

A novel hairless mouse model on an atopic dermatitis-prone genetic background generated by receptor-mediated transgenesis

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Abstract Current mouse models for atopic dermatitis (AD) have a serious drawback, being the existence of dense hair on the body. Thus, a hairless

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animal model on an AD-prone genetic background will be a powerful tool to investigate the basis of and therapy for this complex disease. We applied the Toxin Receptor-mediated Cell Knockout (TRECK) method to generate a hairless transgenic (Tg) mice on the NC/Nga background, an AD-prone inbred strain. A minigene with the mouse *Keratin71* (*Krt71*) promoter and human diphtheria toxin receptor, which intrinsically functions as the heparin-binding EGF-like growth factor, was introduced into the pronucleus of NC/Nga oocytes. Unexpectedly NCN24, one NC/Nga Tg line, showed a dominant hairless phenotype without diphtheria toxin administration. Furthermore, the atopic dermatitis-like predisposition and IgE elevation was observed in both NCN24 and the NC/Nga wildtype strain. NCN24 mice, which we have newly developed, will be useful to assess drugs for AD therapy, being able to monitor skin inflammation without shaving.

Keywords Receptor-mediated transgenesis · NC/Nga inbred strain · Hairless mouse model · Atopy

Introduction

Atopic dermatitis (AD, eczema) is a pruritic, chronic and relapsing inflammatory skin disease with predominantly childhood onset, but the symptoms can persist or begin in adulthood (Hanifin 1993). Atopic

dermatitis is associated with various immunological abnormalities and multiple environmental factors. For AD, animal models are also indispensable tools to understand the pathogenic basis of and therapy for this complex disease. Up to now, at least three spontaneous mouse models for AD have been developed (Gutermuth et al. 2004) and the inbred strain NC/Nga is the most widely used model for the disease. Although this strain was established from Japanese fancy mice in 1957 (Kondo et al. 1969), our recent molecular-genetic survey of the NC/Nga genome revealed that its background mainly belongs to the European subspecies of *Mus musculus*, *M. m. domesticus*, which includes the widely used laboratory (classical inbred) strains, such as C57BL/6J, among others (Sakai et al. 2005). The phenotypes of skin lesions in NC/Nga match the symptoms of human atopic dermatitis, such as itching, erythema and hemorrhage, with high titers of immunoglobulin E (IgE) for various allergens (Matsuda et al. 1997). Genetic approaches have indicated that many genetic and environmental factors are responsible for the induction of skin inflammation (Kohara et al. 2001).

We have now generated a hairless mouse model for AD on the NC/Nga genetic background to study the pathophysiology of the disease and to use it for the screening of ointment compounds for novel skin-lesion therapies. To generate the hairless mice, we applied a novel method which we have been recently developing for cell lineage-specific ablation using diphtheria toxin (DT), also known as the TRECK (Toxin receptor mediated cell knockout) method (Saito et al. 2001). To achieve the specific ablation of hair shafts we used the promoter of the *keratin71* (*krt71/krt2-6g/mK6irs1*) gene, which encodes a type 2 keratin filament protein. *Krt71* is involved in hair development and also affects the morphology of coat hair by its mutation, because the gene product is expressed in cells of the inner root sheath (IRS). Several allelic mutations found in the Caracul (*Ca*) phenotype morphologically are very similar to the classical wavy coat mutation in laboratory mice (Kikkawa et al. 2003). Therefore, we constructed a minigene in which the expression of the human DT receptor, the heparin-binding EGF (Naglich et al. 1992), is driven by the promoter of the *Krt71* gene. The minigene was introduced directly into pronucleus-stage eggs of the NC/Nga strain to generate NC/Nga-Krt71-TRECK-transgenic (Tg) mice. An

