

RESEARCH ARTICLE

Functional Study of Ectodysplasin-A Mutations Causing Non-Syndromic Tooth Agenesis

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Abstract

Recent studies have demonstrated that ectodysplasin-A (EDA) mutations are associated with non-syndromic tooth agenesis. Indeed, we were the first to report three novel EDA mutations (A259E, R289C and R334H) in sporadic non-syndromic tooth agenesis. We studied the mechanism linking EDA mutations and non-syndromic tooth agenesis in human embryonic kidney 293T cells and mouse ameloblast-derived LS8 cells transfected with mutant isoforms of EDA. The receptor binding capability of the mutant EDA1 protein was impaired in comparison to wild-type EDA1. Although the non-syndromic tooth agenesis-causing EDA1 mutants possessed residual binding capability, the transcriptional activation of the receptor's downstream target, nuclear factor κB (NF-κB), was compromised. We also analyzed the changes of selected genes in other signaling pathways, such as WNT and BMP, after EDA mutation. We found that non-syndromic tooth agenesis-causing EDA1 mutant proteins upregulate *BMP4* (bone morphogenetic protein 4) mRNA expression and downregulate *WNT10A* and *WNT10B* (wingless-type MMTV integration site family member 10A and 10B) mRNA expression. Our results indicated that non-syndromic tooth agenesis causing EDA mutations (A259E, R289C and R334H) were loss-of-function, and suggested that EDA may regulate the expression of *WNT10A*, *WNT10B* and *BMP4* via NF-κB during tooth development. The results from our study may help to understand the molecular mechanism linking specific EDA mutations with non-syndromic tooth agenesis.

Introduction

Tooth agenesis is a term used to describe the failure to develop all normally developing deciduous or permanent teeth, and is one of the most common developmental anomalies in humans [1]. It can occur in isolation or in association with other genetic diseases as part of a recognized clinical syndrome, such as hypohidrotic ectodermal dysplasia (HED), which are defined as syndromic tooth agenesis [2]. The most common type of tooth agenesis occurs in isolation, and is called non-syndromic tooth agenesis (or isolated tooth agenesis) [2].

To date, non-syndromic tooth agenesis has been reported to be associated with mutations or polymorphisms in *MSX1*, *PAX9*, *AXIN2*, *WNT10A*, *EDA*, *EDAR* and *EDARADD* [3–16]. Among which, *EDA* mutations could cause X-linked hypohidrotic ectodermal dysplasia (XLHED) [17, 18], and have recently been linked to non-syndromic tooth agenesis [3, 4]. *EDA* is located on chromosome Xq12–q13.1 and encodes ectodysplasin-A (EDA), a member of the tumor necrosis factor (TNF) family [19]. EDA is a type II transmembrane protein with a small N-terminal intracellular domain, a furin cleavage site and a larger C-terminal extracellular domain containing a collagen-like repeat with a single interruption and a C-terminal TNF homology domain [20]. It has been demonstrated that eight isoforms of the EDA transcript can be created by differential splicing of the gene's 12 exons; however, only two isoforms differing by two amino acids, EDA1 (391 aa) and EDA2 (389 aa), contain a TNF homology domain [18, 20, 21]. Although EDA1 and EDA2 are closely related splice variants, the respective proteins appear to have different patterns of expression and receptor binding specificity in the mouse tooth placode, skin and hair follicles [21]. EDA1 has been shown to specifically bind to EDAR, a member of the TNF receptor superfamily, and activates the NF- κ B pathway [21]. EDA2 exclusively binds to XEDAR, another member of the TNF receptor superfamily, and also activates the NF- κ B pathway [21].

Previous investigations into the impact of syndrome-causing EDA mutations have shown that most syndrome-causing EDA mutations are predicted to cause an elimination of receptor signaling ultimately [22–25]. Interestingly, Mues *et al.* analyzed a EDA missense mutation in a family with X-linked recessive, non-syndromic tooth agenesis, and found that expression, receptor binding and signaling capability of the mutant EDA1 proteins were only impaired, rather than abolished [25]. We have described three novel EDA mutations (A259E, R289C and R334H) associated with sporadic non-syndromic tooth agenesis, with all the mutations located in the TNF domain [4]. In this study, we performed a functional analysis of these EDA mutations in comparison with another non-syndromic tooth agenesis-causing mutation S374R [25], and the HED-causing mutations H252L and Y343C [22], to determine the molecular mechanism through which they contribute to the pathogenesis of non-syndromic tooth agenesis.

Materials and methods

Construction of EDA expression vectors

Mammalian expression vectors for secreted FLAG-tagged forms of wild-type EDA1 and EDA2, EDA1 and EDA2 with HED-causing mutations H252L and Y343C, and expression vectors for human EDAR:Fc and human XEDAR:Fc, were kindly provided by Professor Pascal Schneider (Department of Biochemistry, University of Lausanne, Switzerland) [22]. Mammalian expression vectors for secreted Flag-tagged forms of EDA1 and EDA2 with the non-syndromic tooth agenesis-causing mutations A259E, R289C, R334H and S374R were generated using a QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies Company, Santa Clara, CA, USA) to conduct site-directed mutagenesis on wild-type EDA1 and EDA2 expression vectors.

