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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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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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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.

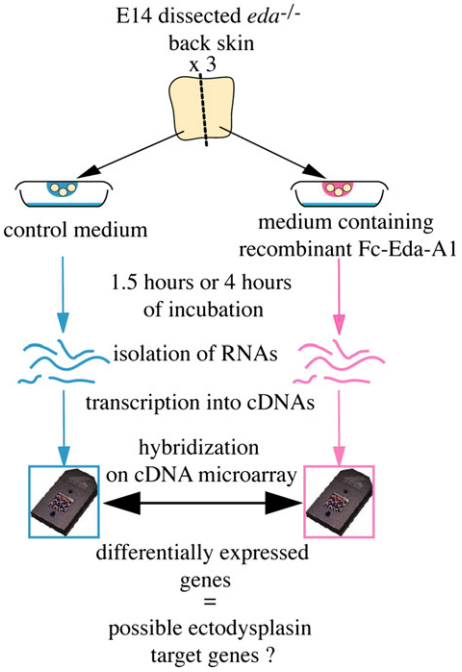


Fig. 1. Schematic representation of the experimental procedure used to identify direct targets of Eda-A1/Edar signalling pathway. E14 *eda*$^{-/-}$ back skins were dissected and cut in two parts along the midline. Pools of 3 half-skins were cultured in a hanging drop of control medium, whereas the 3 other halves were cultured in medium supplemented with 2 μg/ml of Fc-Eda-A1 recombinant protein. After 1.5-hour or 4-hour incubation, total RNA was extracted from the samples, and processed for hybridization on Affymetrix® Mouse genome 430 2.0 Arrays. Three replicates were used for each condition.

Promoter analysis and luciferase reporter assays

Mouse and human upstream promoter sequences (−5000 to +1; A of the first ATG codon being +1) of *dkk4* and *lrp4* were aligned and analyzed for the presence of putative NF-κB binding sites with the CONSITE program (http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite) using 60% conservation cut-off and window size 25. For *lrp4*, sites with an 80% transcription factor score threshold were retained. For *dkk4*, transcription factor score thresholds from 75 to 80% were used in the analysis.

For the cloning of mouse *dkk4* promoter region containing the putative NF-κB response elements, a 0.8 kb region upstream of the translation initiation codon was amplified with the following primers: forward 5′-TGTTTATGCGCCCCACTATT-3′ and reverse primer including a Hind3 site 5′-CTAGCAAGCTTCCTCAGTCACTCTGGTCTCTCG-3′. The resulting PCR product was digested with SmaI/Hind3 and cloned into pGL3 basic luciferase reporter vector (Promega, Madison). For transfection, 293T cells were seeded at 1×10^5 cells per well on gelatin coated six well plates. The following day, 950 ng of each luciferase reporter plasmid was co-transfected with 50 ng of *Renilla* luciferase plasmid (pRL-TK, Promega) and 500 ng of expression plasmids for wild-type *edar*, mutant *edar* (*edar*^{Sleek}) lacking the death domain, empty vector (pEF1/Myc-His A) (Invitrogen) (Koppinen et al., 2001), or 50 ng of wild-type β-catenin in pIRES2-AcGFP1-Nuc vector and 50 ng of human Lef1 in pcDNA/AmpI vector (kindly provided by Hannu Sariola) using Fugene6 (Roche) as recommended. The amount of DNA in each transfection was kept constant by adding pEF1/Myc-His A. The luciferase assays were performed 24 h after transfection using the Dual-Luciferase Reporter Assay System as specified by the manufacturer (Promega) and *firefly* luciferase activities were measured using Bio Orbit 1253 luminometer and normalized to the *Renilla* luciferase values. Data shown are mean±SEM of two independent experiments with duplicate/triplicate samples.

In situ hybridization

Cultured skins were treated with cold methanol before fixation. Whole embryos, isolated mandibles and methanol treated skins were fixed in 4% paraformaldehyde overnight, and processed for whole-mount in situ hybridization as described earlier (Mustonen et al., 2004) by using the InSituPro robot (Intavis AG, Germany). Digoxigenin-labeled probes were detected with BM Purple AP Substrate Precipitating Solution (Boehringer Mannheim Gmbh, Germany). The following probes were used: *edar* and *shh* (Laurikkala et al., 2002); a 994 base pairs probe specific to the *dkk4* sequence (nt 114–1107 of BC018400); a 824 base pairs probe specific to the *lrp4* sequence (nt 60–883 of NM_172668). Some stained samples were embedded in 0.5% gelatin, 30% albumin, 20% sucrose, 2% glutaraldehyde in PBS to be sectioned in 30 μm thick slices using a vibratome.

Results

Search for ectodysplasin-induced genes by microarray analysis

We have shown earlier that the formation of the first wave of hair placodes is restored in *eda*$^{-/-}$ back skin cultures when recombinant Fc-Eda-A1 protein is administered to the culture medium (Mustonen et al., 2004). In addition, we reported that Eda-A1/Edar target genes *ctgf/ccn2* and *shh* are induced already after 1 h of incubation with Fc-Eda-A1 protein, their expression levels peaking by 4 h (Pummila et al., 2007). We made use of these findings in designing the protocol for identification of immediate target genes of Edar (Fig. 1). Back skins of E14 *eda* -deficient mouse embryos were cut in two halves along the midline and these were cultured in hanging drops of culture medium with or without recombinant Fc-Eda-A1. The explants were harvested after 1.5 and 4 h and processed for hybridization on Affymetrix® microarray chips containing approximately 14,000 well characterized mouse gene probes. Genes presenting an at least 1.5-fold intensity difference of expression in treated compared to non-treated skins were considered as putative Eda target genes. Altogether, 22 genes were found upregulated after 1.5 h, and 142 genes after 4 h, and 16 of them were present in both arrays. Among the genes upregulated upon Eda treatment, we found *ctgf/ccn2* in both arrays, and *follistatin* and *shh* in the 4-hour array. These genes were previously identified as targets of Eda-A1 (hereafter Eda) (Mou et al., 2006; Pummila et al., 2007), indicating that our experimental procedure had been successful. In this study, we focus on genes related to Wnt signalling.

Dkk4 and lrp4 are induced by Eda whereas several other Wnt associated placodal genes are not

Several Wnt pathway genes have been proposed as possible Eda targets (Cui et al., 2006). Interestingly, our microarray experiment sorted out only two genes with prior association with Wnt signalling: *dkk4* and *lrp4*. *Dkk4* was the most highly differentially expressed gene (~30-fold increased) after 4 h of Eda treatment while *lrp4* was only moderately upregulated (~2-fold increased).

In order to validate the accuracy of the microarray data, we monitored the induction kinetics of *dkk4* and *lrp4* transcripts after treatment of *eda*-deficient skin explants with recombinant Eda protein by quantitative RT-PCR technique. E14 skin explants were cut into two halves along the dorsal midline and cultured with or without Fc-Eda-A1 (Pummila et al., 2007). RNAs were isolated after 1, 3, 5, 7 and 9 h of culture, and the number of *dkk4* or *lrp4* transcripts was measured. *Dkk4* expression increased rapidly and prominently, reaching about 2-fold within the first hour, and rising almost exponentially at least until 9 h when it was 130-fold. *Lrp4* expression increased more moderately showing a rather constant 2–3-fold induction at all time points (Fig. 2A). The induction of *dkk4* at 4 h was lower than in the microarray data set, most likely due to slight batch-to-batch variation observed with recombinant Fc-Eda-A1 (see also Fig. 6L).

