., 2002; Chu et al., 2004) indicating an absolute requirement of Wnt signalling for the initiation of ectodermal appendage development. *Lef1*$^{-/-}$ mice lack whiskers, teeth, and mammary glands, but exhibit a reduced number of pelage hairs (Van Genderen et al., 1994; Kratochwil et al., 1996), suggesting a redundancy of LEF/TCF factors during ectodermal organ development.

Although both Eda and Wnts are high in the hierarchy of signals required for ectodermal organ development, the relationship between Wnt and Eda-A1/Edar signalling has remained unclear. Recent studies have suggested that Eda-A1/Edar signalling modulates other signal pathways involved in hair development. We and others have identified two BMP inhibitors, *ccn2/ctgf* and *follistatin*, as well as *shh* as putative targets of the Eda-A1/Edar pathway (Mou et al., 2006; Schmidt-Ullrich et al., 2006; Pummila et al., 2007). These genes and many others were also found in a microarray analysis comparing transcriptomes from *eda*$^{-/-}$ and wild-type skin at the stage of hair placode formation (Cui et al., 2006). Since primary hair placodes fail to form in *eda*$^{-/-}$ skin, it is expected that many markers of the epidermal placode and dermal condensate emerge from this type of screen, and therefore it is challenging to differentiate between the direct Eda target genes from those that are secondarily activated.

In an attempt to identify direct target genes of the ectodysplasin pathway, we performed a microarray profiling of genes differentially expressed in *eda*$^{-/-}$ skin after a short exposure to Fc-EdaA1 in vitro. Surprisingly, among these were only two genes with an apparent connection to the Wnt pathway: *dkk4* and *lrp4*. In this report, we show that *dkk4* and *lrp4* are expressed in ectodermal organ placodes, and that they are likely to be novel target genes of Eda-A1/Edar pathway. Using a quantitative approach, we demonstrate that *dkk4* is strongly, and *lrp4* moderately upregulated upon administration of Eda-A1 protein. We also provide evidence indicating that the expressions of *dkk4* and *lrp4* depend on Wnt signalling present prior to Eda-A1/Edar activity in developing hair placodes. Moreover, our data suggest that Eda is dispensable for hair follicle induction but is essential for establishing a correct pattern of primary hair follicles.

Materials and methods

Animals

Wild-type females from the NMRI strain were kept by breeding with NMRI males. The generation and maintenance of the mouse strains used in this study have been described earlier: *eda*-deficient mice (also referred as *eda*$^{-/-}$; *tabby* mice, Jackson Laboratories stock #JR0314) (Pispa et al., 1999); *K14-eda* mice (Mustonen et al., 2003); *NF-κB*^{REP} (Bhakar et al., 2002). *K14-eda* and *NF-κB*^{REP} embryos were identified by PCR. The appearance of a vaginal plug was taken as embryonic day (E) 0, and embryos were carefully staged according to limb morphogenesis. *K14-eda* mice are of FVB background and *NF-κB*^{REP} of C57Bl/6 background. *NF-κB*^{REP} mice were bred into *eda*$^{-/-}$ background to monitor NF-κB activity in the absence of Eda.

Skin culture

Back skin from E13 NMRI wild-type embryos was dissected and grown on nuclepore filters at 37 °C for 24 h in a Trowell-type culture containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS), glutamine and penicillin–streptomycin. Recombinant mouse Dkk4 (R&D Systems) was administered to the culture medium as indicated in the text.

X-gal staining

Embryos were fixed for 30 min in 4% paraformaldehyde, then washed three times for 10 min in Dulbecco's PBS pH 7.5 containing 2 mM $MgCl_2$ and 0.02% Nonidet P-40. Samples were stained overnight at room temperature with X-gal staining solution (1 mg/ml X-gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ and 10% Nonidet P-40 in PBS, pH 7.5).

Hanging drop cultures and quantitative RT-PCR

To analyze the induction of gene expression after different treatments indicated in the text, E14 *eda*$^{-/-}$ back skins were grown submerged in hanging drops as described earlier (Pummila et al., 2007). Each skin was dissected in Dulbecco's PBS pH7.5 and cut in two halves along the midline. For each treatment, one half was used as control and the other one was exposed to different molecules indicated in the text. Each skin half was cultured individually in one drop of 40 μl pre-warmed medium supplemented with purified recombinant 1 to 2 μg/ml of Fc-Eda-A1 (Gaide and Schneider, 2003), 6-bromoindirubin-3′-oxime (BIO; Calbiochem), both molecules, or equivalent proportion of molecule dissolvent for controls. Three different batches of Fc-Eda-A1 protein were used in the current study. The only difference noticed between the batches was that the onset of induction of the genes analyzed was somewhat delayed with batch 2, which was used in most of the experiments.

To manipulate Wnt signalling, skin halves were pre-treated for 4 h with the indicated concentrations of CKI-7, a potent caseine kinase 1 inhibitor (United States Biological) or the equivalent concentration of the dissolvent. Skin halves were then transferred into fresh CKI-7 containing medium such that one half was exposed to 2 μg/ml of recombinant Fc-Eda-A1 for 4 h while the other one was used as a control. A minimum of triplicate samples was analyzed each time.

After the number of hours indicated in the text, tissues from hanging drops were placed into 350 μl lysis buffer of the RNeasy mini kit (Qiagen) containing 1% β-mercaptoethanol (Sigma). Total RNA was isolated as specified by the manufacturer's instruction and quantified using a nanodrop spectrophotometer. 500 ng of total RNA was reverse transcribed using 500 ng of random hexamers (Promega) and 100 units of Superscript II (Invitrogen) following the manufacturer's instructions. For the time-course experiment, quantitative PCR was performed using 2X SYBR-green PCR master mix (Applied Biosystems), using the default PCR conditions for the ABI 7000. Data were normalized against *ranbp1* and analyzed with Applied Biosystems' Prism software. For the experiment comparing Fc-Eda-A1 and BIO effects on gene induction, Lightcycler DNA Master SYBR Green I (Roche) was used with a Lightcycler 480, and the software provided by the manufacturer was used for analysis; data were normalized against *K14*. Primer sequences are available upon request. Gene expression was quantified by comparing the sample data against a dilution series of PCR products of the gene of interest.

Microarray experiment and analysis

Pools of three E14 *eda*$^{-/-}$ half-skins pairs were submerged in hanging drops and cultured for 1.5 h or 4 h in the absence or presence of 2 μg/ml Fc-Eda-A1 followed by total RNA isolation as described above (Fig. 1). Biological triplicates for each condition were performed. RNA quality and concentration was monitored using a 2100 Bioanalyzer (Agilent Technologies). RNAs were processed and hybridized on Affymetrix® Mouse Genome 430 2.0 arrays, and data were analyzed in the Turku Centre for Biotechnology, National Microarray Centre Affymetrix Service (Finland). Data were preprocessed by RMA (Robust Multi-Array Average) using R/Bioconductor, allowing background correction, quantile normalization, probe specific correction and summary value computation. 2-base log-transformed intensity ratio (treated/control) was calculated for each sample pair. Average of replicate log-ratios and *t*-test of replicates were calculated. Genes over 1.5-fold differences and with a *p*-value below 0.05 were chosen for further analysis.