Expression of soluble EDA in cells and supernatants

Vectors encoding soluble, wild-type or mutant FLAG-tagged EDA1 or EDA2 protein, EDAR:Fc or XEDAR:Fc were transfected into human embryonic kidney 293T cells, and the cells were maintained in serum-free OptiMEM (Invitrogen Corporation, Carlsbad, CA, USA) culture medium for 7 days. Subsequently, the cells and supernatants were collected separately. The detection of FLAG-tagged EDA1 or EDA2 proteins in cell lysates and supernatants was then performed by anti-FLAG western blot.

Immunoprecipitation assay

FLAG ligands (~1 mg in 100–400 μ l of cell supernatant) were added to 5 μ l of M2-agarose affinity matrix (Sigma-Aldrich, Louis, MO, USA), and mixed with receptors:Fc (~500 ng in 100–400 μ l of cell supernatant). All samples were diluted to 1 ml with PBS and incubated on a rotating wheel overnight at 4°C. Beads were washed three times with 1 mL PBS after centrifugation at 1,000 rpm for 2 min at 4°C. After the final wash, the pellets were resuspended in an equal volume of 2 \times sodium dodecyl sulfate (SDS) loading buffer and boiled for 5 min. Protein samples were analyzed using SDS-polyacrylamide gel electrophoresis. After separation by electrophoresis, the protein were transferred to a Millipore Immobilon-P membrane (EMD Millipore Corporation, Billerica, MA, USA), and incubated with an anti-FLAG (Sigma-Aldrich, Louis, MO, USA), anti-EDAR or anti-XEDAR antibody (R&D Systems, Minneapolis, MN, USA) for western blot analysis. For semi-quantitative analysis of western blot images, the bands were densitometrically analyzed via the ImageJ 1.49v software. The integrated density was the area of the band multiplied with the mean gray value. Results were assessed for statistical significance using Student's *t*-test.

Immunofluorescence

The mouse ameloblast-derived cell line LS8 was kindly provided by Professor Malcolm L. Snead (Center for Craniofacial Molecular Biology, University of Southern California, USA). The LS8 cells were maintained in DMEM (Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 mg/mL streptomycin. The pCR3 vector and vectors encoding wild-type or mutant EDA1 were transfected into LS8 cells using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions. Immunofluorescence was performed using primary antibody against NF- κ B p65 (Santa Cruz Biotechnology, Dallas, TX, USA). Alexa Fluor 594 (Molecular Probes, Invitrogen Corporation, Carlsbad, CA, USA) was used for detection. Slides were mounted with Vectashield Mounting Medium (VECTOR LABS, Burlingame, CA, USA) and imaged by fluorescence microscopy. The counting was performed with ImageJ 1.49v software. Results were assessed for statistical significance using Student's *t*-test.

Luciferase assay

LS8 cells were cotransfected with three plasmids (pNF- κ B-luc plasmid, EDA mutant plasmid, and pRL-TK plasmid) and cultured for 48 hours. The pCR3 vector and wild-type EDA vector were used as negative and positive controls, respectively. Following culturing, firefly and *Renilla* luciferase activities were measured using a dual luciferase assay system (Promega Corporation, Madison, WI, USA) on a Synergy 2 Multi-Detection Microplate Reader (BioTek, Winooski, VT, USA). Firefly luciferase activity was standardized against *Renilla* luciferase control activity. Each experiment was performed in triplicate and repeated at least four times. Results were assessed for statistical significance using Student's *t*-test.

Gene expression analysis

LS8 cells were cultured in 6-well plates and transfected with wild-type and mutant EDA1 using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA from untreated or transfected LS8 cells was extracted using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA), and reverse transcription was performed using the ThermoScript RT-PCR System (Invitrogen Corporation, Carlsbad, CA, USA). Real-time PCR was conducted using 2×SYBR-green PCR master mix on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA). Gene-specific primer sequences were obtained from the Primer Bank [26]. Values were normalized against *GAPDH* using the $2^{-\Delta\Delta C_t}$ method [27]. The global gene expression analysis was performed as previously described [28]. Results were assessed for statistical significance using Student's *t*-test.

Results

Non-syndromic tooth agenesis-causing mutant EDA possesses residual receptor-binding capability

Four non-syndromic tooth agenesis-causing EDA mutations A259E, R289C, R334H [4] and S374R [25], and two HED-causing EDA mutations H252L and Y343C [22] were tested for expression, receptor binding and signaling ability. All of these mutations were located in the TNF domain of the EDA protein. The results showed that the expression of wild-type and mutant EDA1 or EDA2 proteins could be detected in 293T cell lysates (Figs 1A and 2A) and supernatant (Figs 1B and 2B), indicating that all the protein constructs could be expressed and secreted out of the transfected cells. In order to investigate the receptor binding capability of the mutant EDA, we performed immunoprecipitation *in vitro*. The results showed that the binding of all non-syndromic tooth agenesis-causing EDA1 proteins to the receptor EDAR was variably reduced compared with wild-type, but not abolished (Fig 1C and 1D). In contrast, EDA1 carrying either of the two HED-causing mutations H252L and Y343C lost all receptor binding capability (Fig 1C and 1D). The binding affinity of all non-syndromic tooth agenesis-causing or HED-causing EDA2 proteins to XEDAR was variably reduced, compared with wild-type (Fig 2C and 2D). In contrast to EDA1, the HED-causing mutations H252L and Y343C in EDA2 retained residual receptor binding capability to XEDAR (Fig 2C and 2D).

