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Prenatal therapy in developmental disorders: drug targeting via intra-amniotic injection to treat X-linked hypohidrotic ectodermal dysplasia

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Short title: Drug targeting via intra-amniotic injection

TO THE EDITOR

Pathologies associated with genodermatoses and other genetic disorders can irremediably affect fetuses, making early stage therapies desirable. Prenatal maternal drug administration, however, exposes mothers to potential drug toxicity and is limited by the variability in transplacental drug delivery. Alternative approaches to fetal treatment should entail low risk drug delivery with reproducible pharmacokinetics. X-linked hypohidrotic ectodermal dysplasia (XLHED), the most common inherited disorder of ectoderm development, is caused by a lack of the signaling molecule ectodysplasin A1 (EDA1) which is essential for ectodermal placode formation and subsequent development of various skin appendages, glands, and teeth (Mikkola, 2009). Patients with XLHED have less hair, fewer or no eccrine sweat, sebaceous and meibomian glands, and malformed or absent teeth. Insufficient thermoregulation can lead to perilous hyperthermic episodes during infancy (Blüschke et al, 2010) and remains an important issue throughout life (Hammersen et al, 2011). Many affected individuals suffer from recurrent airway and eye problems (Dietz et al, 2013). To date only symptomatic treatment is available for these patients.

Causative therapeutic approaches are expected to be most effective if applied already *in utero*, with the additional benefit that immune tolerance of a replacement protein may be induced, facilitating postnatal re-application (Schneider et al, 2002; Waddington et al, 2003). In the *Tabby* mouse, a well-characterized animal model of XLHED (Falconer, 1952), prenatal exposure to EDA1 via serial intravenous administrations to the dam corrected developmental abnormalities to a far greater extent than postnatal administration (Gaide and Schneider, 2003). This approach may, however, be suboptimal for achieving reproducible therapeutic drug concentrations in human fetuses, and would expose the mother to high serum levels of an exogenous molecule. We hypothesized that EDI200, an EDA1 replacement protein consisting of the receptor-binding domain of EDA1 and the Fc part of IgG1, may enter the fetal circulation also after injection into the amniotic fluid (AF), because the fetus swallows AF regularly and the neonatal Fc receptor which is present in rodent and human fetal intestine (Shah et al, 2003) may facilitate intestinal absorption of Fc-containing proteins. AF could thus serve as a drug reservoir and provide for continuous drug uptake. Here we report striking reversal of the XLHED phenotype of *Tabby* mice following a single intra-amniotic injection of EDI200.

Long-term stability of this recombinant protein in AF, *i.e.* the retention of binding to its cognate receptor, was confirmed *in vitro* under various conditions (Figure S1).

Pharmacokinetics following intra-amniotic injection of 35 µg EDI200 per amniotic sac (= 100 µg/g of estimated fetal body weight) was studied in wild-type mice at day 15 of gestation (E15). EDI200 serum levels were measured at different time points after injection both in treated and untreated fetuses as well as in the dams. All maternal animals recovered well from the intervention; 93% of the treated fetuses survived. Intra-amniotic injection of EDI200 resulted in mean fetal serum levels of 9.0 µg/ml and 1.2 µg/ml at 6 and 96 hours, respectively. After 6 hours, this corresponds to 180 ng of EDI200 per fetus, or 0.5% of the injected protein, assuming a total serum volume of approximately 20 µl in an E15 mouse fetus. Interestingly, there was a low level of EDI200 transfer into the circulation of untreated siblings and that of the pregnant dam (Figure 1). The drug was partially and slowly redistributed from treated fetuses, in which EDI200 concentration diminished over time, to untreated siblings that witnessed a parallel increase of the serum levels (up to 0.57 µg/ml). Maternal EDI200 serum levels remained <0.1 µg/ml at the time points investigated. Thus, intra-amniotic administration of EDI200 at E15 resulted in substantial fetal uptake with minimal maternal exposure.

