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

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

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.

Keywords

Anhidrotic ectodermal dysplasia; Ectodysplasin; Edar; Tbx1; Bmp7; Jag1

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

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

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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 down regulated 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 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

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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 membrane (GE Healthcare, NJ, USA). The membrane was then blocked with 5% non-fat dry milk in 1 × phosphate buffered saline containing 0.1% Tween-20 and incubated with primary antibodies overnight at 4 °C. The antibodies included rabbit polyclonal anti-Tbx1 (Abcam, MA, USA), dilution 4 μg/ml; goat polyclonal anti-Pitx1 (N-15) (Santa Cruz Biotechnology, CA, USA), dilution 1:200; and rabbit polyclonal anti-Sox11 (H-290) (Santa Cruz Biotechnology), dilution 1:200. After washing in PBS containing 0.1% Tween-20, the membranes were incubated with HRP-conjugated donkey anti-goat or donkey anti-rabbit antibodies (Santa Cruz Biotechnology) for 2 h at room temperature. Immunoreactive bands were then visualized with an enhanced chemiluminescence (ECL) kit (Amersham Buckinghamshire, UK).

3. Results and discussion

EDA signaling regulates initiation and progression of skin appendages during early developmental stages and hair shaft formation at later stages. Shh, BMP, Wnt, and LTβ pathways have all been implicated in these processes downstream of *EDA* signaling (Cui and Schlessinger, 2006). Recent findings suggested that *EDA* signaling also regulates hair follicle cycling during postnatal life, through the apoptosis-related XIAP (X-linked inhibitor of apoptosis protein) (Fessing et al., 2006). Thus *EDA* signaling likely regulates a variety of genes in its action in different appendages at different stages.

A number of downstream targets of *EDA* signaling have been revealed by comparing gene expression profiles of whole skin samples from *Eda*-expressing (wild-type) and *Eda*-null (Tabby) animals (Cui et al., 2006). However, the expression of *EDA* pathway genes is restricted to epidermis and the epidermal part of skin appendages (Cui and Schlessinger, 2006); and because the epidermis comprises only about 1/10th of whole skin, we reasoned that RNA species from many *EDA*-responsive genes might be diluted in whole skin RNA preparations to a level too low to be seen easily. Furthermore, whole skin is difficult to manipulate for in vitro studies of *EDA* signaling. We therefore established primary keratinocytes from wild-type and Tabby skin as a possible cellular model to extend the studies with whole skin.

3.1. Expression profiling of wild-type and Tabby primary keratinocytes

Before starting transcription profiling, we carried out Q-PCR for *Eda* and *Edar* to confirm that *EDA* pathway members are expressed in the primary keratinocytes. Both *Eda* and *Edar* were highly expressed in wild-type primary keratinocytes. As expected, *Eda* expression was significantly downregulated in Tabby keratinocytes; however, *Edar* expression level was comparable to or even slightly higher in Tabby than in wild-type (Fig. 1). Expression levels of

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further downstream genes, Edaradd, Nemo and Rela in Tabby keratinocytes were also comparable to wild-type in expression profiles (data not shown). These results suggested that the *EDA* pathway is active in wild-type keratinocytes and might be primed to function even in Tabby keratinocytes.

Microarray hybridization of RNAs from passage 4 cells yielded a list of 385 genes with significantly altered expression (see Materials and methods). The list included 208 genes downregulated and 177 genes upregulated in Tabby keratinocytes (Supplementary Table 1). We designated these genes as "preliminary candidate" genes.

Because of the differences of the cell system from intact skin and the possible drift during cell culture, additional criteria were applied to identify the most likely true targets of *Eda* action. The keratinocytes proved to be resistant to transfection procedures, so that we could not simply ask what expression differences were reversed by introduction of an *Eda* gene into Tabby cells. Also, although TNF-alpha activated NF-kB as expected, recombinant ectodysplasin from two commercial sources did not stimulate the NF-kB pathway in the cells, perhaps because of poor multimerization or post-translational modification of the recombinant protein.