Non-syndromic tooth agenesis-causing EDA1 mutant proteins impair the transcriptional activation of NF- κ B in LS8 cells

Eda is expressed in dental epithelium during tooth development [29]. In order to analyze the mechanism of EDA mutations affected the function of dental epithelial cells *in vitro*, we used an epithelium-derived ameloblast cell line LS8. It has been established that binding between Eda-A1 and its receptor induces activation of the Eda/Edar/NF- κ B signaling pathway [30, 31]. First, we checked whether LS8 cells express the Eda-A1 receptor, Edar. The results showed that LS8 cells express Edar (Fig 3). Because non-syndromic tooth agenesis-causing EDA1 proteins retain residual receptor binding activity, we performed immunofluorescence of NF- κ B subunit p65 and dual luciferase assay to investigate whether this reduced binding could induce transcriptional activation of NF- κ B in LS8 cells. The results showed that the red fluorescence of p65 in the nucleus was intense in most wild-type EDA1 transfected LS8 cells (Fig 4A–4C). However, the nuclear translocation of p65 was significantly reduced in non-syndromic tooth agenesis-causing and HED-causing EDA1 transfected LS8 cells (Fig 4G–4Y). Moreover, there was a significant difference in the number of p65 translocated nuclei between non-syndromic tooth agenesis-causing EDA1 mutants and HED-causing EDA1 mutants transfected LS8 cells