This approach was then evaluated in pregnant *Tabby* mice with doses of 100, 10, and 1 µg/g of estimated fetal body weight. All treated *Tabby* mouse fetuses of the high and intermediate dose cohorts survived the E15 intra-amniotic injection and were born without complications. They were easily distinguishable from native *Tabby* mice already in the second week after birth. Later, normal eye opening, retro-auricular and guard hair as in wild-type mice, and a normally shaped tail tip (Figure 2 a, b) were evident. Starch-iodine tests revealed regular sweat production at the paws (Figure 2 c-e). Normal eccrine sweat glands (Figure 2 f-h) were detected in footpads of these animals. In addition, size and shape of the molars resembled those of wild-type mice (Figure 2 i-k).

Thus, in contrast to a single maternal intravenous injection of 400 µg of EDI200 in pregnant *Tabby* mice at E15, which corrected the XLHED phenotype in the offspring only partially (unpublished own data), a single intra-amniotic dose of 3.5 µg or above resulted in complete phenotypic correction. No adverse effects were observed. All treated *Tabby* mice survived to adulthood and showed normal behavior and fertility. The lower dose of 1 µg/g body weight yielded only a partial restoration of normal ectoderm development, with less guard and/or tail hair and fewer sweat glands present (Table S1). Interestingly, but less relevant to human singleton gestations, a dose-dependent correction was also observed for untreated littermates (Table S1) – explained by partial leakage of the drug to neighboring fetuses. As expected from previous studies (Gaide and Schneider, 2003), the maternal *Tabby* phenotype was not

visibly altered by EDI200 administration, regardless of the dose. All dams remained fertile and no adverse effects of the treatment could be detected during an observation period of 6 to 9 months.

The EDA1 signaling pathway is well conserved among vertebrates (Pantalacci et al, 2008) and findings in animal models should therefore be transferable to human XLHED patients. Early corrective treatment would increase their life expectancy, would have a high impact on the quality of life and substantially reduce medical expenses. Intra-amniotic drug delivery might be most beneficial if attempted during mid-gestation, when sweat gland development is not yet completed (Ersch and Stallmach, 1999). This approach is likely to have a good benefit/risk ratio, supported by the broad experience with amniocentesis, and may represent a novel paradigm for treatment of disorders in early human development.

POTENTIAL CONFLICT OF INTEREST

Kenneth Huttner is an employee of Edimer Pharmaceuticals, Inc., Pascal Schneider holds shares in this company. Holm Schneider is a member of the clinical advisory board of Edimer Pharmaceuticals.

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FIGURE LEGENDS

Figure 1: EDI200 pharmacokinetics after intra-amniotic administration to mice

EDI200 was injected into amniotic sacs of 4 pregnant wild-type mice carrying a total of 23 fetuses at day E15 (100 µg/g of estimated fetal body weight). Some fetuses of each mother were left untreated. Another pregnant mouse and the fetuses of her served as negative controls. EDI200 serum concentrations were measured at different time points by receptor-binding ELISA. Highest serum levels were detected 6 hours after the injection. There was some leakage to untreated siblings and, to a lower extent, into the mother's circulation. *n.a.*, not applicable; * $p<0.05$; ** $p<0.01$.

Figure 2: Phenotype after prenatal treatment with EDI200

Tabby mice were treated *in utero* (E15) by a single intra-amniotic injection of EDI200 (here 100 µg/g) and investigated postnatally. Age-matched native *Tabby* mice (a) could be distinguished clearly from treated animals (b), which had a darker and denser coat, showed normal eye opening and hair around the eye, retro-auricular and guard hair like wild-type mice, and a hairy, normally shaped tail. Starch-iodine tests of the paws revealed numerous dark spots in a characteristic pattern indicating functional sweat glands (c). Histological analysis of footpad sections confirmed the presence of fully developed eccrine sweat glands in the treated animals (f). Native *Tabby* mice (d, g) and wild-type mice (e, h) served as controls. Molars of treated *Tabby* mice (i) were altogether larger than those of native *Tabby* mice (j) and showed similar shape and cusps pattern as in wild-type animals (k).