Using the same experimental procedure, we monitored the fold of induction of some other Wnt pathway components which are co-expressed with *edar* in nascent hair placodes and have been proposed to be Eda targets (Andl et al., 2002; Cui et al., 2006), but did not show any significant changes in our microarray screen (Fig. 2B). These included the Wnt signalling components *lef1*, *β-catenin* and *wnt10b*, which appear in placodes when hair primordium morphogenesis is rescued by Fc-EdaA1 treatment in cultured *tabby* (*eda*$^{-/-}$) skin (Mustonen et al., 2004, and our unpublished data). In addition we examined the induction of *sostdc1* (also known as *ectodin* and *wise*), a secreted modulator of Wnt and BMP signalling (Itasaki et al., 2003; Laurikkala et al., 2003; O'Shaughnessy et al.,

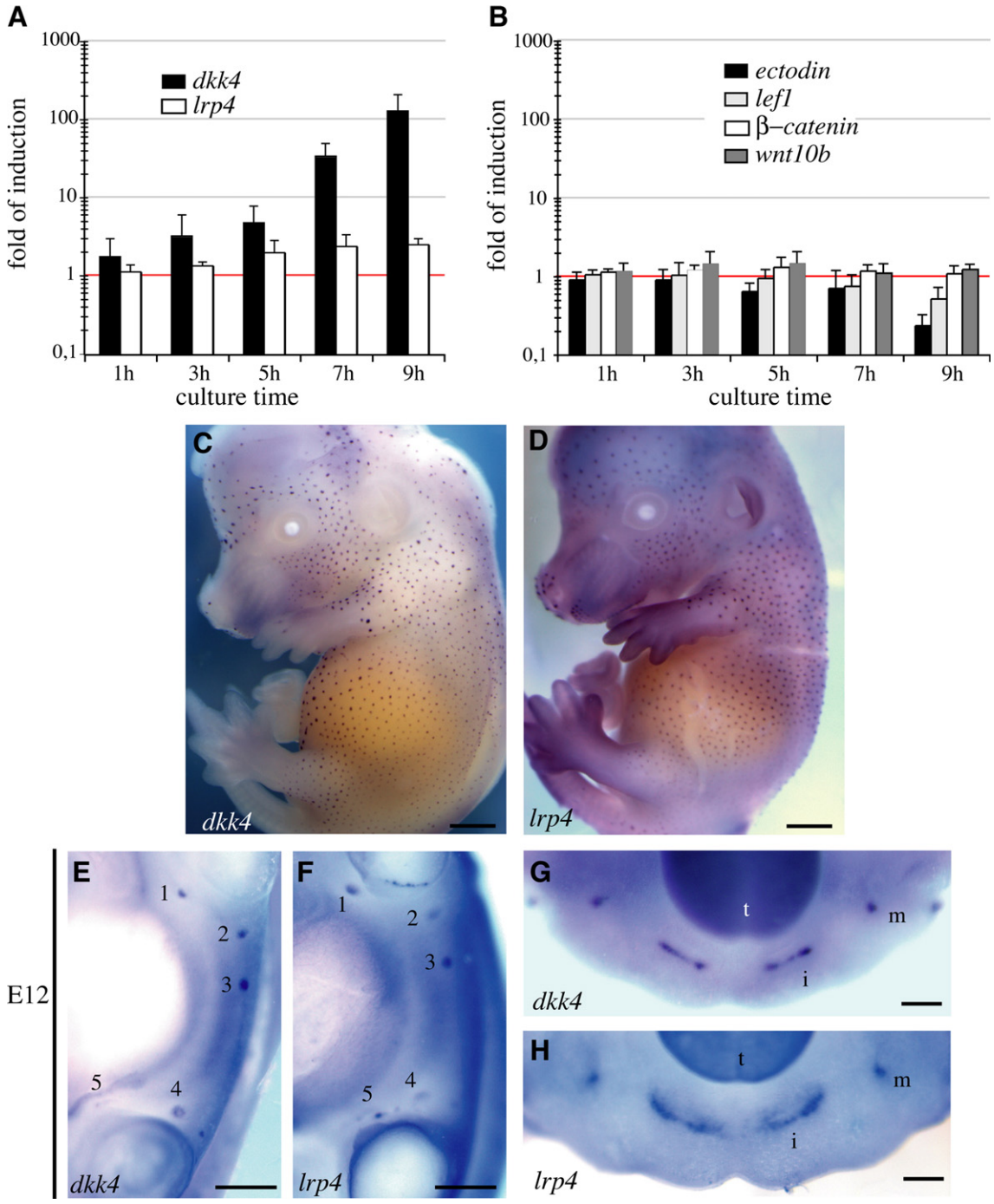


Fig. 2. Validation of *dkk4* and *lrp4* as putative target genes of Eda-A1/Edar signalling. (A, B) *Eda*$^{-/-}$ skin explants were cultured in hanging drops in the presence or absence of 2 μg/ml Fc-Eda-A1, and a time-course analysis induction of *dkk4* and *lrp4* (A) as well as of other Wnt pathway-associated genes (B) was performed by quantitative RT-PCR. *Dkk4* transcription constantly increased from 1 h to 9 h upon Fc-Eda-A1 stimulus. *Lrp4* was upregulated after 3 h of treatment and remained at fairly constant levels thereafter whereas no changes were observed in *ectodin*, *lef1*, *β-catenin* and *wnt10b* transcripts. (C–H) *Dkk4* and *lrp4* expression was monitored in E14 skin, and E12 lower jaw and mammary buds in wild-type embryos by in situ hybridization. (C, E, G) *Dkk4* and (D, F, H) *lrp4* were expressed in E14 wild-type primary hair follicles placodes, as well as in tooth and mammary buds (numbered) at E12. i, incisor placode; m, molar placode.; t, tongue. Scale bar: 1 mm in panels C, D; 500 μm in panels E, F; 200 μm in panels G, H.

2004) which was previously shown to be overrepresented in wild-type compared to *eda*$^{-/-}$ skin (Cui et al., 2006). In contrast to *dkk4* and *lrp4*, none of these genes was upregulated during the course of the 9-hour experiment. *Lef1*, and in particular *Sostdc1* levels even tended to decrease upon prolonged exposure to Fc-EdaA1. The fact that these placode markers were not induced in the time window analyzed supports the conclusion that genes upregulated upon brief exposure to recombinant Eda are likely to be direct transcriptional targets of Edar.