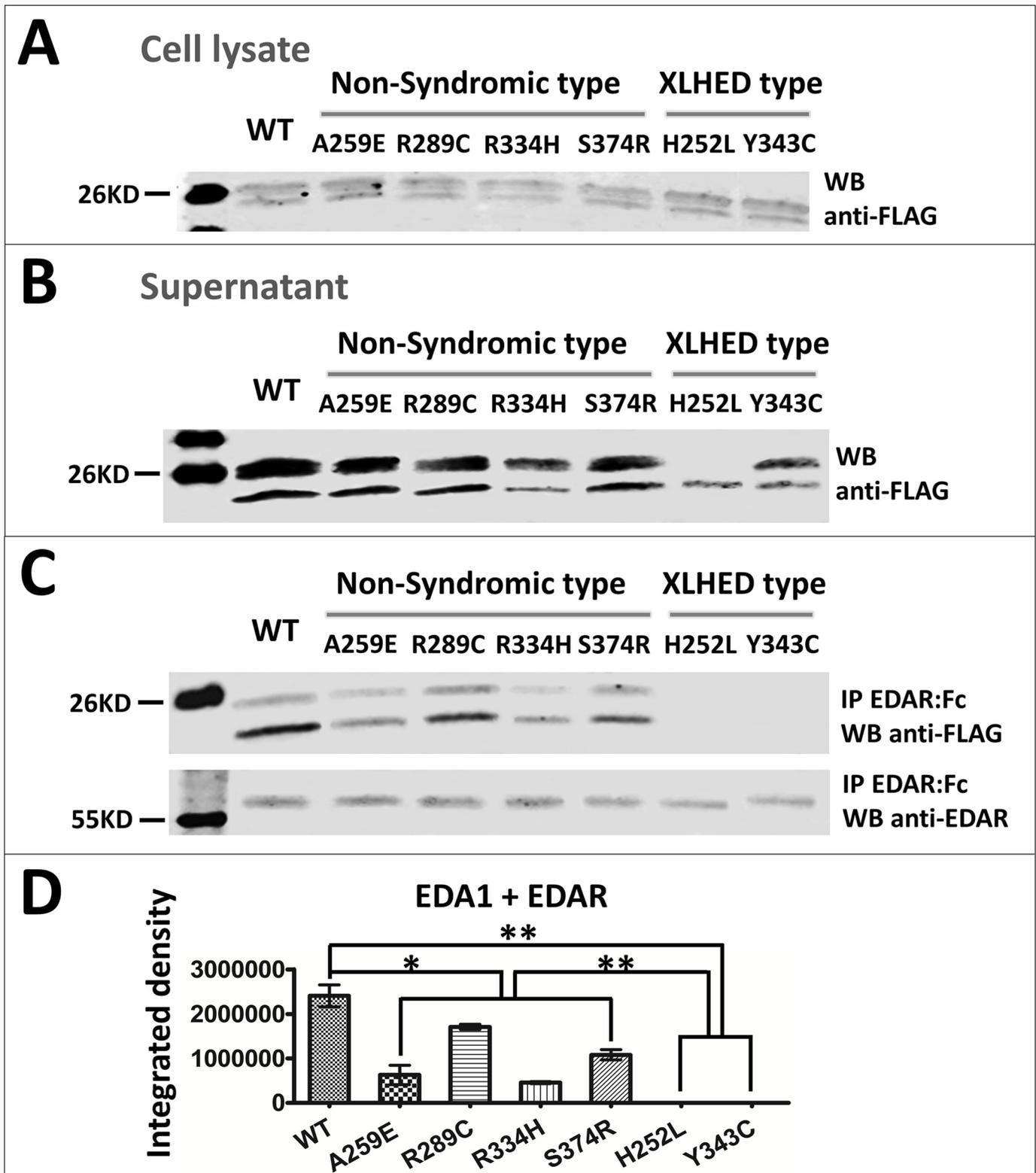


Fig 1. Expression and receptor binding of wild-type and mutant EDA1 proteins. Representative western blots depict the expression level of FLAG-tagged wild-type and mutated EDA1 in (A) cell lysate and (B) supernatant of transfected 293T cells. (C) Ligands in supernatants were immunoprecipitated with EDAR:Fc and analyzed by western blot. Precipitated EDAR:Fc is shown in the bottom panel. (D) Semi-quantitative analysis of the western blots image in Fig 1C upper panel. *, $P < 0.05$; **, $P < 0.01$; WB, western blot; IP, immunoprecipitated; WT, wild-type.

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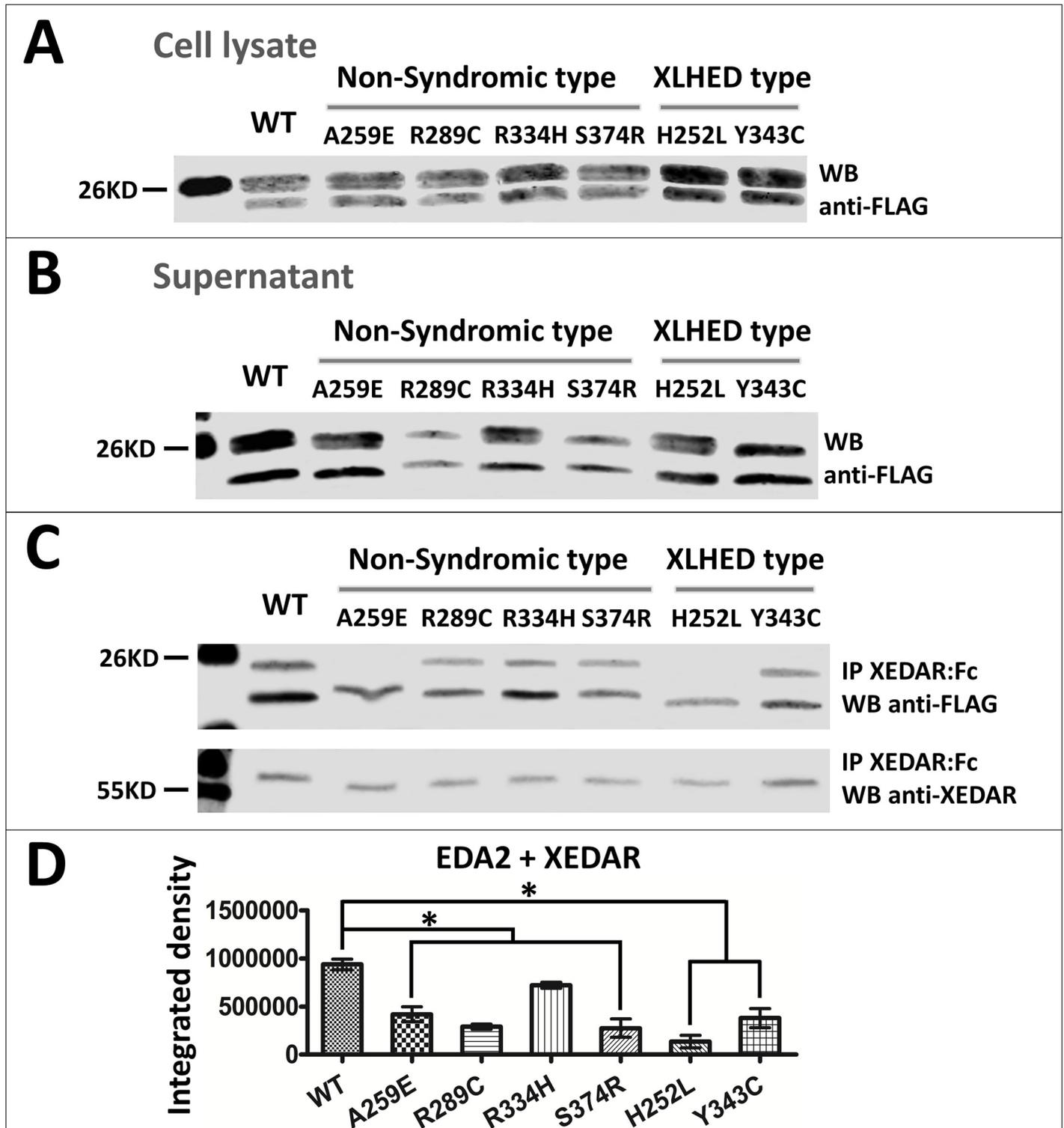


Fig 2. Expression and receptor binding of wild-type and mutant EDA2 proteins. Representative western blots depict the expression level of FLAG-tagged wild-type and mutated EDA2 in (A) cell lysate and (B) supernatant of transfected 293T cells. (C) Ligands in supernatants were immunoprecipitated with XEDAR:Fc and analyzed by western blot. Precipitated XEDAR:Fc is shown in the bottom panel. (D) Semi-quantitative analysis of the western blots image in Fig 2C upper panel. *, $P < 0.05$; **, $P < 0.01$; WB, western blot; IP, immunoprecipitated; WT, wild-type.