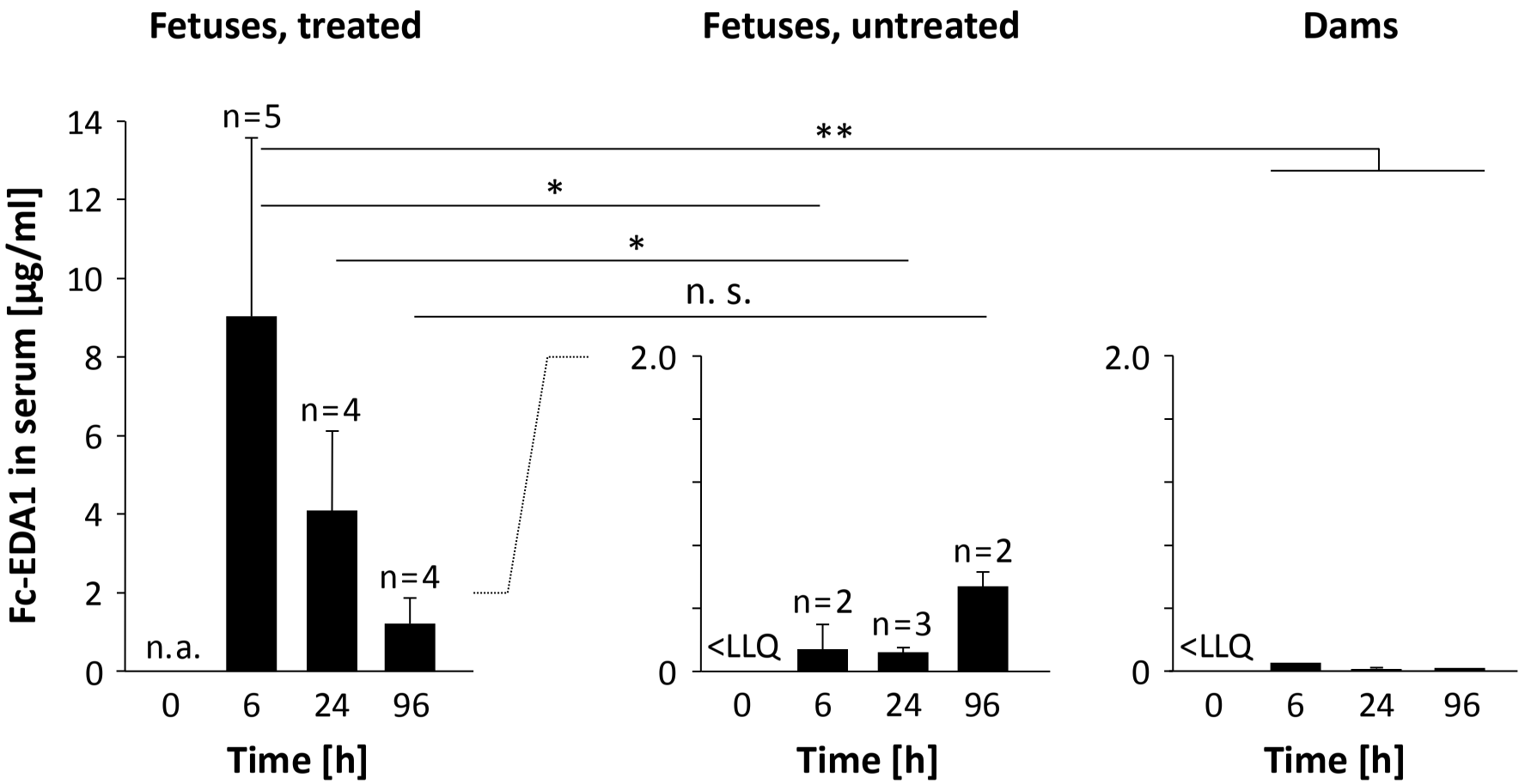
Fetuses, treated
Fetuses, untreated
Dams
Fc-EDA1 in serum [µg/ml]
14
12
10
8
6
4
2
0
n=5
n=4
n=4
n.a.
**
*
*
n. s.
2.0
0
n=2
n=3
n=2
<LLQ
2.0
0
<LLQ
0
6
24
96
Time [h]
Time [h]
Time [h]

