Gene 427 (2008) 42–46

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Candidate *EDA* targets revealed by expression profiling of primary keratinocytes from Tabby mutant mice

Diana Esibizione [a,b], Chang-Yi Cui [a], David Schlessinger [a,*]

[a] *Laboratory of Genetics, National Institute on Aging, NIH Biomedical Research Center, 251 Bayview Boulevard, Suite 100, Baltimore, MD 21224, USA*
[b] *Department of Histology, Embryology and Applied Biology, University of Bologna, Bologna 40126, Italy*

ARTICLE INFO

Article history:
Received 10 June 2008
Received in revised form 23 July 2008
Accepted 16 September 2008
Available online 24 September 2008

Keywords:
Anhidrotic ectodermal dysplasia
Ectodysplasin
Edar
Tbx1
Bmp7
Jag1

ABSTRACT

EDA, the gene mutated in anhidrotic ectodermal dysplasia, encodes ectodysplasin, a TNF superfamily member that activates NF-kB mediated transcription. To identify *EDA* target genes, we have earlier used expression profiling to infer genes differentially expressed at various developmental time points in Tabby (*Eda*-deficient) compared to wild-type mouse skin. To increase the resolution to find genes whose expression may be restricted to epidermal cells, we have now extended studies to primary keratinocyte cultures established from E19 wild-type and Tabby skin. Using microarrays bearing 44,000 gene probes, we found 385 preliminary candidate genes whose expression was significantly affected by *Eda* loss. By comparing expression profiles to those from *Eda-A1* transgenic skin, we restricted the list to 38 "candidate *EDA* targets", 14 of which were already known to be expressed in hair follicles or epidermis. We confirmed expression changes for 3 selected genes, *Tbx1*, *Bmp7*, and *Jag1*, both in keratinocytes and in whole skin, by Q-PCR and Western blotting analyses. Thus, by the analysis of keratinocytes, novel candidate pathways downstream of *EDA* were detected.

1. Introduction

Ectodermal dysplasias (EDs) are a heterogeneous group of hereditary genetic disorders comprising nearly 200 clinically distinguishable forms, with a combined frequency of about 7 in 10,000 births (Itin and Fistarol, 2004). EDs are defined by the deficiency of at least two ectodermal derivatives among hair, sweat glands, teeth and nails (Priolo et al., 2000). Anhidrotic/hypohidrotic ectodermal dysplasia (EDA/HED) is the most frequent form of ED, affecting the development of sweat glands, hair follicles and teeth in human patients and in animals (reviewed in Cui and Schlessinger, 2006).

EDA is caused by mutations in any of several members of *EDA* signaling pathway genes. The pathway includes the ligand ectodysplasin, receptor EDAR, and receptor adaptor protein EDARADD (Kere et al., 1996; Headon and Overbeek, 1999; Headon et al., 2001). *EDA* signaling accesses the canonical NF-kB cascade through TRAF6, NEMO and IkBα, and thus represents a new TNF subfamily for skin appendage development (Cui and Schlessinger, 2006). Accordingly, mutations in *EDA*, *EDAR* and *EDARADD* cause deficiencies in skin appendages, with mutations in the downstream TRAF6, NEMO, IkBα, and NF-kB genes also causing additional immune malfunction (Döffinger et al., 2001; Naito et al., 2002; Courtois et al., 2003).

The regulatory hierarchy of the *EDA* signaling pathway has proven to be complex. Shh, Wnt/Dkk, Bmp and LTβ pathway genes were shown to be located downstream of *EDA*-NF-kB (Andl et al., 2002; Cui et al., 2006, 2007; Headon and Overbeek, 1999; Mou et al., 2006; Närhi et al., 2008). Among various candidate target genes, some were downregulated in a wide range of skin appendages, some only in certain organs, and some only at delimitated times. This variety suggests that there are general and time- or organ specific targets of *EDA* for skin appendage development (Cui et al., 2006). However, none of the inferred target genes could carry out the entire range of *EDA* functions, and knowledge of the full spectrum of *EDA* targets and their cooperative interactions remains incomplete.

Genome-wide expression profiling of whole skin RNA from embryonic and adult mice has inferred a number of *EDA* target genes (Cui et al., 2002, 2006). In a complementary effort to discover target genes, we have now profiled gene expression pattern of cultured primary keratinocytes from wild-type and Tabby mice. This approach has revealed a number of candidate *EDA* target genes, including *Tbx1*, *Bmp7* and *Jag1*, that were not previously detected.