Dkk4 and lrp4 are expressed in placodes during ectodermal organ morphogenesis and upregulated in placodes of mice overexpressing eda

We next analyzed the expression of *dkk4* and *lrp4* by whole-mount in situ hybridization in E14.5 mouse skin, when the placodes of the first wave are well developed and express many placodal marker genes including *edar* (Laurikkala et al., 2002; Headon and Overbeek, 1999). *Lpr4*, and *dkk4* transcripts were restricted to hair placodes, further supporting their regulation by Edar-dependent signalling

(Figs. 2C and D). We then studied their expression in placodes of other ectodermal organs where *edar* transcripts are also present, i.e. mammary placodes and tooth placodes (Pispa et al., 2003; Pummila et al., 2007). At E12, *dkk4* and *lrp4* were both intensely expressed in mammary placodes (Figs. 2E and F) as well as in molar and incisor placodes (Figs. 2G and H). Thus, *dkk4* and *lrp4* seem to be general placode markers of ectodermal appendages and their colocalization with Edar is in line with regulation by Ectodysplasin signalling.

Next, we examined the expression patterns of *dkk4* and *lrp4* in *K14-eda-A1* mice characterized by overexpression of the ligand Eda-A1 throughout the developing ectoderm (Mustonen et al., 2003). In this transgenic line, hair placodes of the first wave are enlarged and sometimes fused at E14, as revealed by broader expression of known placodal markers (Mustonen et al., 2004). Expression of both *dkk4* and *lrp4* was more intense and in a larger area in hair placodes of *K14-eda-A1* embryos (Figs. 3A–D, C', D') as compared to wild-type litter mates (Figs. 3E, E' and F, F'). This suggests that stimulation of Edar signalling results in increased production of *dkk4* and *lrp4* transcripts also in vivo.

Dkk4 and lrp4 promoters possess NF-κB binding sites and dkk4 promoter is responsive to Edar

Since several studies have shown that Eda-A1/Edar signalling activates the NF-κB pathway (Yan et al., 2000; Koppinen et al., 2001; Kumar et al., 2001) we next examined the promoter regions of *dkk4* and *lrp4* for the presence of potential NF-κB responsive elements by computational analysis using mouse and human sequences to find evolutionary conserved sites (see Materials and methods for details) (Fig. 4A). Within the *lrp4* promoter sequence, 6 distinct putative NF-κB binding sites were found with a high score. Among those sites, one located 2271 base pairs upstream of the ATG was 100% identical between mouse and human and matched perfectly with the NF-κB consensus binding sequence. For *dkk4*, one putative site, located 446 base pairs upstream of the ATG was identified by the software, and by applying a slightly lower transcription factor score threshold 2 more conserved NF-κB recognition sequences were revealed. Hence these results suggest that *dkk4* and *lrp4* might be regulated by NF-κB further supporting the hypothesis that *dkk4* and *lrp4* are direct targets of Eda-A1/Edar.

To test whether the upstream promoter region of *dkk4* that included the conserved NF-κB sites was responsive to Edar, we cloned a 0.8 kb region of *dkk4* into a luciferase reporter vector. It is well established that TNF receptors can activate downstream signalling cascades in a ligand-independent fashion upon over-expression in transfected cells. We have previously shown that transfection of 293T cells with wild-type Edar induces the expression of a NF-κB luciferase reporter while mutant forms of Edar associated with the ectodermal dysplasia phenotype lead to severely compromised responses (Koppinen et al., 2001). After 24 h,

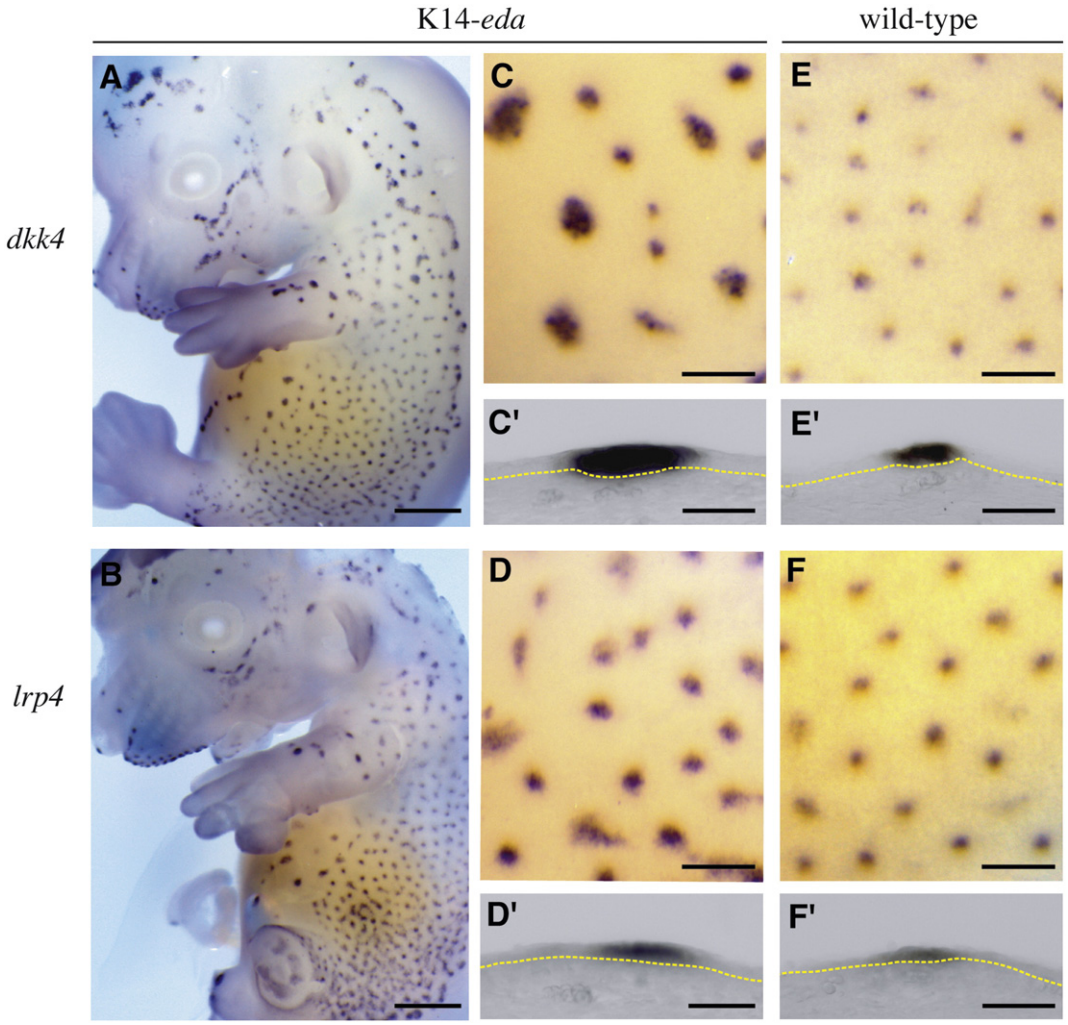


Fig. 3. *Dkk4* and *lrp4* expression is increased in K14-*eda* mice. Whole-mount in situ hybridization with probes specific to *dkk4* (A, C, E) and *lrp4* (B, D, F) at E14.5. *K14-Eda* embryos (A, B, C, D) revealed a more intense and broader expression of both genes compared to the expression pattern in wild-type litter mates (E, F). (C'–F') Vibratome sections of embryos stained with in situ hybridization. Scale bars: 1 mm in panels A, B; 200 μm in panels C–F; 50 μm in panels C'–F'.