We therefore further discriminated the candidate *EDA* targets based on in vivo results, comparing the keratinocyte profiles with previous expression profiles of adult stage transgenic skin in which *Eda-A1* was expressed at a very high level (Cui et al., 2006). From this analysis, among the initial 385 genes, 38 were also upregulated at least 1.5-fold when the *Eda-A1* transgene was over-expressed and downregulated at least 1.5-fold when the transgene was not expressed. The subsets are designated as "candidate *EDA* targets" (Table 1). The 38 selected genes, classified according to their known or probable functions, include transcription factors, signaling proteins and protease inhibitors (Table 1). Most had not been associated with the *EDA* pathway in earlier studies with whole skin.

3.2. Confirmation of expression changes in keratinocytes by Q-PCR and Western blot assays for selected genes

We selected 4 genes from the "candidate *EDA* target" group (*Tbx1, Bmp7, Jag1* and *Prss12*), and 4 additional genes from the "preliminary candidate" group (*Pitx1, Foxg1, Sox11* and *Plau*) for further tests by Q-PCR and/or Western blotting approaches with primary keratinocytes.

Q-PCR analyses confirmed microarray results for 7 of the genes, *Tbx1, Bmp7, Jag1, Prss12, Pitx1, Foxg1* and *Plau*, representing both "candidate *EDA* target" and "preliminary candidate" groups (Figs. 2A, B). *Sox11* was not efficiently amplified by Q-PCR, but Sox11 protein was downregulated in Tabby keratinocytes, consistent with the microarray results (Fig. 2C). Notably, although the differences between wild-type and Tabby were unequivocal, positive Q-PCR signals for most of genes were observed only after about 35 cycles rather than the 30 or fewer that are sufficient for highly expressed genes. The results thus support the notion that genes expressed at low level can be more easily scored in keratinocytes then in whole skin. As expected, Western blot analysis confirmed that Tbx1 and Pitx1 were also significantly downregulated in Tabby keratinocytes (Fig. 2C). Pitx1 protein was detectable, but only at very low levels even in wild-type keratinocytes (Fig. 2C). Thus, Q-PCR and Western blot assays corroborate the microarray results for both "preliminary candidate" and "candidate *EDA* target" groups in primary keratinocytes. However, the shorter candidate gene list was more reliably confirmed in Q-PCR assays on whole skin, as follows.

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3.3. Q-PCR confirmed expression changes between wild-type and Tabby whole skin for selected genes from the "candidate EDA target" group

To assess expression levels of selected genes in vivo we carried out additional Q-PCR assays for 3 "candidate *EDA* target" genes, *Tbx1, Bmp7* and *Jag1*, and 3 "preliminary candidate" genes *Pitx1, Foxg1* and *Sox11* using RNA samples from skin of littermate wild-type and Tabby embryos at E15.5, 16.5, 18.5 and postnatal day 1 (P1).

Tbx1 was slightly downregulated in E15.5 Tabby skin and more sharply at E16.5, 18.5 and p1 (Fig. 3A). Downregulation of *Bmp7* in Tabby skin was less pronounced, but was significant at E16.5, 18.5 and p1 (Fig. 3B). *Jag1* was slightly downregulated in Tabby skin at E15.5 and significantly at E16.5 and p1 (though it was similar to wild-type at E18.5) (Fig. 3C). These results suggested that all genes are affected over a considerable developmental period for hair follicles in Tabby mice.

In contrast, 3 genes from the "preliminary candidate" group showed confirmatory levels in keratinocytes, but were either too low to detect (*Pitx1*) or gave non-significant expression changes (*Foxg1* and *Sox11*) in whole skin (data not shown). Further validation efforts will therefore be necessary to determine how many of the "preliminary candidate" genes from the list are truly involved in the response to *EDA* in vivo.

3.4. Implication of novel candidate target genes

The candidate target genes, especially those encoding transcription factors and signaling proteins, are most likely located downstream of the *EDA*-NF-kB cascade. NF-kB inactive mice showed phenotypes almost identical to Tabby mice (Schmidt-Ullrich et al., 2001, 2006; Cui et al., 2003), suggesting that NF-kB is the primary transcriptional player in *EDA* signaling pathway.