2. Materials and methods

2.1. Primary keratinocyte isolation and in vitro culture

Timed mating was set up with C57BL/6J male and Tabby female mice (C57BL/6j-A^{W-J}-Ta^{6J}). Fresh skin was harvested from the backs of E19 embryos just before delivery, and the epidermis/upper-follicle

Abbreviations: EDA, anhidrotic ectodermal dysplasia; FDR, false discovery rate; NF-kB, nuclear factor kappa B; Q-PCR, quantitative real-time PCR.

* Corresponding author. Tel.: +1 410 558 8337; fax: +1 410 558 8331.
E-mail address: SchlessingerD@grc.nia.nih.gov (D. Schlessinger).

0378-1119/$ – see front matter © 2008 Published by Elsevier B.V.
doi:10.1016/j.gene.2008.09.014

Table 1
"Candidate *EDA* target" genes from expression profiling of primary keratinocytes

Altered genes	Fold-changes in PK [a] (Ta/WT)	Fold-changes in WTTG skin (TG/WT)	Sublocalization in skin [b]	References
Transcription factors				
Tbx1	0.1 ▼	2.0 ▲	ORS	Zoupa et al. (2006)
Tsc22d3	2.2 ▲	0.3 ▼	n.d.	
Ifi204	2.3 ▲	0.6 ▼	n.d.	
Signal transduction				
Bmp7	0.1 ▼	1.5 ▲	Mx, DP	Rendl et al. (2005)
Ror2	0.1 ▼	2.7 ▲	n.d.	
Jag1	0.4 ▼	1.6 ▲	Epi, ORS, Mx	Estrach et al. (2006)
Edn1	0.4 ▼	1.6 ▲	Epi	Kim et al. (2007)
Inhba	0.5 ▼	1.7 ▲	Epi, DP	Cruise et al. (2004); Nakamura et al. (2003)
Cytokine/kinase/hormone				
Il23a	0.2 ▼	2.5 ▲	Epi	Piskin et al. (2006)
Areg	0.3 ▼	2.0 ▲	Epi	Yoshida et al. (2008)
Mark1	0.4 ▼	1.9 ▲	n.d.	
Nek6	0.4 ▼	1.9 ▲	n.d.	
Nppb	0.4 ▼	1.7 ▲	n.d.	
Enzyme/inhibitor				
Prss12	0.1 ▼	2.1 ▲	DP	Rendl et al. (2005)
Pla2g7	0.2 ▼	1.6 ▲	ORS	Rendl et al. (2005)
Stfa3	0.2 ▼	2.1 ▲	ORS	Rendl et al. (2005)
Glul	6.3 ▲	0.6 ▼	n.d.	
Transporter				
Oabpl3	0.4 ▼	1.6 ▲	n.d.	
Slc16a6	0.4 ▼	2.0 ▲	n.d.	
Zinc finger protein				
Zfp518b	0.4 ▼	1.6 ▲	n.d.	
Zdhhc2	2.5 ▲	0.4 ▼	n.d.	
Fhl1	9.6 ▲	0.6 ▼	n.d.	
Ca^{++}/protein binding				
S100a8	0.3 ▼	7.0 ▲	Epi, Mdu	Schmidt et al. (2001)
Whrn	0.3 ▼	2.0 ▲	n.d.	
Pdzrn3	0.4 ▼	1.7 ▲	n.d.	
Nrp2	0.4 ▼	2.1 ▲	n.d.	
Cd44	0.4 ▼	1.7 ▲	Epi, DP	Tuhkanen et al. (1999)
Tnnt2	0.5 ▼	2.1 ▲	n.d.	
Ahnak	2.1 ▲	0.5 ▼	Epi	Masunaga et al. (1995)
Tubulin/cell cycle/heat shock				
Tubb2a	0.3 ▼	1.6 ▲	n.d.	
Mad1l1	0.4 ▼	1.6 ▲	n.d.	
Dnaja1	0.5 ▼	1.6 ▲	n.d.	
Unknown function				
Palmd	0.3 ▼	1.5 ▲	ORS	Rendl et al. (2005)
Ler5	0.4 ▼	1.5 ▲	n.d.	
Prkrip1	0.5 ▼	1.6 ▲	n.d.	
Herpud1	2.4 ▲	0.6 ▼	n.d.	
Arrdc3	2.7 ▲	0.5 ▼	n.d.	
0610010D20Rik	3.4 ▲	0.5 ▼	n.d.	

