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The Clouston syndrome mutation connexin30 A88V leads to hyperproliferation of sebaceous glands and hearing impairments in mice

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ABSTRACT

Distinct mutations in the gap junction protein connexin30 (Cx30) can cause the ectodermal dysplasia Clouston syndrome in humans. We have generated a new mouse line expressing the Clouston syndrome mutation Cx30A88V under the control of the endogenous Cx30 promoter. Our results show that the mutated Cx30A88V protein is incorporated in gap junctional plaques of the epidermis. Homozygous Cx30A88V mice reveal hyperproliferative and enlarged sebaceous glands as well as a mild palmoplantar hyperkeratosis. Additionally, homozygous mutant mice show an altered hearing profile compared to control mice. We conclude that the Cx30A88V mutation triggers hyperproliferation in the skin and changes the cochlear homeostasis in mice.
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1. Introduction

Clouston's hidrotic ectodermal dysplasia (HED, also called Clouston syndrome, OMIM #129500) is a rare autosomal dominant genetic disorder characterized by alopecia, nail dystrophies and palmoplantar hyperkeratosis. Some patients develop hyperpigmentation of the skin over the large joints [1]. Additionally, sensorineural hearing loss has been reported in a few cases [2,3]. The extent of the symptoms can be highly variable even within the same family, i.e. the hair loss can be partial or total and can be manifested at birth or later [4]. Molecular genetic screening of Clouston syndrome patients revealed that the patients carried an autosomal dominant mutation in the GJB6 gene, which encodes the gap junction protein connexin30 (Cx30) [5–7].

Gap junctions are transmembrane channel proteins, which mediate cell–cell communication. They form conduits in the plasma membrane of two adjacent cells allowing the diffusional exchange of molecules up to a mass of 1.8 kDa [8]. Gap junctions are composed of connexin protein subunits. Six connexins build a hemichannel (connexon), which can interact with a hemichannel of a contacting cell to form a complete intercellular gap junction channel [9]. Connexins are members of a multigene family. So far, 20 different connexin isoforms have been described in mice and 21 have been found to be expressed in humans [10]. Each connexin isoform shows a cell type specific expression and distinct contribution to channel properties [11].

So far, four different Clouston syndrome-causing human Cx30 mutations have been identified: Cx30G11R, Cx30A88V, Cx30V37E and Cx30D50N [5–7]. Moreover, another Cx30 mutation (Cx30T5M) has been described to lead to hearing impairments in humans. However, in contrast to the Clouston syndrome associated mutations, patients harboring the Cx30T5M mutation exhibit no hair and skin symptoms [12]. Mutations in connexins can alter the channel properties of gap junctions. Coupling analyses of the mutations Cx30G11R and Cx30A88V in HeLa cells and in paired *Xenopus* oocytes revealed that these mutations do not influence the formation of functional intercellular channels, but cause open hemichannels as indicated by a leakage of ATP into the extracellular medium [13].

In order to analyse the effects of Cx30A88V *in vivo*, we inserted this mutation into the mouse genome. After ubiquitous

Abbreviations: Cx, Connexin; Cx30A88V, substitution of the amino acid residue alanine to valine at position 88 of Cx30; SG, sebaceous gland; ABR, Auditory Brainstem Responses; DPOAE, Distortion Product Otoacoustic Emissions; NLS, Nuclear Localization Signal; IRES, Internal Ribosomal Entry Site

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Cre-mediated deletion of the Cx30 wildtype coding region, the Cx30A88V mutation is expressed under control of the endogenous Cx30 promoter. Homozygous Cx30A88V mice exhibit several symptoms of the highly variable Clouston syndrome, for example a mild hyperkeratosis of the palmoplantar skin and hearing impairment, whereas other characteristics like hair loss and nail defects are not displayed by this mouse line.

2. Material and methods

The material and methods are summarized. Detailed descriptions of all methods can be found in the Supplemental material.

2.1. Treatment of mice

All experiments were performed with littermates of >87.5% CD-1 background.

All mice used in this study were kept under standard housing conditions with a 12 h/12 h dark-light cycle and with food water *ad libitum*. All experiments were carried out in agreement with local and state regulations for research with animals.

2.2. Histological and immunofluorescence analyses

For histological and immunofluorescence analyses skin biopsies were taken from the dorsal mid-thoracic region and from the paws.

For histological analyses the skin biopsies were fixed in Bouin's solution and embedded in paraffin or in 2-Hydroxyethylmethacrylate (Technovit 7100, Heraeus Kulzer, Wehrheim, Germany). The 5 μm thick sections were stained with haematoxylin/eosin (HE).