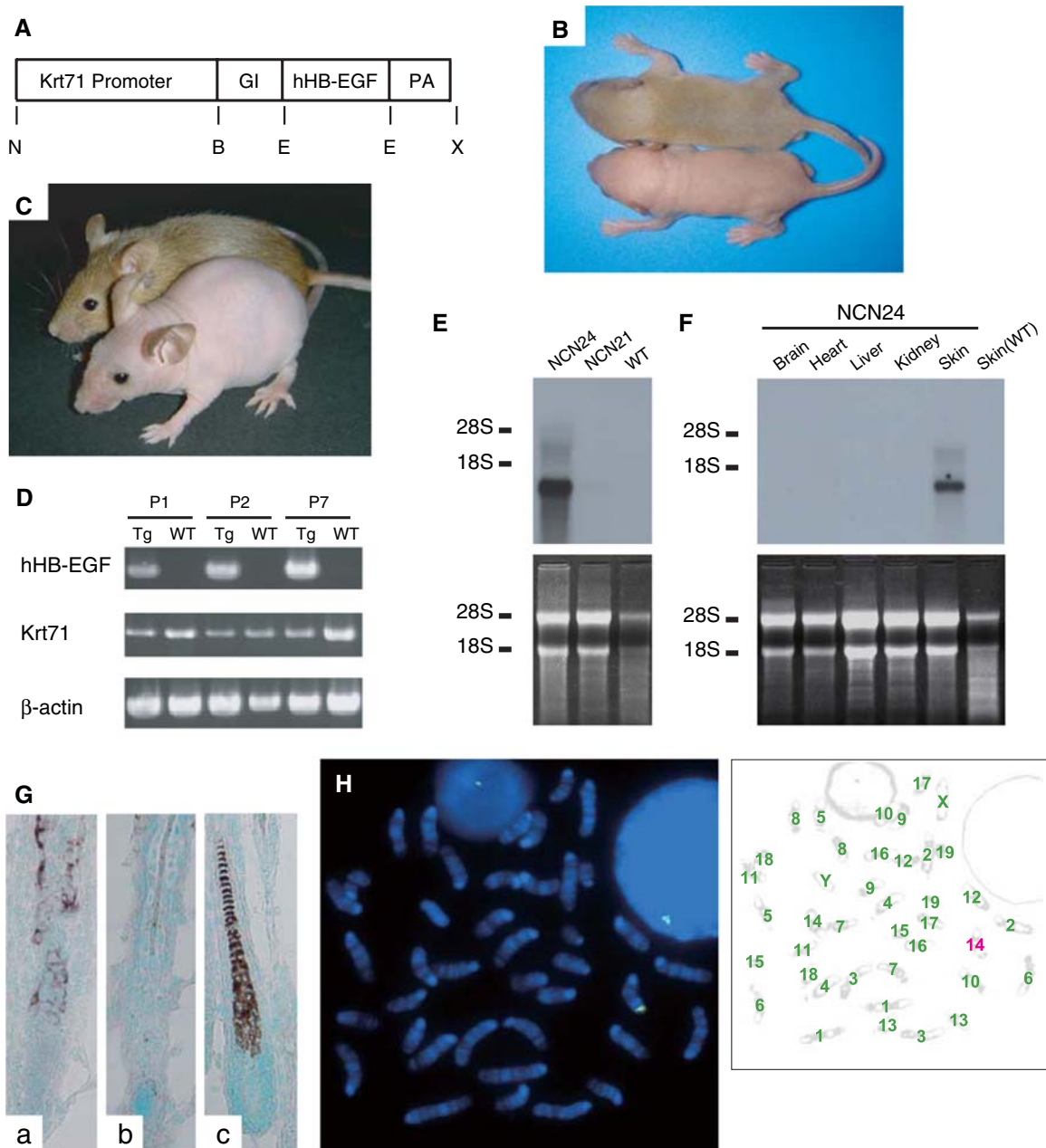
Fig. 1 The transgene construct, phenotype of NCN24 mice and transgene expression. **(A)** The Tg construct contains the 8.7 kb promoter region of *Krt71*, rabbit β -globin second intron [GI], human *HB-EGF* cDNA [*hHB-EGF*], rabbit β -globin and simian virus 40 (SV40) early gene polyadenylation (poly A) signal [PA]. B, *Bam*HI; E, *Eco*RI; N, *Nor*I; X, *Xho*I. **(B)** NC/Nga-Tg(*Krt71*-HBEGF)24Rin (NCN24) mice at postnatal day 7 (P7) exhibit a hairless phenotype without DT treatment throughout the whole body, compared with the wildtype littermate. **(C)** A 6-week-old NCN24 mouse (in front), relative to a control littermate (back). **(D)** RT-PCR amplification of the human *HB-EGF*, *Krt71* at postnatal day 1 (P1), day 2 (P2) and day 7 (P7) from NCN24 (Tg) and Wildtype (WT). Amplification of β -actin was used as an internal control. **(E)** Northern blot analysis. Expression levels of human HB-EGF mRNA in skins of transgenic mice lines (top). **(F)** Human HB-EGF mRNA expression in the NCN24 transgenic mouse line (top). Wildtype mouse skin, negative control. Ethidium bromide staining shows that equal amounts of total RNA were electrophoresed in each lane (bottom). **(G)** In situ hybridization of the transgene was performed in P12 dorsal skin hair follicles of NCN24 (**a**, **b**) or Wildtype (**c**). Expression of *hHB-EGF* mRNA in the IRS from NCN24 mice was detected in samples tested with antisense (**a**) but not sense (**b**) probes. No positive signals were detected in skin sections from wildtype mice using antisense probes (**c**). **(H)** In situ hybridization to show the chromosomal localization of the transgene. Chromosome numbers are denoted to the right. The transgene was detected in only one arm of chromosome 14 (magenta)