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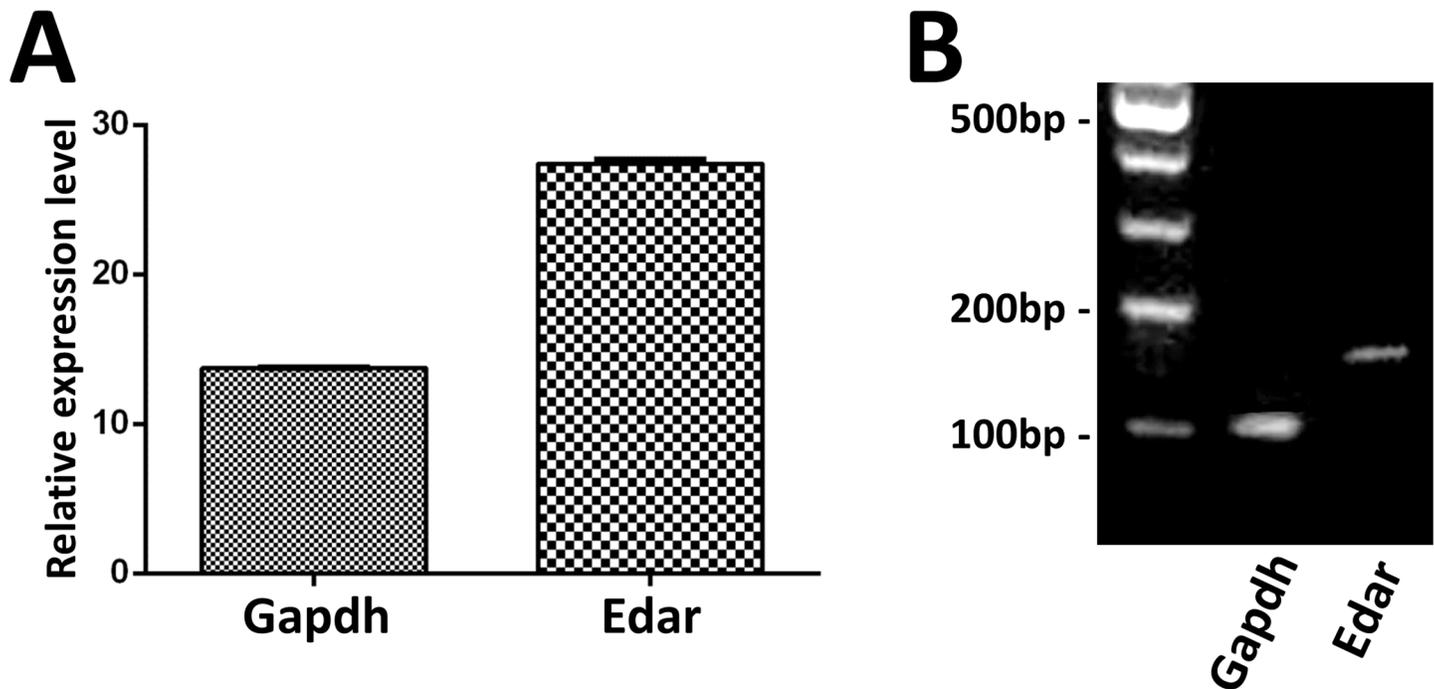


Fig 3. LS8 cells express the Eda-A1 receptor, Edar. Real-time PCR (A) and RT-PCR (B) were used to detect the expression of *Edar* in LS8 cells.

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(Fig 4Y). The result of the dual luciferase assay was coincident with our discovery from immunofluorescence of NF- κ B subunit p65, but there was no significant difference between non-syndromic tooth agenesis-causing EDA1 mutants and HED-causing EDA1 mutants (Fig 4Z). Taken together, our results indicated that non-syndromic tooth agenesis-causing EDA1 mutant proteins impair the transcriptional activation of NF- κ B in LS8 cells.

Non-syndromic tooth agenesis-causing EDA1 mutant proteins promote BMP4 expression in LS8 cells

The interaction of Eda-Edar and the Bmp signaling pathways is crucial for tooth morphogenesis [29], hair placode formation [32] and hair follicle patterning [33]. Bmp4 is a downstream target of Eda/Edar pathway during tooth development [29]. Therefore, we performed real-time PCR analysis to investigate *BMP4* expression levels in LS8 cells after transfection with non-syndromic tooth agenesis-causing or HED-causing EDA1 mutant proteins. The result revealed that both non-syndromic tooth agenesis-causing and HED-causing EDA1 mutants significantly increased *BMP4* expression in transfected LS8 cells (Fig 5A). Moreover, there was a significant difference in *BMP4* expression between non-syndromic tooth agenesis-causing EDA1 mutants and HED-causing EDA1 mutants in transfected LS8 cells (Fig 5A). This result suggested that either non-syndromic tooth agenesis-causing EDA1 mutants, or HED-causing EDA1 mutants, could promote *BMP4* expression in LS8 cells.