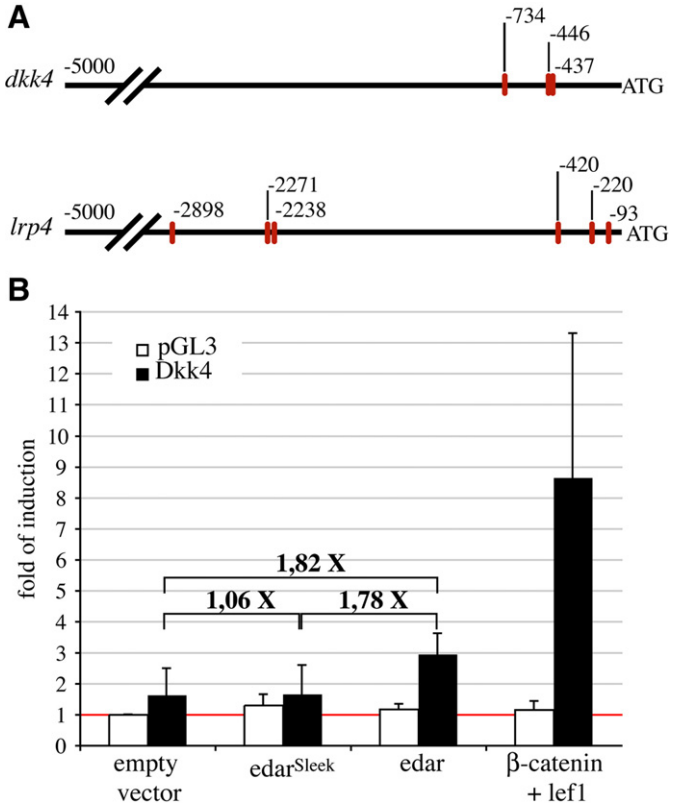


Fig. 4. Computational search for NF-κB binding sites in *dkk4* and *lrp4* promoter regions and luciferase reporter assays of *dkk4* promoter activation. (A) Several putative conserved NF-κB consensus binding sequences were identified within the 5000 bp region upstream of ATG in both *dkk4* and *lrp4* genes. Numbers refer to mouse promoter sequences. (B) 0.8 kb fragment of mouse *dkk4* and upstream promoters encompassing the NF-κB consensus elements were cloned into a promoter-less pGL3 luciferase reporter. Wild-type Edar-induced *Dkk4* promoter sequence drove the expression of the reporter moderately when co-expressed with wild-type Edar into 293T cells, and more strongly when co-transfected with *β-catenin* and *lef1* plasmids.

transfection of *edar* led to a moderate but noticeable 2-fold induction of *dkk4* promoter reporter construct as compared to the control plasmid or $edar^{Sleek}$ mutant (Fig. 4B). The modest activation of the 0.8 kb proximal *dkk4* promoter by Edar suggests that other regulatory regions either further upstream or downstream and not revealed by computational analysis are essential for the efficient Eda-A1-dependent induction of *dkk4* in vivo. Alternatively, other co-factors not present in 293T cells may be required for efficient induction by Edar.

Dkk4 and lrp4 are focally expressed in $eda^{-/-}$ skin in the absence of NF-κB activity

Since our results suggested that the Eda-A1/Edar pathway is upstream of *dkk4* and *lrp4*, we next examined the expression of these two genes in $eda^{-/-}$ mice. As mice lacking Eda-A1/Edar signalling do not show hair placodes at E14, nor present patterned expression of any placodal marker so far analyzed, including *edar* (Headon and Overbeek, 1999; Laurikkala et al., 2002), we expected absence of localized *dkk4* and *lrp4* expression in $eda^{-/-}$ skin. Surprisingly, both genes we expressed in pointed irregular patterns in the E14.5 $eda^{-/-}$ skin, albeit at much lower intensity than in wild-type skin (Figs. 5A, D; compare to Figs. 2C and D). The pattern of both genes in $eda^{-/-}$ embryos (Figs. 5B, E) was markedly different from that seen in E14 wild-type skin where the expression was more intense and decorated the regularly arranged and rather uniformly sized placodes of the first wave hair follicles (Figs. 5C, F). To our knowledge, *dkk4* and *lrp4* are the first placode markers showing a clear periodic expression pattern in *eda* mutant skin at E14. These *dkk4*- and *lrp4*-positive foci probably represent the same structures that were recently identified in histological sections of *Edar*-deficient E14.5 skin and termed as 'pre-placodes' (Schmidt-Ullrich et al., 2006).

We next studied whether the observed expression of *dkk4* and *lrp4* in the $eda^{-/-}$ embryos was dependent on the NF-κB pathway. We crossed $eda^{-/-}$ mice with mice reporting NF-κB activity via β-galactosidase expression (Bhakar et al., 2002), allowing us to monitor in the same embryos the NF-κB activity with X-gal staining, and the expression patterns of *dkk4* and *lrp4* by whole-mount in situ hybridization. At E14, while $NF\text{-}\kappa B^{REP}/eda^{-/-}$ embryos exhibited the same *lrp4* and *dkk4* expression pattern as $eda^{-/-}$ embryos (data not shown), they did not show patterned *edar* expression (Fig. 5G) nor NF-κB activity in skin (Fig. 5H), in contrast to $NF\text{-}\kappa B^{REP}$ mice in a wild-type C57Bl/6 background, where positive Xgal staining was evident in hair placodes (Fig. 5I). This indicates that NF-κB activity in primary hair placodes depends on Eda-A1/Edar signalling, as earlier suggested using an independent NF-κB reporter (Schmidt-Ullrich et al., 2006).

Dkk4 expression was dramatically reduced also in dental placodes of the lower jaw in $eda^{-/-}$ background. The staining patterns varied, however, between embryos. In some specimens, the expression was absent from incisors but faint expression was seen in the molar placodes (Fig. 5J, compare to Fig. 2G), whereas in other samples weak staining was present only in incisor placodes (Fig. 5K). *Lrp4* expression did not show any significant difference either in molar or incisor placodes as compared to the wild-type mice (Fig. 5L compare to 2H). In addition, *dkk4* and *lrp4* transcripts were also detected in $eda^{-/-}$ mammary placodes at E12 (data not shown). These observations indicate that *dkk4* and *lrp4* are still induced, although at a lower extent, after disruption of NF-κB signalling in ectodermal placodes, suggesting that other signals in addition to ectodysplasin regulate their expression in vivo.

Dkk4 and lrp4 show similar expression pattern in wild-type and $eda^{-/-}$ embryos at the time of hair follicle induction

Because *dkk4* and *lrp4* both showed localized expression in $eda^{-/-}$ pre-placodes at E14.5, we next examined their expression at the time when the first hair follicles are induced. At E13.5, both transcripts were readily observed in the first rows of hair placodes that appear parallel to mammary line (Figs. 6A–D). The same foci were also positive for NF-κB reporter activity (Fig. 6E). In addition, some other discrete and more disorganized stained foci were observed around the regularly patterned rows. At the same stage, both genes were also detected in vibrissae placodes and buds (Fig. 6). Interestingly, a similar pattern of both *dkk4* and *lrp4* was observed in E13.5 $eda^{-/-}$ embryos (Figs. 6F–I), whereas again no NF-κB activity was discernible (Fig. 6J). Taken together, our data suggest that this early placodal expression of *dkk4* and *lrp4* is independent of Edar/NF-κB signalling but subsequently, the establishment of the normal pattern of primary hair placodes and upregulation of *dkk4* and *lrp4* fails in *eda* mutant embryos.