For immunofluorescence analyses skin biopsies were frozen in Tissue-Tek embedding medium (Sakura, Zoeterwonde, The Netherlands), fixed in 4% paraformaldehyde and cryosectioned (14 μm). The following primary antibodies were used: mouse anti-Cx26 (monoclonal, 1:500, Invitrogen, Carlsbad, CA, USA, Cat. No. 13-8100), rabbit anti-Cx30 (diluted 1:250, Invitrogen, Cat. No. 71-2200), rabbit anti-keratin1 (diluted 1:500, Covance CRP, Muenster, Germany, Cat. No. PRB-149P), guinea pig anti-keratin K2e (diluted 1:200, Progen, Heidelberg, Germany, Cat. No. GP-CK2e), mouse anti-keratin 6 (monoclonal, diluted 1:100, Progen, Cat. Co. 61090), rat anti-Ki67 (monoclonal, diluted 1:50, DakoCytomation, Glostrup Denmark, Cat. No. M7249), rabbit anti-ß-galactosidase (diluted 1:500, MP Biomedicals, Santa Ana, CA, USA, Cat. No. 55976). The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG (diluted 1:1000, Invitrogen, Cat. No. A-11029, USA), Alexa Fluor 594 goat anti-rabbit IgG (diluted 1:1000, Invitrogen, Cat. No. A-11037, CA, USA), Alexa Fluor 488 goat anti-rat IgG (diluted 1:1000, Invitrogen, Cat. No. A-11006, USA), Alexa Fluor 488 goat anti-rabbit IgG (diluted 1:1000, Invitrogen, Cat. No. A-11008,), Cy2 goat anti-guinea pig IgG (diluted 1:1000, Jackson ImmunoResearch, West Grove, PA, USA).

For Nile Red stainings, cryosections were fixed in 4% paraformaldehyde, incubated for 2 h with 5 μg/ml Nile Red solution (Sigma–Aldrich, Cat. No. 72485, St. Louis, MO, USA) and mounted with Glycergel mounting medium (DakoCytomation, Glostrup, Denmark).

2.3. Immunoblot analyses

Total protein were extracted from paw skin. Extraction, electrophoresis, blotting and detection is described in the supplement. The following primary antibodies were used: mouse anti-Cx26 (monoclonal, 1:500, Invitrogen, Cat. No. 13-8100), rabbit anti-keratin1 (diluted 1:500, Covance CRP, Cat. No. PRB-149P), guinea pig anti-keratin K2e (diluted 1:1000, Progen, Cat. No. GP-CK2e) and mouse anti-keratin 6 (monoclonal, diluted 1:100, Progen, Cat. Co. 61090). Loading controls were performed with mouse anti-GAPDH (diluted 1:10,000, Millipore, Temecula, CA, USA) antibodies.

Horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse, goat anti-rabbit, goat anti-giunea pig, diluted 1:10000, Jackson ImmunoResearch, West Grove, PA, USA) were used.

2.4. Hearing analyses

ABR (Auditory Brainstem Responses) were recorded as described in [14]. In brief, mice aged 6–9 weeks were anesthetized with Ketamine/Xylazine i.p. and the difference potential between subcutaneous electrodes at the mastoid and vertex was recorded during free-field stimulation. ABR threshold was determined with 10 dB precision as the lowest stimulus intensity that evoked a reproducible response waveform. For DPOAE we used the System III and the MF1 speaker system (Tucker Davis Technologies) driven by custom-written Matlab software (Mathworks) to deliver two continuous primary tones (frequency $f2 = 1.2 \times f1$, intensity $l2 = l1$-10 dB SPL) and sampled the signal using a MKE-2 microphone (Sennheiser) and sound card (DMX 6Fire, Terratec).

2.5. Statistical analyses

For statistical analyses a two-tailed Student's *t*-test or individual Mann Whitney *U* tests were used. A P-value < 0.05 was regarded as statistically significant. Asterisks indicate a P-value of <0.05 (*), <0.01 (**) or <0.001 (***). Error bars indicate standard error of the mean (S.E.M.).

3. Results

3.1. Generation of a Cx30A88V mouse line

Cx30A88V mice were generated by targeting the Cx30 locus in mouse embryonic stem (ES) cells via homologous recombination (Supplemental Fig. 1A). The conditional targeting vector contains the wildtype Cx30 sequence flanked by loxP sites and the coding region of the mutated Cx30A88V. The point mutation in the Cx30A88V gene, leading to a substitution of an alanine residue on position 88 by a valine residue, was inserted together with a ScaI restriction site by mutagenesis PCR. Therefore the ScaI restriction site is not present in the wildtype sequence of Cx30. The expression of Cx30A88V is linked to the expression of a lacZ reporter gene by an internal ribosomal entry site (IRES) between the coding region of Cx30A88V and a lacZ gene. The lacZ gene is coding for ß-galactosidase which, due to a nuclear localization signal (NLS), is transported to the nucleus and can be used to monitor the Cx30A88V expression in vivo. For selection of homologously recombined ES-cells, the vector includes cDNA coding for neomycin resistance, which is flanked by frt-sites.

HM1 ES-cells were transfected with the conditional Cx30A88V vector. Five out of 800 clones were positive (verified by PCR and Southern blot analyses, data not shown) and could be used for blastocyst injections. The heterozygous Cx30 +/floxA88V chimeras were mated with Flp-recombinase expressing mice to delete the neomycin resistance cDNA (Supplemental Fig. 1B) and with phosphoglycerate kinase (pgk)- cre recombinase expressing mice for ubiquitous deletion of the wildtype Cx30 sequence (Supplemental Fig. 1C). Cx30A88V mice were genotyped by PCR analyses (Supplemental Fig. 1D). To verify the presence of the mutated Cx30A88V sequence a PCR reaction amplifying the coding region of Cx30wt and Cx30A88V was performed, followed by a ScaI restriction (Supplemental Fig. 1E). The different genotypes of transgenic Cx30A88V

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