Non-syndromic tooth agenesis-causing EDA1 mutant proteins inhibit WNT10A and WNT10B expression in LS8 cells

The reciprocal interaction between Wnt/ β -catenin and Eda/Edar/NF- κ B signaling pathways is crucial for the development of ectodermal appendages [34, 35]. Wnt10a and Wnt10b show specific expression pattern and play important roles during tooth development [36–38].

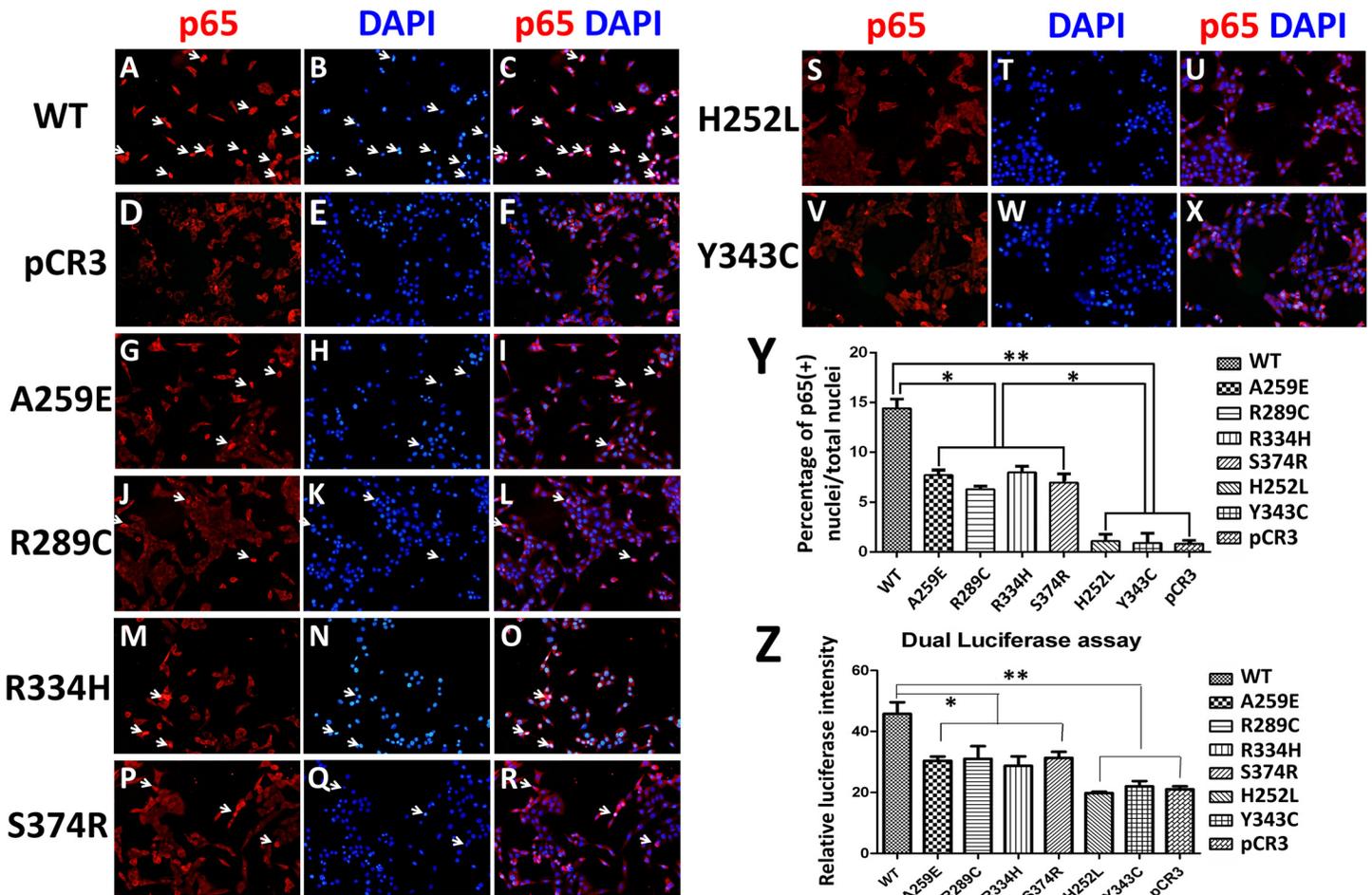


Fig 4. Transcriptional activation of NF-κB in LS8 cells transfected with wild-type and mutant EDA1 proteins. (A-X) Immunofluorescence of NF-κB subunit p65 (p65, red; DAPI, blue) in LS8 cells transfected with wild-type EDA1 (A-C), pCR3 (D-F), non-syndromic tooth agenesis-causing EDA1 mutants (G-R), and HED-causing EDA1 mutants (S-X). White arrows indicate the nuclear staining of p65. (Y) Quantitation of the ratio of p65- positive nuclei number. (Z) Dual luciferase assay revealed that transcriptional NF-κB activation induced by mutant EDA1 proteins was significantly reduced compared with that of wild-type EDA1. *, $P < 0.05$; **, $P < 0.01$; WT, wild-type.