Wnt signalling induces dkk4 and lrp4 expression and acts in parallel with the Eda-A1/Edar pathway

Dkk4 transcription has been reported to be increased in the fore-brain of mice carrying a dominant-active β-catenin ($N^{cre/b\text{-}cat\Delta ex3+/-}$ embryos), suggesting that it could be downstream of canonical Wnt activity (Diep et al., 2004). Because Wnt signalling has an established early function in placode formation, we tested whether Wnt signalling might be responsible for the *dkk4* and *lrp4* expression occurring in $eda^{-/-}$ skin. We compared the fold of induction of *dkk4* and *lrp4* by quantitative real-time PCR in E14 $eda^{-/-}$ back skin treated for 4 h with

either Fc-Eda-A1 or 6-bromoindirubin-3′-oxime (BIO), a pharmacological inhibitor specific to glycogen synthase kinase-3β (GSK-3β) (Meijer et al., 2003) that stimulates canonical Wnt pathway or both factors together (Fig. 6K). BIO induced *dkk4* expression in a dose-dependent manner from 2.4 to 5.3-fold with BIO concentrations from 5 μM to 20 μM. The addition of both factors together led to an overall higher induction, corresponding approximately to the sum of each treatment alone. Similar findings were observed with *lrp4* but at a lower extent.

To address the question whether the induction of *dkk4* by Eda-A1 was dependent on intact Wnt/β-catenin signalling, we used the casein kinase 1 inhibitor CKI-7 (Chijiwa et al., 1989; Price, 2006) to inhibit canonical Wnt activity. First, E14 *eda*$^{-/-}$ back skin halves were treated with 0 to 400 μM of CKI-7 for 4 h to block casein kinase 1 activity. After the pretreatment, the explants were transferred into fresh CKI-7 containing medium so that one half was exposed to Fc-Eda-A1 whereas the other one was used as a control (Fig. 6L). Low concentration (25 μM) of CKI-7 did not have a gross effect on *dkk4* induction by Eda-A1 although prominent variation between samples was observed. However, higher concentrations of CKI-7 lead to decreased induction of *dkk4* yet a more than 10-fold induction was evident even at a very high (400 μM) concentration of CKI-7. These data propose that Wnt signalling has a prominent contribution to the ability of Eda-A1 to induce *dkk4* yet Eda-A1 appears to regulate *dkk4* also independent of Wnt activity.

These results indicate that *dkk4* and *lrp4* are both targets of Eda-A1/Edar as well as Wnt signalling in developing skin appendages. In line with these data, *dkk4* promoter was recently shown to contain several LEF/TCF consensus binding sites and was responsive to ectopically expressed β-catenin and Lef1 in a luciferase reporter assay (Fig. 4B; Sick et al., 2006; Bazzi et al., 2007).

Dkk4 blocks hair placode formation

Although Dkk1 has an established role as a Wnt inhibitor and its overexpression in skin prevents completely hair placode formation (Andl et al., 2002), the function of Dkk4, a poorly characterized member of Dkk family, in skin appendage development is poorly understood. Therefore we examined the effect of Dkk4 on hair placode formation. We cultured E13.0 (i.e. prior to the formation of the first wave of hair follicles) wild-type skin for 24 h in medium supplemented with increasing doses of recombinant Dkk4 (Figs. 7A–D). Placode formation was monitored by in situ hybridization analysis of *shh* expression. Dkk4 inhibited the appearance of placodes in a dose-dependent manner (Fig. 7E) and the highest Dkk4 concentration (1 μg/mL) almost completely blocked placode formation. This result indicates that Dkk4 acts as an inhibitor of Wnt signalling in developing skin.

Discussion

Previous genome wide studies aiming at discovering Eda target genes have either compared the expression of genes in adult skin of mice with altered levels of Eda expression (Cui et al., 2002) or more recently have analyzed differentially expressed genes at various developmental stages in control vs. *eda*-deficient skin (Cui et al., 2006). However, interpretation of data derived from such experiments is not straightforward as all genes secondarily induced by primary Eda targets are likely to be differentially expressed as well. Here we report a novel approach for the identification of direct Eda target genes. We performed a microarray screen on embryonic *eda* null skin after a short-term exposure to recombinant Eda at the time when primary hair placodes form in wild-type skin.

Dkk4 and lrp4 are likely to be direct Eda target genes

Intriguingly, in our microarray screen we detected only two differentially expressed genes, *dkk4* and *lrp4*, with an obvious connection to Wnt signalling while the expression levels of a number of other Wnt pathway-related genes such as *kremen2*, *dkk1*, *wnt5a*, *wnt6*, *wnt10b*, *wnt11*, and *sostdc1* previously identified as candidate target genes of Eda (Cui et al., 2006) were not changed. As Eda and Wnt pathways are both activators of placode formation, we further analyzed in detail those Wnt-related genes co-expressed with Edar in developing skin appendages and with an established or proposed positive role in placode formation. In line with the microarray data, we found no alterations in β-*catenin*, *wnt10b*, or *lef1* mRNA levels upon Eda treatment indicating that these genes, although not focally expressed in *eda* mutant skin at E14 (Laurikkala et al., 2002; Andl et al., 2002), are not directly regulated by Edar. Interestingly, we noticed a downregulation of *sostdc1* after prolonged Eda treatment, a finding that is in fact consistent with the largely non-overlapping expression patterns of *sostdc1* and *edar* in vivo (Laurikkala et al., 2003).