unexpected result emerged: one Tg founder strain had a hairless phenotype without the administration of DT. DT is highly toxic to mice and humans and therefore it is not an appropriate agent in experimental models intended to investigate pathogenic aspects relevant to human disease. Such DT treatment and attention to DT administration in mice are no longer needed when the novel hairless Tg mice are used.

Experimental procedures

Generation of *Krt71* promoter/human HB-EGF transgenic NC/Nga mice, genotyping and RT-PCR

NC/Nga mice were purchased from SLC (Tokyo, Japan). DNA sequence information was obtained from the Mouse Genome Sequencing Consortium (NCBI Build 32: <http://www.ncbi.nlm.nih.gov/genome/guide/mouse/index.html>). Approximately 9 kb of the *Krt71* promoter region was amplified by PCR using genomic C57BL/6J mouse DNA using the following primer sets: 5'-AGC GGC CGC CAG ATT ATG AAA GGA



ACTCCTGCAAC-3', 5'-AGAGAGCAC TGC GGA GCA ACC ACT GAA GC-3' and 5'-TGG ATC CGT TGG TAG AGG ATG GAG TAA AGG TGC-3'. The *Krt71* promoter was cloned into the *Bam*HI and *Not*I sites of the TRECK vector (Saito et al. 2001). The 11-kb *Not*I/*Xho*I fragment containing the *Krt71* promoter, β -globin intron, human *HB-EGF* cDNA was excised, purified and used for microinjection. The construct used for transgenesis is outlined in Fig. 1A. PCR

genotyping was performed on genomic DNA isolated from tail biopsies of transgenic and wildtype mice. Genomic DNA was extracted with phenol-chloroform after treatment with proteinase K and precipitated in isopropanol. Human *HB-EGF* cDNA sequences served as positive controls for genotyping of Tg animals. PCR was performed using the following primer sets: 5'-GTA TCG AAA GTG ACT GGT GCC TCG C-3' and 5'-CTT CTG ATA GGC AGC CTG CAC CTG AGG-3'. Total

RNA was isolated from the dorsal skin by using TRIzol reagent (Invitrogen). Double-stranded cDNA was synthesized by Sensiscript RT Kit (QIAGEN), according to the manufacturer's manual. RT-PCR was performed using the following primer sets: human *HB-EGF*, 5'-GCT CTT TCT GGC TGC AGT TCT CTC GGC ACT GG-3' and 5'-GGT ATA TAA GCG ATT TTC CAC TGG GAG GCT C-3'; *Krt71*, 5'-GTG AGT GAT TGC CAG GCT TGG TGA TCA G-3' and 5'-GTG CTG TTT ATT GAG AAT GCA ACA GG GAG-3'. Hemizygous Tg mice were used and compared with control wildtype NC/Nga mice in this study. All animal experiments were approved by the institutional committees for animal experiments of Tokyo Metropolitan Institute of Medical Science.

Northern hybridization

Human *HB-EGF* transgene expression was detected using the previously described method with slight modifications (Saito et al. 2001).