The newly associated transcription factors and signaling genes imply some further features of the mechanism of *EDA* action. *Tbx1*, a member of T-box transcription factor family, is involved in the development of many organs, and has been recently shown to be expressed in developing tooth germs and the outer root sheath of hair follicles, though with no known function (Zoupa et al., 2006; Rendl et al., 2005). *Tbx1* was significantly downregulated in E16.5 and E18.5 Tabby skin in our previous whole skin expression profiling (Cui et al., 2006), and we here confirmed the expression changes in vivo and in vitro by Q-PCR and Western blot assays. Significant downregulation of *Tbx1* in Tabby primary keratinocytes demonstrates that the in vivo expression changes are not a secondary effect of a different timing of hair follicle development in Tabby compared to wild-type mice. These results thus strongly suggest *Tbx1* as a proximal target of *EDA*.

Bmp7 was previously shown to be involved in feather placode formation in chicken (Harris et al., 2004) and was found to be expressed both in epidermal matrix region and dermal papillae of mice (Rendl et al., 2005). It was recently shown that dermal *Bmp7* was rapidly upregulated in skin organ cultures stimulated with ectodysplasin (Mou et al., 2006). *Bmp7* did not show significant changes in our previous expression profiling of whole Tabby skin (Cui et al., 2006), but is now revealed as a likely target of *EDA*, providing an additional link between the *EDA* and BMP pathways.

We also found that *Jag1*, a Notch pathway ligand, the critical factor for late stage hair follicle development (Millar, 2002), is regulated by the *EDA* pathway, with an in vivo expression pattern that suggested stage-specific regulation. This is the first indication of a possible functional interaction between the *EDA* and Notch pathways.

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Keratinocytes have several intrinsic limitations as a model system because they are heterogeneous; because their status compared to epidermal stem cell differentiation is not well defined; and because their gene expression profile may shift in culture compared to in vivo. However, they do express the *Eda* receptor, and compared to whole skin, they increase the sensitivity to recognize a number of additional *Eda* targets, particularly those expressed at a very low level in epidermal tissue. The cell system or cloned sublines may also provide a route to further analyses — for example, to discriminate primary vs. secondary targets of *EDA* action.

4. Conclusions

Defining the full spectrum of downstream target effectors is critical to understand *EDA* function in skin appendage development. Here we showed that primary keratinocytes from wild-type and mutant Tabby skin constitute an alternative source to explore *EDA* target genes. By genome-wide expression profiling, we inferred a list of 385 genes that are possible candidate *EDA* targets and a subset including *Tbx1, Bmp7, Jag1* and another 35 genes that are likely *EDA* targets. Keratinocytes may thus provide an in vitro model to study details of events involved in *EDA* signaling.

Abbreviations

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gene.2008.09.014.

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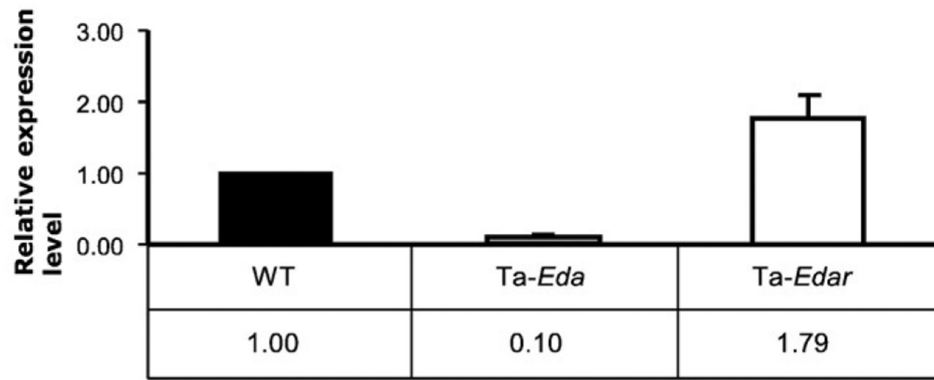

Fig. 1.

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*).