Figure 1

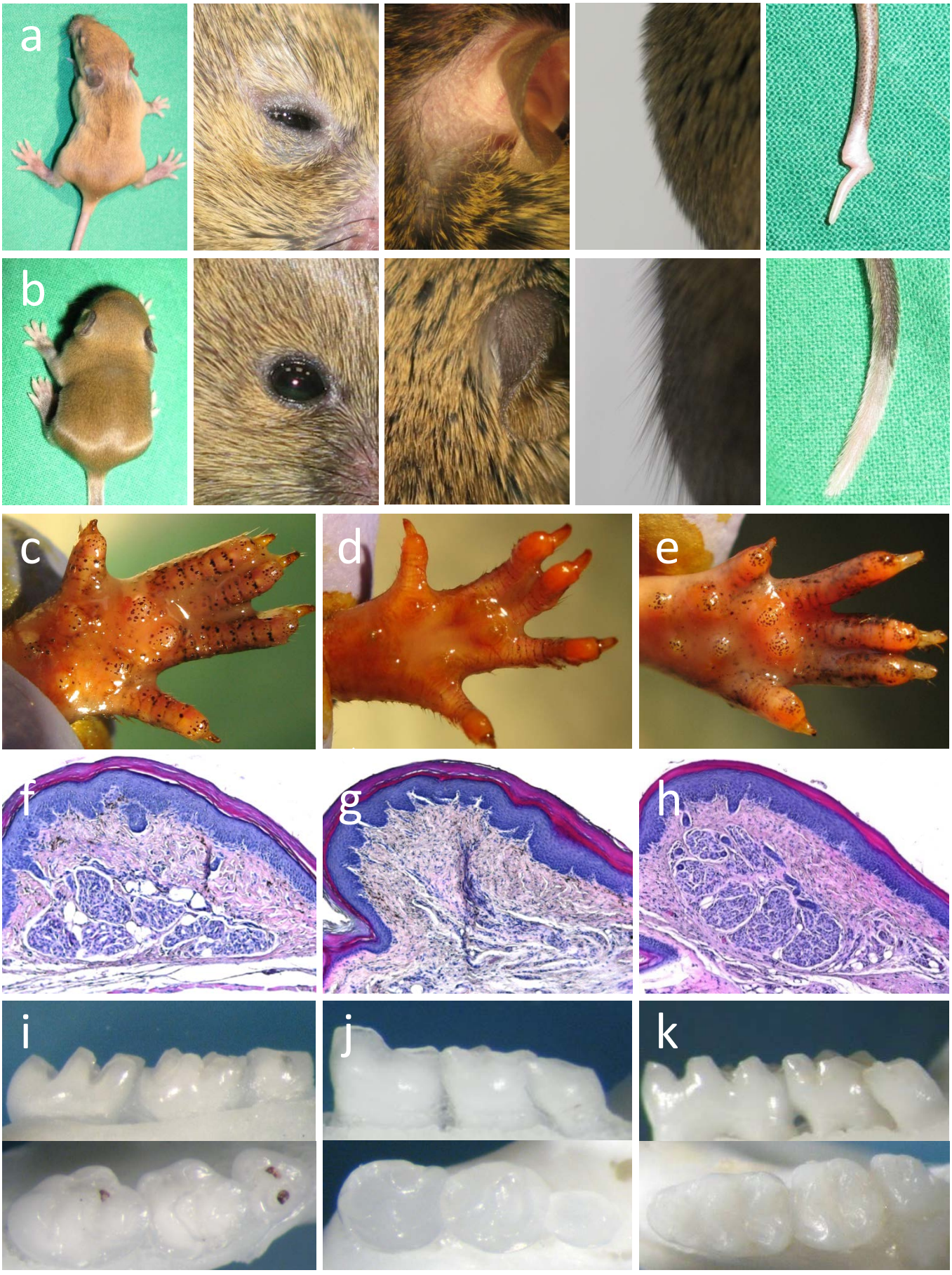

Figure 2

Supplementary information

MATERIALS AND METHODS

Reagents and human samples

EDI200, a recombinant fusion protein consisting of the receptor-binding domain of EDA1 (aa 238-391) and the Fc part of IgG1, was provided by Edimer Pharmaceuticals, Inc. (Cambridge, USA). The antibodies mAbEDAR1, an anti-EDAR mouse IgG1 antibody (Kowalczyk et al, 2011), EctoD2 and biotinylated EctoD3, two murine antibodies recognizing EDA1 (Kowalczyk-Quintas et al, 2014), and hEDAR-hFc (Kowalczyk et al, 2011) were described previously. To produce hEDAR-mFc, an appropriate expression plasmid [hEDAR (aa 1-183)-mIgG1 (aa 235-461 of GenBank accession number AAA75163.1)] was generated and introduced into CHO cells (Sigma), yielding a stable hEDAR-mFc-secreting cell clone. The protein was purified from conditioned supernatants by affinity purification on immobilized mAbEDAR1, which was coupled to NHS-activated Sepharose (GE Healthcare) at a ratio of 5 mg antibody per ml of Sepharose according to the manufacturer's instructions. Fresh human amniotic fluid (pooled remnants from two elective amniocentesis samples) was obtained with informed consent, kept at 4°C and used within 24 hours.

Incubations in amniotic fluid

Protein stability in AF was evaluated essentially as described previously (Schmidt et al, 2004). EDI200 was incubated in fresh human AF, from which cellular components had been removed or not by centrifugation, at concentrations of 10 or 500 µg/ml at 37°C in the presence of penicillin (50 U/ml) and streptomycin (50 µg/ml). Aliquots were removed at different time points (0, 0.5, 4, 24, 72, and 168 h) and immediately stored at -80°C until use. A comparable series of samples was obtained after exposure of EDI200 to fresh human AF at 4°C. All samples were then investigated in parallel by receptor-binding ELISA.