doi:10.1371/journal.pone.0154884.g004

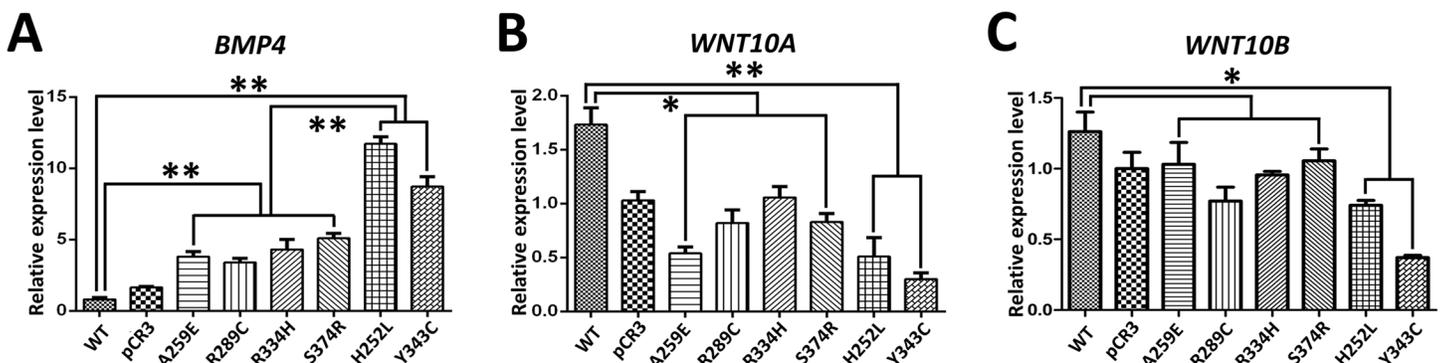


Fig 5. Effect of EDA1 mutations on BMP4, WNT10A and WNT10B relative expression levels. Real-time PCR was used to determine the relative expression level of *BMP4* (A), *WNT10A* (B) and *WNT10B* (C) in LS8 cells transfected with wild-type EDA1 and EDA1 mutants. *, $P < 0.05$; **, $P < 0.01$; WT, wild-type.

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Therefore, we performed Real-time PCR analysis to investigate *WNT10A* and *WNT10B* expression in LS8 cells after transfection with non-syndromic tooth agenesis-causing or HED-causing EDA1 mutant proteins. The result revealed that *WNT10A* expression was significantly downregulated in both non-syndromic tooth agenesis-causing and HED-causing EDA1 mutants transfected LS8 cells (Fig 5B). *WNT10B* expression was significantly downregulated in LS8 cells transfected with HED-causing EDA1 mutants (Fig 5C). *WNT10B* expression was also downregulated in LS8 cells transfected with non-syndromic tooth agenesis-causing EDA1 mutant proteins, but there was no significant difference when compared with that of wild-type EDA1, or HED-causing EDA1 mutants (Fig 5C). Our data suggested that either non-syndromic tooth agenesis-causing EDA1 mutants, or HED-causing EDA1 mutants, could inhibit *WNT10A* and *WNT10B* expression in LS8 cells.

Discussion

Recently, EDA mutations have been linked with non-syndromic tooth agenesis [3, 4, 39, 40]. We have reported three novel *EDA* mutations in sporadic non-syndromic oligodontia cases, all of which were located in the TNF domain [4]. Here, we investigated the molecular mechanism underlying non-syndromic tooth agenesis-causing EDA mutations. While the impact of known HED-causing EDA mutations located in the TNF domain are different, they all affect the receptor binding ability of EDA, leading to an abolished binding capability with specific receptors, and a compromised NF- κ B response [22]. In contrast to previous studies into HED-causing EDA mutations, we found that non-syndromic tooth agenesis-causing EDA mutant proteins possessed residual receptor binding capability. This finding is consistent with the recent report by Mues et al. [25]. Furthermore, using bioinformatics analyses, we previously showed that the A259E and R289C mutations were both located at the surface of the protein, away from the key receptor binding region of Glu308, and the R334H substitution only influenced the stability of the homotrimers, thus these non-syndromic tooth agenesis-causing mutations partially affected EDA function [3, 4]. The results from the current study strongly support our previous conclusions. Therefore, it seems that the presence of non-syndromic tooth agenesis appears to attenuate signaling through the NF- κ B pathway, rather than completely block it, as occurs in XLHED.

During early development of the ectodermal organs, the *Eda* signaling pathway plays a crucial role in placodal cell fate determination [41]. The activation of the transcription factor NF- κ B is crucial for *Eda* signaling [42]. NF- κ B suppression results in severe defects at the early stages of epidermal appendage development; with an epidermal phenotype that is analogous to HED in humans and identical to the phenotypes of *Eda*^{-/-}, *Edar*^{-/-}, or crinkled mice [43]. Our results are consistent with these previous studies and show that transcriptional activation of NF- κ B was activated by overexpression of wild-type EDA1 in LS8 cells, but was impaired in LS8 cells transfected with non-syndromic tooth agenesis-causing mutant EDA1 proteins.