In situ hybridization for human *HB-EGF* expression for skin samples

For the preparation of RNA probes, the 500 bp human *HB-EGF* cDNA fragment amplified by RT-PCR was subcloned into the pDrive cloning vector (QIAGEN). Antisense or sense RNA of human *HB-EGF* probes were labeled with digoxigenin (DIG)-labeled UTP (Roche) according to the manufacturer's protocol. Skin cryosections (10 µm thick) were fixed and dried, and then prehybridized for 1 h at 37°C with hybridization buffer (10% dextran sulfate Na, 20 mM Tris–HCl, pH 8.0, 0.3 M NaCl, 0.2% sarcosyl, 0.02% denatured salmon sperm DNA, 500 ng/ml yeast tRNA, 1× Denhardt's solution, and 50% formamide). Samples were hybridized at 55°C for 16 h in hybridization buffer containing 1 µg/ml DIG-labeled riboprobe. The DIG nucleic acid detection kit (Roche) was used for immunological detection following the manufacturer's protocol. After color development, the reactions were stopped and slides were counterstained with Methyl Green (Lab Vision).

Chromosomal localization of the transgene

In situ hybridization to detect integration of the human *HB-EGF* transgene was performed as

described previously (Harada et al. 1995) by using a male spleen NCN24 sample.

Dermal histology and immunostaining

The skin samples were fixed in 4% paraformaldehyde, sectioned (5 µm), and stained with either Hematoxylin-Eosin (HE) or Giemsa solution by standard methods.

ELISA

Total serum IgE was measured by ELISA as described previously (Hirano et al. 1988).

Flow cytometry analyses

For cell population analyses, bone marrow, spleen, and thymus of 4 week old mice were removed and passed through mesh screens to make single-cell suspensions. Cells were stained with the appropriate combinations of the following antibodies and reagents: CD19-PE, CD11c-FITC, NK1.1-PE, DX5-biotin, CD3-FITC, CD4-APC, CD4-biotin, CD8-APC, CD25-biotin, Streptavidin-APC, Streptavidin-PE and SA-APC (Becton-Dickinson) and Gr-1-FITC (eBioscience). In all experiments, cells were analyzed on FACSCalibur (Becton-Dickinson) using CellQuest software.

Results and discussion

Transgenesis using a *Krt71*-DTR minigene (Fig. 1A) resulted in two transgenic founder mice (NCN21 and NCN24). Unexpectedly, one transgenic line, NCN24 [NC/Nga-Tg(*Krt71*-*HB-EGF*)24Rin], showed a hairless phenotype without DT treatment. The hairless phenotype was obvious in newborns and body hair was completely absent (Fig. 1B, C) during the lifetime of the Tg mice (data not shown). The hairless phenotype was inherited in a dominant fashion. The human *HB-EGF* mRNA driven by the IRS-specific *Krt71* promoter was expressed and detected in skin tissue at the anagen phase of the hair cycle, from postnatal days 1, 2 and 7 of NCN24 mice (Fig. 1D). Northern blot analysis showed the transgenic lines NCN24 expressed higher levels of human *HB-EGF* mRNA than the other transgenic line, NCN21, in skin tissue (Fig. 1E). To confirm tissue-specific expression of the introduced human *HB-EGF* genes, we used northern blot analyses

of various tissues (brain, heart, liver, kidney, and skin) and in situ hybridization analyses of skin. In the transgenic line NCN24, expression of human HB-EGF mRNA was detected by both northern blotting (Fig. 1F) and in situ hybridization (Fig. 1G) only in skin tissue. The human HB-EGF gene had integrated into the E4 region of chromosome 14 (Fig. 1H). We confirmed that NCN24 Tg mice are co-isogenic with the parental NC/Nga strain. Thus, the phenotype can be ascribed entirely to the transgene and not to any other chromosomal region.

A histological study of NCN24 skin showed abnormalities in the distribution and morphology of hair follicles, when compared with wildtype littermates particularly in the anagen stage of the first hair cycle. At the P1 stage, the density of hair bulbs in the epidermis was much lower than in the wildtype (Fig. 2A-a, b). At the P7 stage, the hair follicles in NCN24 were evidently immature, at a low-density and irregular, and were located beneath the epidermis, compared with wildtype littermates with normal hair development (Fig. 2A-c, d). At the P12 stage (Fig. 2A-e, g), melanin granules in the hair follicles of the NCN24 mice showed an irregular distribution with weak and degraded fibers (Fig. 2A-e, f). The skin thickness in NCN24 mice was obviously less than that of wildtype littermates (Fig. 2A-e, g). These abnormalities in NCN24 were associated with impaired differentiation of hair shaft cells, e.g., the shape and structure of hair follicles in the hairless mutant was disorganized (Fig. 2A-e, f) when compared with wildtype littermates (Fig. 2A-g, h).