Receptor-binding ELISA

For the detection of EDI200, ELISA plates (Nunc Maxisorb) were coated with hEDAR-mFc (0.2 µg/ml PBS) and blocked with a solution of skimmed milk powder (4%) and Tween-20 (0.5 %) in PBS. Serial dilutions of EDI200 samples prepared in a separate, pre-saturated plate were added, and binding was revealed with horseradish peroxidase-coupled goat anti-human-IgG

(JIR 109-005-033), followed by addition *o*-phenylenediamine substrate (Sigma). The reaction was stopped with acid and absorbance was read at 492 nm. Binding of mAbEDAR1 to EDAR was evaluated in plates coated with hEDAR-hFc (0.2 µg/ml PBS). After blocking of the plate, serial dilutions of mAbEDAR1 were added and the binding was revealed with horseradish peroxidase-coupled goat anti-mouse IgG (JIR 115-036-166). For the quantification of EDI200 by sandwich ELISA (Kowalczyk-Quintas et al, 2014), ELISA plates were coated with EctoD2 (5 µg/ml of PBS) and blocked. Twenty-fold diluted serum samples (100 µl) were added to each well, and captured EDI200 was revealed with biotinylated EctoD3 (1 µg/ml) followed by peroxidase-coupled streptavidin. Concentrations of EDI200 were calculated by comparison with a standard curve of EDI200 in PBS. The lower limit of quantitation (LLQ) was 0.8 ng/ml.

Animals and surgical procedures

C57BL/6 wild-type mice (Charles River) and white-bellied agouti B6CBAa $A^{w\text{-}J}$/A-Eda^{Ta}/J *Tabby* mice (000314; Jackson Laboratory) were housed in individually ventilated cages under standard conditions with a light/dark cycle of 12 hours and free access to standard chow and tap water. All experimental procedures were conducted in accordance with the German regulations and legal requirements.