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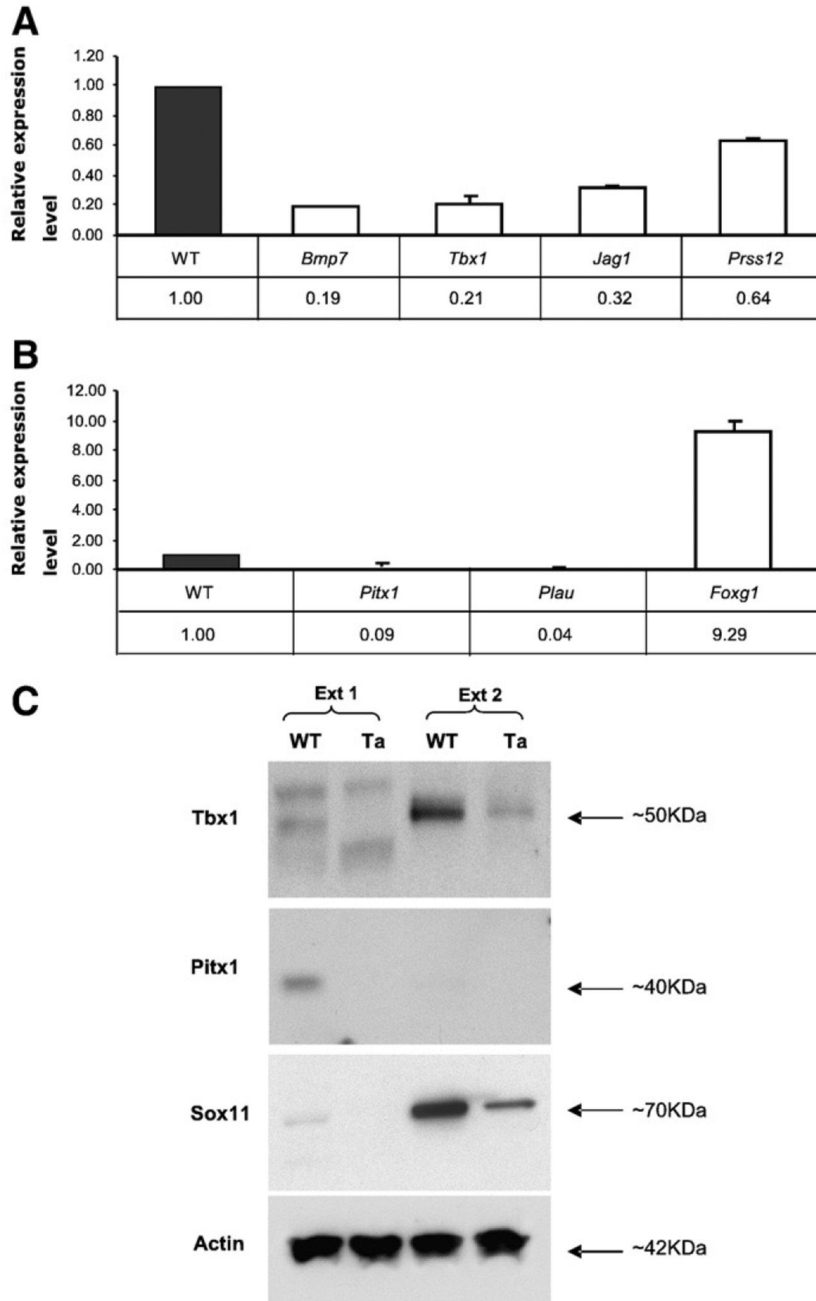

Fig. 2. Relative expression levels for selected genes in Ta primary keratinocytes

(A) *Bmp7, Tbx1, Jag1* and *Prss12* from the "candidate *EDA* target" group showed significant downregulation in Ta keratinocytes, with WT set to 1.0. (B) *Pitx1, Plau* and *Foxg1* from the "preliminary candidate" group also showed significant expression changes that were consistent with microarray results. (C) Western blotting assays showed that Tbx1 protein was significantly downregulated in Ta keratinocytes in the Ext 2 fraction (see Materials and methods). Pitx1 and Sox11 were also downregulated in Ta keratinocytes in Ext 1 and Ext 2 fractions, respectively. Actin was a loading control.