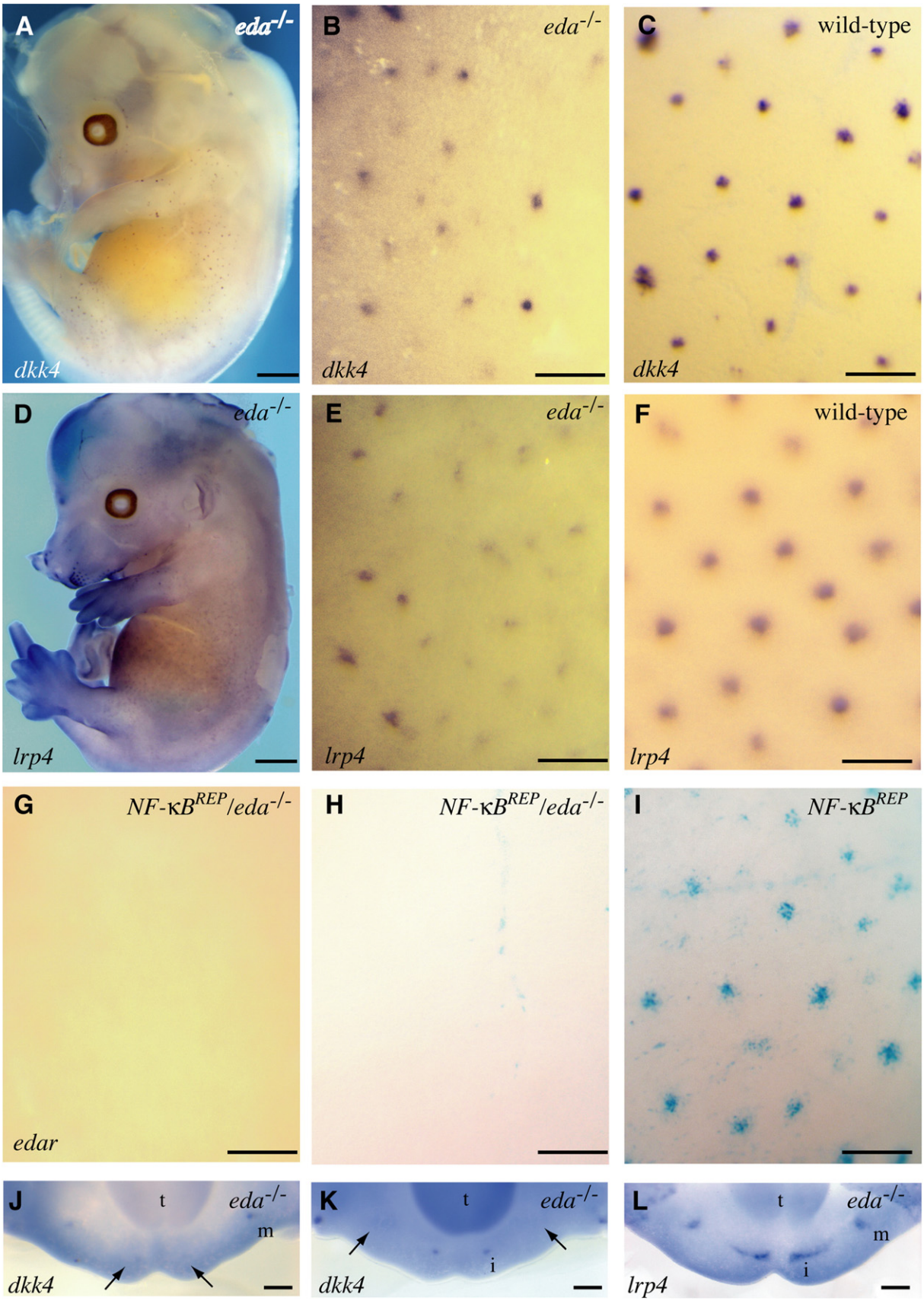
Based on the microarray, RT-qPCR, reporter gene, and expression analyses in mouse embryos with altered levels of Eda signalling we conclude that *dkk4* and possibly also *lrp4* are likely to be direct transcriptional targets of Edar. Our in situ hybridization analysis identified both *dkk4* and *lrp4* as novel markers of all skin appendage placodes. While we were finalizing our manuscript, similar expression patterns were also reported by others (Weatherbee et al., 2006; Sick et al., 2006; Bazzi et al., 2007). Dkk4 is a poorly characterized member of the Dickkopf family of Wnt modulators and in the absence of a *dkk4*-deficient mouse model its biological function has remained elusive. However, TOPFLASH reporter assays have shown that Dkk4 can co-operate with Kremen1/2 to suppress Wnt responses in a manner analogous to Dkk1 (Mao and Niehrs, 2003). In line with these data, our skin explant studies suggest that Dkk4 acts as a Wnt inhibitor during placode formation.

The mode of action of Lrp4 is not well established either. The extracellular domain of Lrp4 resembles that of Lrp5 and Lrp6, the co-receptors of Wnts involved in canonical Wnt cascade (Clevers 2006). Lrp4 was shown to inhibit β-catenin-dependent signalling in vitro (Johnson et al., 2005), and mice bearing null or hypomorphic alleles of *lrp4* present limb defects thought to reflect excess of Wnt activity (Johnson et al., 2005; Simon-Chazottes et al., 2006; Weatherbee et al., 2006) suggesting that Lrp4 acts as a Wnt antagonist in developing ectoderm. However, the kidney abnormalities of *lrp4* mutant mice are reminiscent of Wnt loss-of-function phenotypes. Moreover, Lrp4 is known to have Wnt-independent functions (May et al., 2007). The clarification of the role of Lrp4 in skin appendage development must await the detailed characterization of the *lrp4* null phenotype (Weatherbee et al., 2006).

Wnt activity precedes Eda signalling in nascent hair placodes

The expression of both *dkk4* and *lrp4* was detectable at E13.5 in the first rows of hair placodes that emerge both dorsal and ventral to the mammary line in wild-type embryos. This correlated well

Fig. 5. *Dkk4* and *lrp4* are expressed in *eda*-deficient skin at E14.5 in the absence of NF-kB activity. Whole-mount in situ hybridization with a probe specific to *dkk4* (A–C, J, K), *lrp4* (D–F, L) and *edar* (G), and whole-mount X-gal staining (H, I). *Dkk4* (A, B) and *lrp4* (D, E) are expressed in hair 'pre-placodes' in E14 *eda*$^{-/-}$ skin, but less intensely and in a perturbed pattern compared to primary hair placodes in wild-type E14.5 skin (C, G). Neither *edar* transcripts (G), nor β-galactosidase activity (H) is detected in E14.5 the skin of *NF-*/*eda*$^{-/-}$ embryos, whereas β-galactosidase activity is present in *NF-kB*^{REP} primary hair placodes (I). In E12 *eda*$^{-/-}$ lower jaws, strongly reduced amounts of *dkk4* were observed in dental placodes (J and K; arrows point to the location of incisor and molar placodes), while no gross changes in *lrp4* expression were detected (L). i, incisor placode; m, molar placode; t, tongue. Scale bars: 1 mm in panels A, D; 200 μm in panels B, C, E, F, G–L.