Blood samples from adult animals were obtained by puncture of the retrobulbar venous plexus and post-coagulation serum was stored at -20°C. Intra-amniotic injections were performed essentially as follows: Timed-mated pregnant mice on gestational day 15 (E15) were anesthetized with isofluran (2%). For perioperative analgesia, metamizole (100 µl) was administered subcutaneously. The abdominal cavity was opened by midline laparotomy and both uterine horns were exposed completely. EDI200 was injected into the amniotic sacs of individual fetuses at doses between 1 and 100 µg/g of fetal body weight (16 µl of EDI200 at 2.2 mg/ml, assuming a maximum fetal weight of 0.35 g) using glass syringes with 33 gauge needles (Hamilton). The amniotic sacs of some fetuses were left untreated. The uterus was then placed back in the abdomen, and the abdominal wall was closed by continuous suture of the peritoneal layer and interrupted stitches of the skin. All mice were allowed to recover in a pre-warmed cage. Wild-type mice were sacrificed 6, 24 or 96 h after the intervention. Fetuses were delivered by Cesarian section. Each amniotic sac was dissected individually and the fetus was washed several times in saline prior to decapitation to obtain fetal blood. Treated fetuses were distinguishable from untreated fetuses by their position in the uterus.

Maternal blood was collected by cardiac puncture. Post-coagulation serum was stored at -20°C. *Tabby* mice were allowed to deliver normally and were left with their pups.

Phenotypic characterization

The offspring of *Tabby* mice was investigated at the time points indicated for various morphological features (tail hair, shape of the tail tip, guard hair, eyes, etc.). Treated animals were distinguished from untreated littermates by their phenotypic reversion, assuming that treated pups would be reverted to a greater extent than untreated ones. Starch/iodine sweat tests were performed as described previously (Gaide and Schneider, 2003). Briefly, the palmar side of a hind paw of the immobilized mouse was covered with a solution of 3% (w/v) iodine in ethanol and allowed to dry at the air. Then a suspension of 40% (w/v) starch in mineral oil was applied. Pictures were taken after one minute; sweat pores appeared as dark spots.

Histological analyses

Tissue samples from skin, eyelids, footpads and tail were fixed in 4% paraformaldehyde for 24 hours. After dehydration and paraffin embedding, sections were cut and stained with hematoxylin and eosin for morphological evaluation. Jaws of adult mice were boiled for 2 hours in water containing 10 g of a standard dishwasher tab per 100 ml, cleansed manually of adherent tissue and bleached with 30% hydrogen peroxide.