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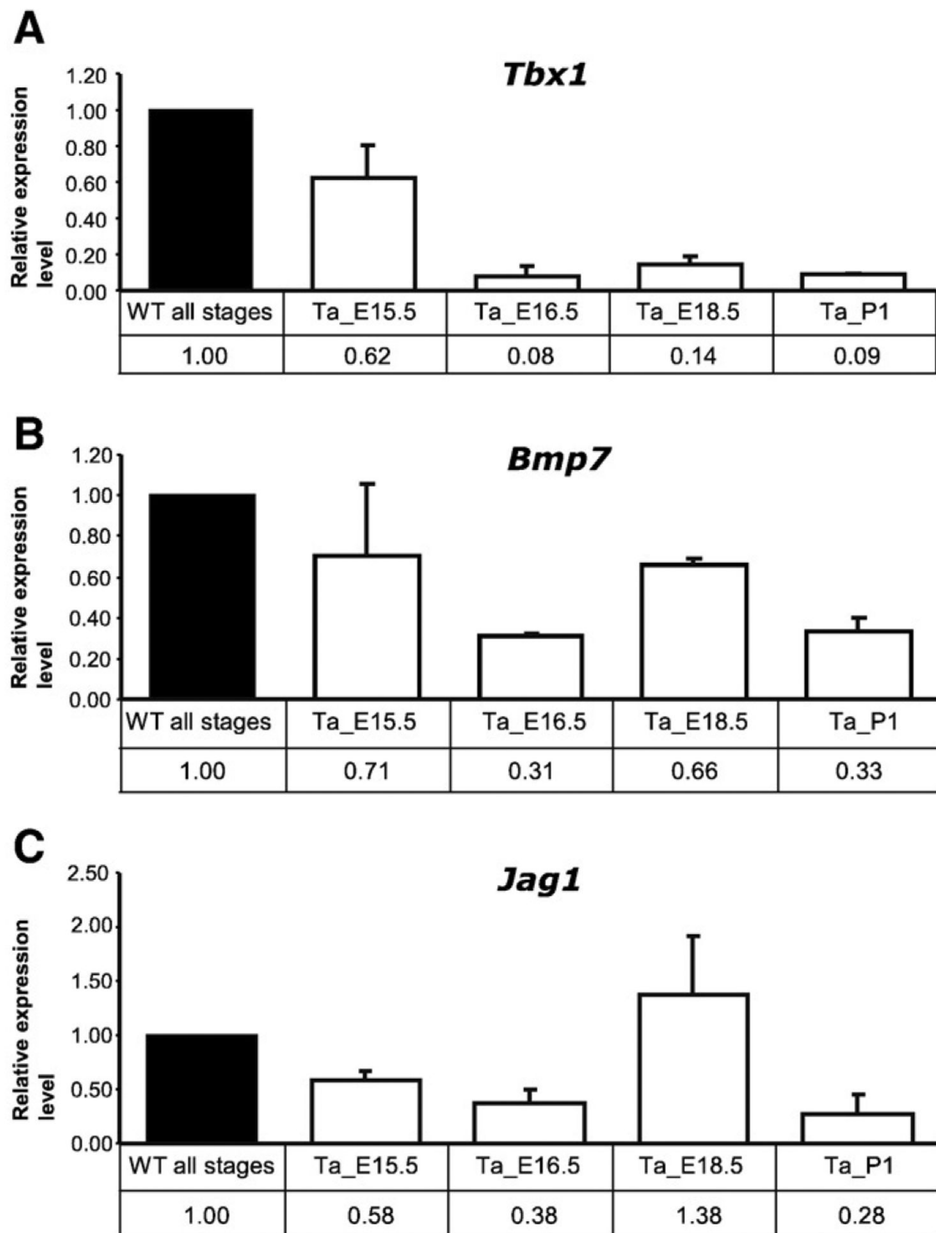

Fig. 3. Relative expression levels of *Tbx1, Bmp7* and *Jag1* in E15.5, 16.5, 18.5 and postnatal day 1 skin, with WT set at 1.0

(A) *Tbx1* was slightly downregulated in Ta skin at E15.5, and significantly thereafter. (B) *Bmp7* was significantly downregulated in Ta skin at E16.5 and thereafter. (C) *Jag1* was significantly downregulated in Ta skin at E15.5, 16.5 and p1, but was comparable to wild-type level at E18.5.

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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	

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Altered genes	Fold-changes in PK [a](Ta/WT)	Fold-changes in WTTG skin (TG/WT)	Sublocalization in skin [b]	References
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.