A
eda-/-
dkk4
B
eda-/-
dkk4
C
wild-type
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D
eda-/-
lrp4
E
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lrp4
F
wild-type
lrp4
G
NF-κBREP/eda-/-
edar
H
NF-κBREP/eda-/-
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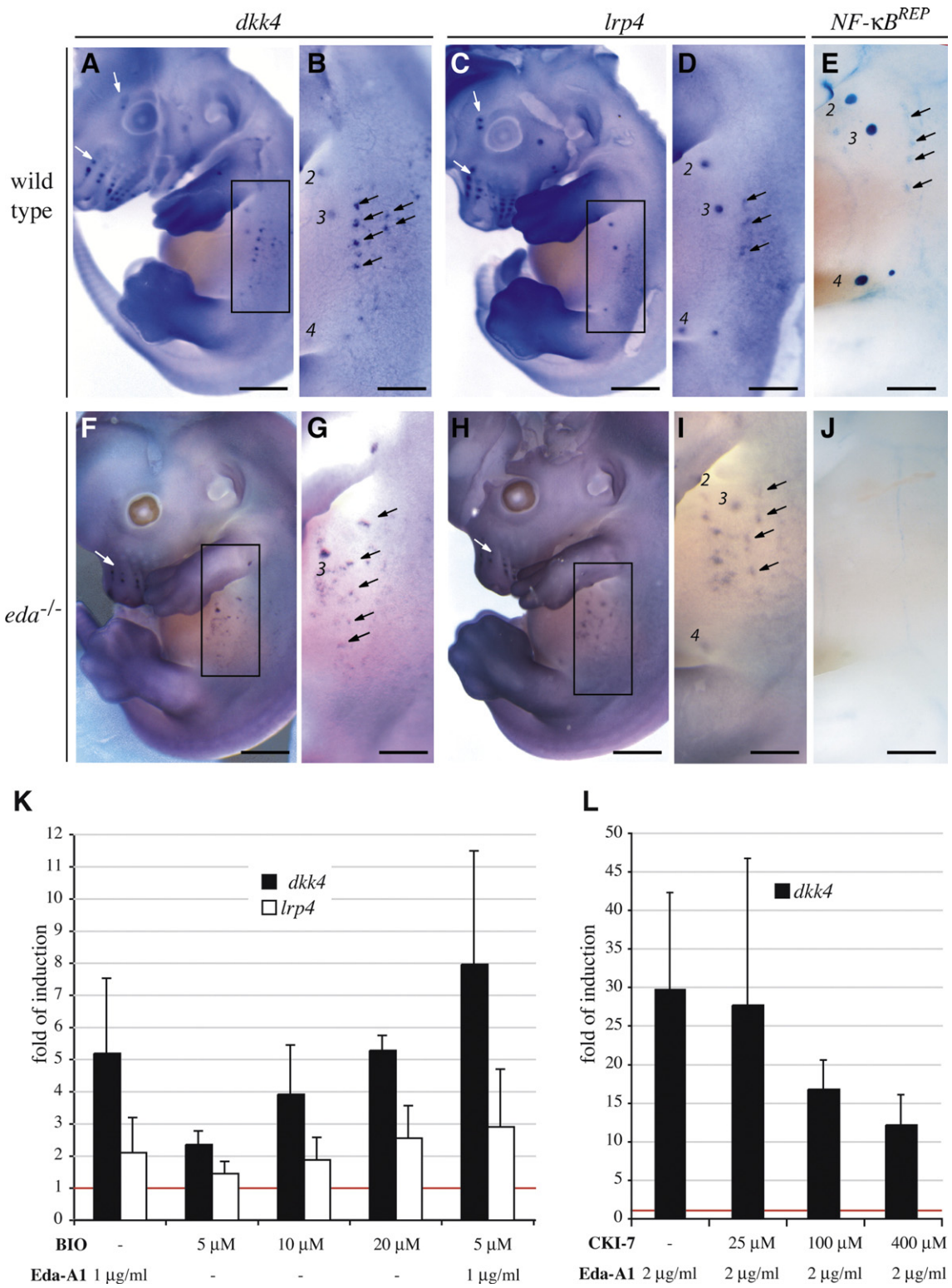

Fig. 6. *Dkk4* and *lrp4* show a similar expression pattern at E13.5 in lateral skin of wild-type and *eda*-deficient embryos, and are upregulated by both Wnt/β-catenin and Eda-A1/Edar signalling. (A–D, F–I) In situ detection of *dkk4* and *lrp4* in wild-type and *eda*$^{-/-}$ E13.5 embryos. Expression was detected in the first hair follicles (black arrows). In addition, mammary buds (indicated by numbers) and whiskers (white arrows) are positive. (E, J) Whole-mount X-gal staining of E13.5 *NF-κB*^{REP} and NF-κB^{REP}/eda$^{-/-}$ embryos. (K) *Eda*$^{-/-}$ skin explants were cultured as depicted in Figs. 1 and 2A in the absence or presence of 1 μg/ml Fc-Eda-A1, of 5 μM to 20 μM of the GSK3 specific inhibitor BIO, or of 1 μg/ml Fc-Eda-A1 and 5 μM BIO together. BIO was able to induce *dkk4* and *lrp4* expression in a dose-dependent manner, and the combination of 1 μg/mlFc-Eda-A1 and 5 μM BIO led to a further increase of *dkk4* transcripts. (L) Fc-Eda-A1 treatment of *eda*$^{-/-}$ skin led to the induction of *dkk4* expression after inhibition of Wnt/β-catenin with 100 μM and 400 μM CKI-7. Scale bars: 1 mm in panels A, C, F, H; 500 μm in panels B, D, E, G, I, J.

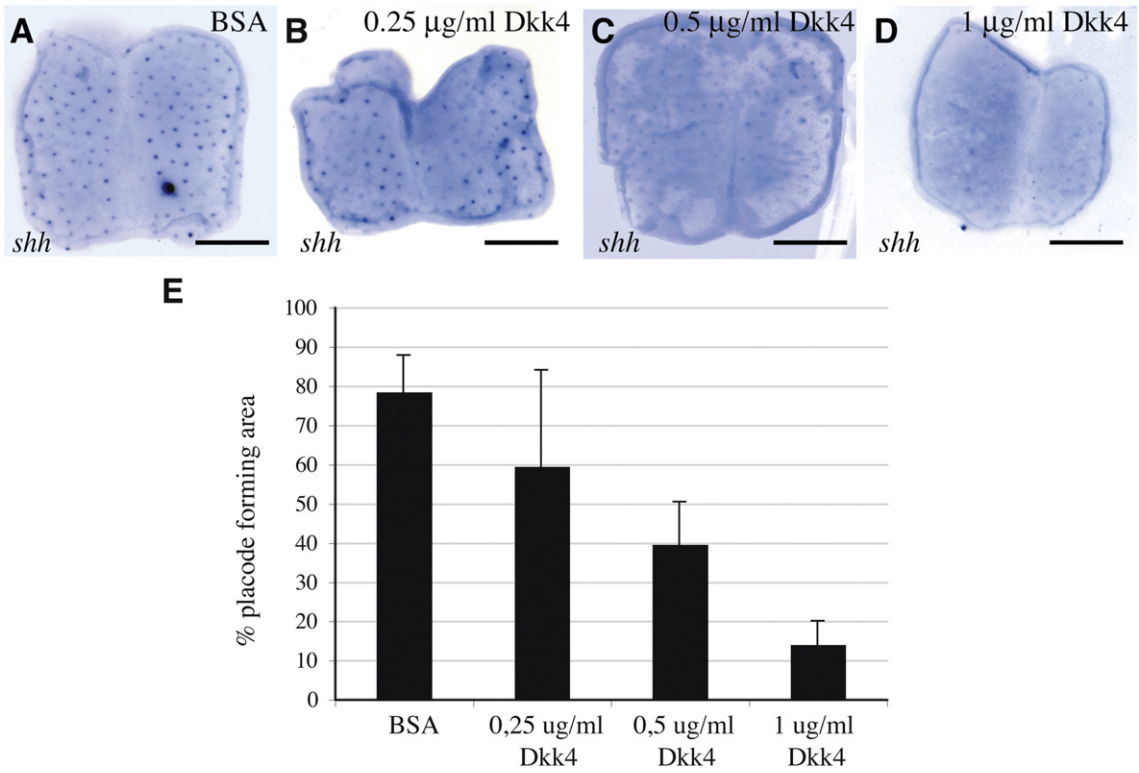

Fig. 7. Treatment of wild-type E13 skin with recombinant Dkk4 blocks primary hair placode formation in a dose-dependent manner. Wild-type E13 skin explants were cultured for 24 h in the absence (A) or presence of 0.25 (B), 0.5 (C) or 1 μg/ml (D) recombinant Dkk4 protein, and placode formation was monitored by *shh* in situ hybridization. A prominent suppression of placode formation was obtained with 1 μg/ml Dkk4. (E) The percentage of placode forming area compared to the whole explant area was calculated for each culture condition. Scale bar: 200 μm.