[a] ▼ and ▲ represent down- and upregulated genes in Ta keratinocytes or *Eda-A1* transgenic (WTTG) skin. The false discovery rate for listed genes are <0.01, corresponding to *P*-values <0.0005.
[b] ORS, outer root sheath of hair follicle; Mx, hair follicle matrix; DP, dermal papillae; Epi, epidermis; Mdu, medulla; n.d., newly-identified genes, sublocalization in skin not yet determined.

segment was isolated from dermis by enzymatic digestion (CellnTec, Bern, Switzerland). At this stage, guard and awl hair follicles are growing and the highly prevalent zigzag hair follicles are being initiated. Thus, isolated keratinocytes are heterogeneous, including epidermal keratinocytes and epidermal cells from the various types of hair follicles.

Primary keratinocytes were cultured in CnT-07 medium (Chemicon International, MA, USA) at 35 °C in 5% CO_2 atmosphere. Genotyping to confirm sex and Tabby mutation status was done by PCR and subsequent enzymatic digestion as previously described (Cui et al., 2006). Both wild-type and Tabby keratinocytes were morphologically heterogeneous and grew slowly until about passage 7, when growth accelerated with the expected spontaneous immortalization.

2.2. RNA isolation, gene expression profiling and Q-PCR

Total RNA was isolated from wild-type and Tabby primary keratinocytes at passage 4 using Trizol (Invitrogen, CA, USA). RNAs were then LiCl precipitated as previously described (Cui et al., 2005). Quality was checked by electrophoresis. RNAs were cyan-3-labeled and hybridized to the 44,000-feature 60-mer-oligo Agilent microarray (Carter et al., 2003). Duplicate hybridization data were obtained from each of two different keratinocyte cultures and analyzed by ANOVA, with the false discovery rate (FDR) set to ≤0.01, fold-differences ≥2, and log intensity ≥3.0 (Carter et al., 2003). To narrow down the list to candidate EDA targets with further support, the resultant 385 "preliminary candidate" genes were further compared to our previous expression profiles of adult stage Eda-A1 transgenic skin (Cui et al., 2006). We selected genes that were downregulated in Tabby keratinocytes but upregulated in Eda-A1 transgenic skin (or upregulated in Tabby keratinocytes and downregulated in Eda-A1 transgenic skin) as "candidate EDA targets". We then categorized them by their possible functions indicated in GO term (Table 1).

Selected candidate genes in resultant lists were confirmed by One-step Q-PCR with TaqMan "Assays on-Demand" probe/primers (Applied Biosystems, NJ, USA). The more readily available cells from passages 7 to 9 were used for this confirmation. To quantify the relative changes in gene expression, the $-2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used and reactions were normalized to GAPDH expression levels. In addition, we isolated RNAs from skin samples of Tabby and wild-type littermates at embryonic stages E15.5, E16.5, E18.5 and postnatal day 1 (P1) for Real-Time PCR analysis.

2.3. Protein isolation and Western blot analysis

Proteins were isolated from primary keratinocytes by vigorous vortexing in ice-cold RIPA buffer [containing 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0.) (Sigma, MO, USA)] and centrifugation. The supernatant was designated as Ext 1. The pellet was then suspended in RIPA buffer +1% sodium dodecyl sulfate and sonicated to extract less soluble proteins (Ext 2). Protein concentrations were measured with the Bradford method, using a Bio-Rad protein assay system (Bio-Rad, CA, USA). Extracted proteins were then denatured by adding β-mercaptoethanol (5% in final volume) and boiling for 5 min. 40 μg of each extract was separated on a 12% Tris-glycine acrylamide gel (Invitrogen) and transferred to a nitrocellulose

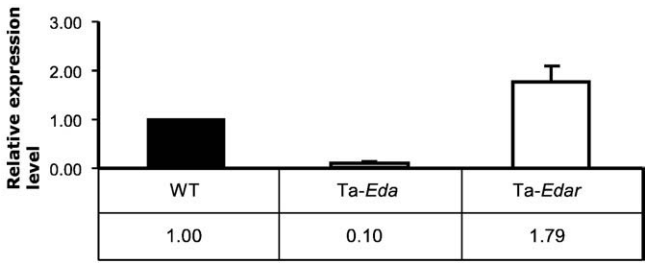

Fig. 1. Expression level of *Eda* and *Edar* in primary keratinocytes from wild-type (set to 1.0) and Tabby mice. *Eda* expression was significantly downregulated, whereas *Edar* was slightly upregulated in Ta keratinocytes (Ta-*Eda* and Ta-*Edar*).