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SUPPLEMENTARY FIGURE

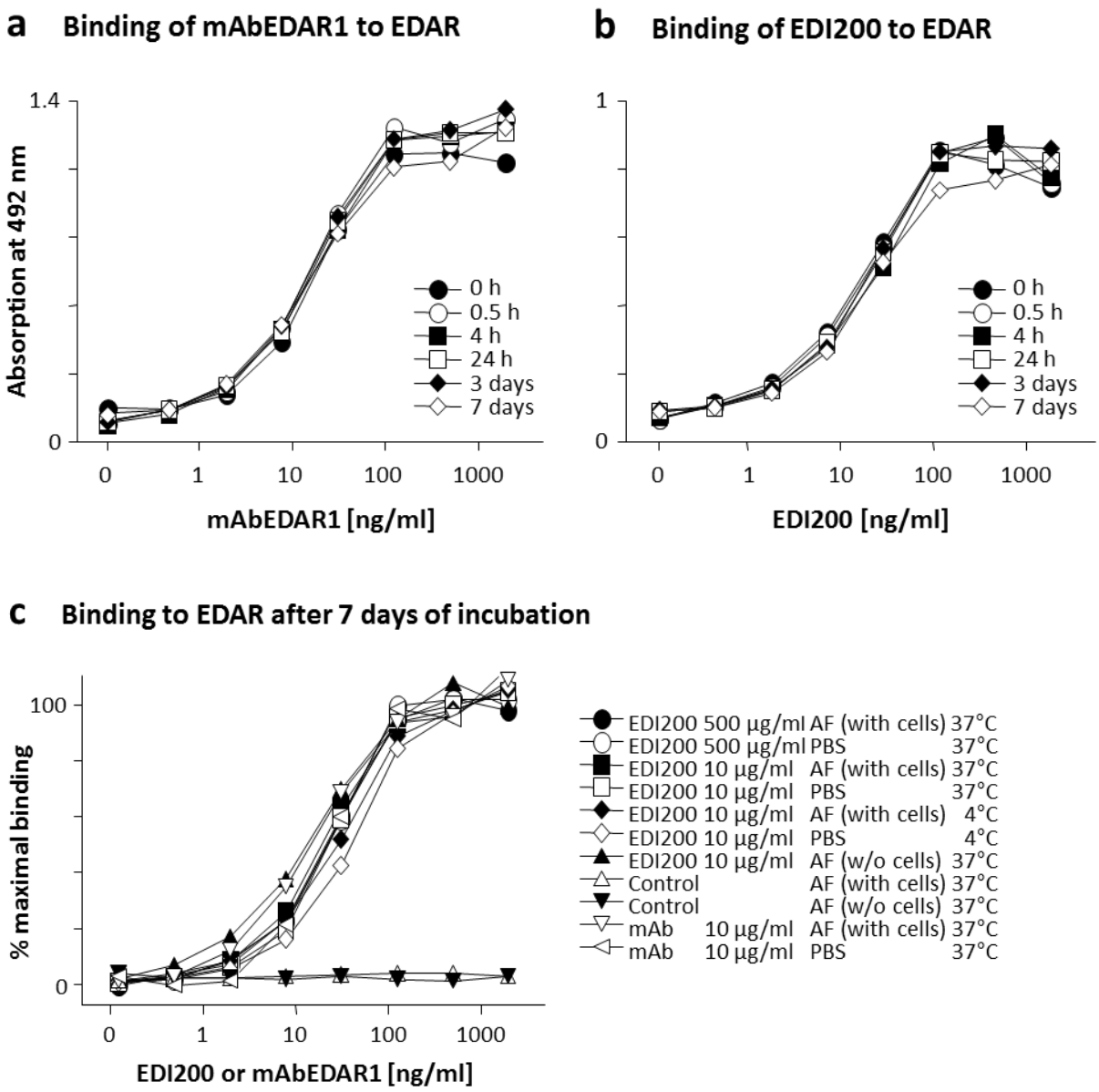

Figure S1: Stability of EDI200 in amniotic fluid

EDI200 and an anti-EDAR antibody (mAbEDAR1) were incubated with fresh human AF at 37°C or left standing at 4°C. Aliquots taken at the time points indicated were tested by receptor-binding ELISA. A. The ability of the anti-EDAR antibody to recognize hEDAR-hFc remained unaffected by incubation in AF at 37°C for up to 7 days. B. The ability of EDI200 to bind to hEDAR-mFc was also not affected by incubation in AF. C. There was no significant alteration of the ability of EDI200 (or mAbEDAR1) to bind to hEDAR-mFc after exposure to AF for 7 days under the conditions indicated.

SUPPLEMENTARY TABLE

	Tail hair	**Tail shape**	**Hair behind the ears**	**Guard hair**	**Eyes**	**Teeth**	**Sweat glands**	**Progeny after mating**
EDI200, 1 µg/g (group A; n=5)	1.6	1.8	2.0	2.0	1.0	1.4	1.0	n.a.
Untreated siblings of group A (n=4)	0.25	0	0	0	0	0	0	n.a.
EDI200, 10 µg/g (group B; n=4)	3.0	3.0	3.0	3.0	3.0	2.75	2.75	4/4
Untreated siblings of group B (n=1)	2.0	2.0	3.0	2.0	2.0	2.0	1.0	1/1
EDI200, 100 µg/g (group C; n=7)	3.0	3.0	3.0	3.0	3.0	3.0*	3.0	7/7
Untreated siblings of group C (n=2)	3.0	2.5	3.0	3.0	3.0	3.0*	3.0	2/2

3 = fully corrected
2 = partially corrected, close to wild-type
1 = partially corrected, close to native *Tabby* mice
0 = no correction
n.a., data not yet available
* only one animal analyzed; the others were kept for long-term follow-up

Table S1: Summary of phenotypic findings after intra-amniotic EDI200 application to fetal *Tabby* mice

EDI200 injection led to a striking, dose-dependent reversion to a normal phenotype. A dose-dependent correction was also observed for “untreated” siblings.