with the appearance of NF-κB reporter activity. Surprisingly, we found normal expression of both genes at E13.5 in the absence of Eda and NF-κB reporter activity. These data unequivocally demonstrate that Eda is dispensable for hair placode induction which is thought to take place in response to dermal cues (Mikkola and Millar, 2006). A day later, both *dkk4* and *lrp4* were still observable in a localized manner in *eda* mutant embryos albeit at strongly reduced levels. The expression of *dkk4* and *lrp4* in *eda*$^{-/-}$ embryos is likely to be due to Wnt activity based on the fact that their expression could be induced in embryonic skin by a brief treatment of BIO, the GSK-3β inhibitor. In line with these data, the proximal promoter of *dkk4* was responsive to β-catenin/Lef1 in reporter assays and contains multiple Tcf/Lef binding sites (Fig. 4B; Sick et al., 2006; Bazzi et al., 2007).

Our findings place the Wnt pathway upstream of Eda/NF-κB, but it remains to be determined whether any of the Eda pathway

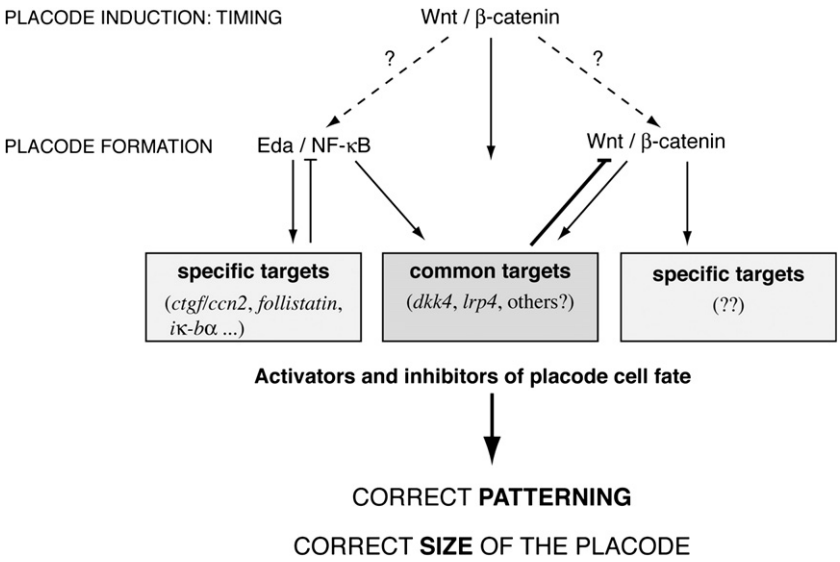

Fig. 8. A model for the interplay between Eda and Wnt pathways during primary hair placode formation. Epithelial Wnt activity is involved in the induction of hair follicles, possibly as an integral part of the response to the primary inductive signal(s) whose molecular identity is still unknown. In the next step, Eda and Wnt pathways may act in parallel in generation of the correct pattern and stabilization of nascent hair placodes. They both are likely to induce the expression of activators and inhibitors of placode cell fate and share some common target genes.

components are directly regulated by Wnt signalling. We propose a model (Fig. 8) where epithelial Wnt activity is essential during the induction stage of hair development, while both Wnt and Eda pathways may act in parallel in stabilization of placodes during the propagation of the first wave of hair follicles. It seems that Eda and Wnt pathways have several common target genes that are characterized by multiple conserved NF-κB and Tcf/Lef binding sites intermingled in their promoters while they apparently have also unique target genes essential for placode formation.

Complex molecular interactions regulate patterning of primary hair follicles

In wild-type embryos the first wave of hair follicles sweeps over the dorsum and head rapidly and a normal follicle pattern is established between E13.5 and E14.5. In *eda* null embryos *dkk4* and *lrp4* were expressed in a punctuate manner at E14.5, but with an obviously irregular spacing indicating a patterning defect. It seems plausible that Edar signalling that gets restricted to emerging placodes strengthens the placode identity of the pre-placode cells and thereby fixes the position of hair follicles. Our in vivo findings are in line with a model based on in vitro manipulation of skin explants with recombinant Eda suggesting that initially a labile, Eda-independent prepattern of primary hair placodes is generated which is however, subject to subsequent modulation by Edar activity (Mou et al., 2006). Unexpectedly, our results also indicate that the reinforcement of placode cell fate by Eda is not due to a direct positive effect on Wnt signalling although we cannot rule out the possibility that Eda indirectly promotes Wnt activity.

Our findings suggest that in addition to its role in expansion of placodes (Mustonen et al., 2004), Eda pathway is essential for the generation of a periodic pattern in the naïve embryonic ectoderm. The prevailing paradigm to explain the appearance of periodicity from an initially homogenous state (such as skin prior to hair follicle development) is the reaction–diffusion model (Nagorcka, 1984; Jung et al., 1998; Maini et al., 2006; Stark et al., 2007) originally formulated by Turing (Turing, 1952). In its simplest form, the model presumes the presence of two soluble factors: an activator that promotes its own production/activity as well as the production of its own inhibitor. When the inhibitor diffuses more rapidly and/or decays faster than the activator, foci (placodes) with high activator and inhibitor concentrations will appear. High local levels of the activator will amplify the signal (in placodal cells) while the inhibitor will prevent the reaction in the surroundings (i.e. impedes neighbouring cells from adopting the follicle fate). The molecular origin of periodic patterning of hair follicles remained obscure for long. Recent studies have suggested that the interplay between Wnts and their inhibitors Dkk1/4 plays a key role (Andl et al., 2002; Sick et al., 2006). However, the identification of Eda as a potent inducer of *dkk4* expression adds more complexity to the simple mathematical equations where hair follicle spacing was explained by Wnt-Dkk specific reciprocal interactions only (Sick et al., 2006). Moreover, it is apparent that the restriction of responsiveness to Eda by BMPs through downregulation of *edar* expression in interplacodal cells is another central aspect involved in hair follicle patterning (Mou et al., 2006). Thus, the reaction–diffusion seems to involve multiple signalling pathways with cross-talks, as already implied by Jiang et al. (2004).

Previous studies have established the role of Eda in several later aspects of skin appendage development such as tooth morphogenesis, nipple formation, and hair shaft production (Mikkola and Thesleff, 2003). More recent findings by us and others combined with those reported here indicate that Eda plays a prominent role in placode spacing and expansion by modulating BMP and Wnt pathways. The identification of Dkk4, a placode inhibitor, as the most highly induced gene in our microarray screen was a startling finding. The next task is to recognize those Eda target genes that mediate its activator functions. Although BMP inhibitors CTGF/CCN2 and follistatin (Mou et al., 2006; Pummila et al., 2007) probably contribute to these functions, other yet uncharacterized target genes are likely to be involved.

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