Correctly timed and placed *Bmp4* expression in the mouse is required for proper tooth development [44]. Recombinant *Eda* counteracted *Bmp4* activity in developing teeth and, importantly, *Bmp4* expression was upregulated over the inner enamel epithelium at E14.5 in *Edar*^{dl/al} mice, indicating that suppression of *Bmp* activity was compromised in the absence of *Eda/Edar* signaling [29]. In the present study, the increased BMP4 expression level in LS8 cells transfected with non-syndromic tooth agenesis-causing EDA1 mutants ranged between that of wild-type EDA1 and HED-causing EDA1 mutants in transfected LS8 cells. We hypothesize that EDA1 lose-of-function mutations lead to compromised suppression of BMP4 activity, resulting in non-syndromic tooth agenesis. Although several studies have implicated a deficit in BMP4 signaling as the evolutionary source of tooth loss in the Aves lineage [45, 46], the

expression level of BMP4 in toothed and toothless turtles indicates that the regulation of tooth formation is more complex than previously thought and likely not simply the result of differential BMP4 expression [47]. Therefore, whether BMP4 expression level correlates with the severity of EDA-associated tooth agenesis phenotypes requires further study.

Recent studies have demonstrated that WNT10A and WNT10B play crucial roles in tooth development, mutations in these two genes are associated with tooth agenesis (our unpublished data and [38]). Maintenance of localized expression of Wnt10a and Wnt10b requires NF- κ B signaling, and Wnt10b was identified as a direct target of NF- κ B [35]. Moreover, Wnt10b is a downstream target of Eda/Edar pathway during tooth development [29]. The expression pattern of *Wnt10b* is expanded in an elongated dental epithelial structure in *Edar^{dl/dl}* mice at E15.0, rather than concentrated in the enamel knot in wild-type mice at the same stage, indicating that compromised Edar expression affects Wnt10b expression pattern during tooth development [29]. Consistent with previous studies, we found that WNT10A and WNT10B expression level were downregulated in LS8 cells transfected with non-syndromic tooth agenesis-causing EDA1 mutant proteins. Thus, in non-syndromic tooth agenesis, compromised EDA1/EDAR/NF- κ B activity results in reduced WNT10A and Wnt10b expression.

In our study, although the HED-causing EDA1 mutant proteins (H252L and Y343C) were unable to bind to their receptors and activate NF- κ B in LS8 cells, the BMP4 expression was increased and WNT10A and WNT10B expression were reduced dramatically. These results suggest that Eda/Edar/NF- κ B seems unlikely to be the only signaling pathway that regulates BMP4, WNT10A and WNT10B expression, other signaling pathways may have the reciprocal interaction with Eda/Edar/NF- κ B in balancing the normal expression level of BMP4, WNT10A and WNT10B during tooth development. This mechanism remains to be determined.

Most of the known EDA mutations affect a variety of ectodermal appendages, such as hair, teeth and glands [1, 2]. From the rescue experiments in the mouse, it is known that Eda-A1 promotes placode enlargement in a dose-dependent manner during early morphogenesis of ectodermal appendages [41, 48]. It appears that a small amount of recombinant Eda-A1 can only rescue hair and salivary gland defects, while a relatively large amount is required to save the number of teeth, suggesting that there is a tissue-specific and dose-dependent requirement of Eda signaling during the development of ectodermal organs [48]. Interestingly, we previously reported that the oligodontia phenotype in EDA-associated isolated tooth agenesis predominantly affects the incisors and canines, but with high possibility of persistence of the first molars [3]. Therefore, our current results strongly suggest that there is a tissue-specific effect, such that residual EDA/EDAR activity can prevent the hypoplasia of most ectodermal appendages, except for the development of teeth, especially the incisors and canines. Considering the tooth agenesis phenotype in EDA-associated HED patients, most of the teeth are missing, but the first molars are most likely to present [49], although the EDA receptor signaling is abolished in this condition. We hypothesize that there is a tooth position-specific requirement of EDA signaling. For example, the development of incisors and canines may require the highest dosage of EDA signaling, but the development of the first molars may either require the lowest dosage or be independent of EDA signaling. Further *in vivo* studies are required to test this hypothesis.

In summary, we have shown that non-syndromic tooth agenesis-causing EDA1 mutants maintain residual receptor binding abilities, resulting in reduced NF- κ B activation, indicating that our non-syndromic tooth agenesis causing EDA mutations (A259E, R289C and R334H) are loss-of-function. Moreover, the compromised NF- κ B activation subsequently leads to compromised suppression of BMP4 and diminished WNT10A and WNT10B expression, suggesting that EDA may regulate the expression of WNT10A, WNT10B and BMP4 via NF- κ B

during tooth development. Our results may help to understand the molecular mechanism linking specific EDA mutations with non-syndromic tooth agenesis.

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Author Contributions

Conceived and designed the experiments: DH HZ HF. Performed the experiments: WS YW YL HL GZ. Analyzed the data: WS YW YL HL DH. Contributed reagents/materials/analysis tools: MLS. Wrote the paper: WS YW DH.

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