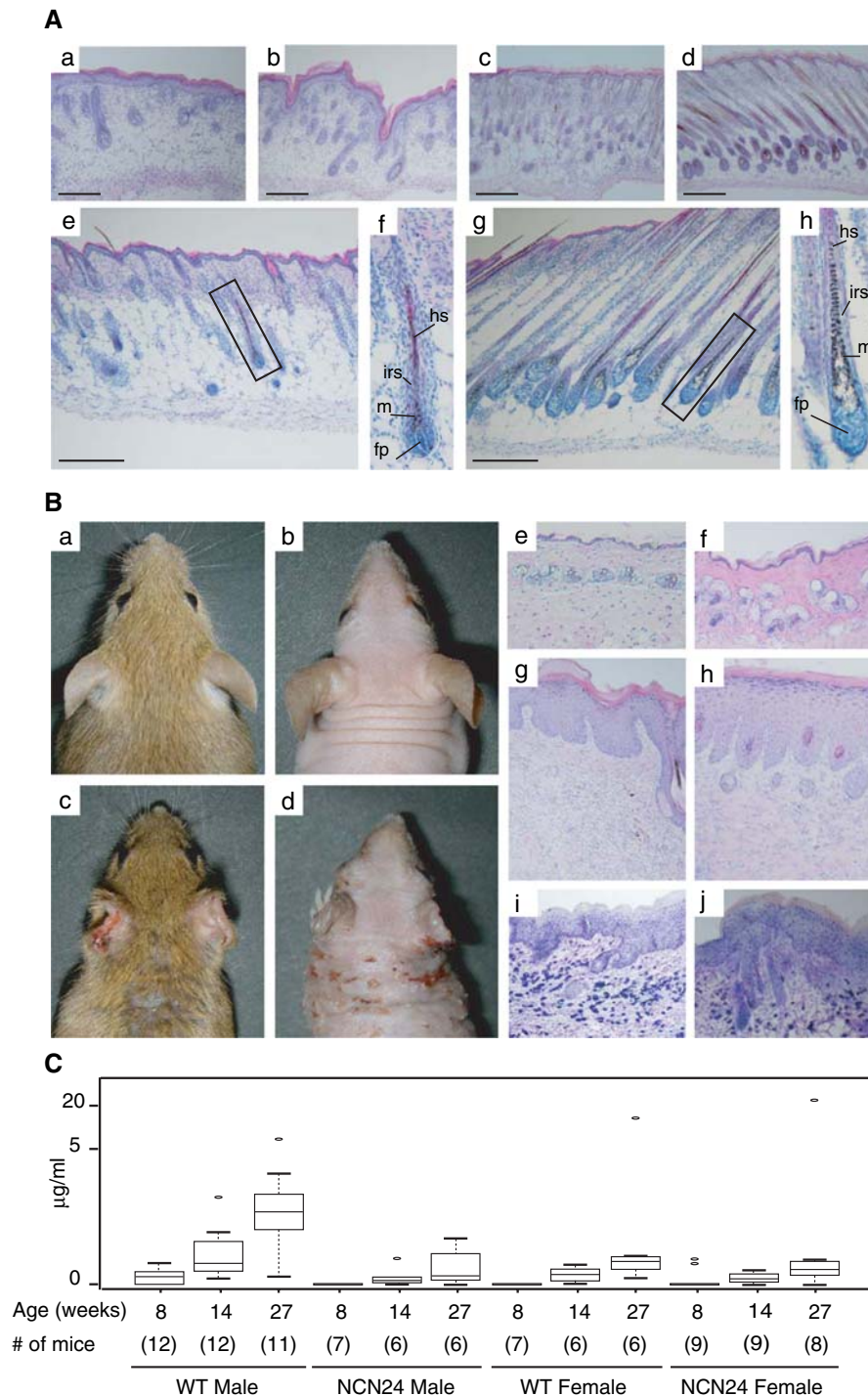
From P1 to P12 stages of NCN24 mice, we only observed immature or irregular hair follicles distributed in skin sections, indicating a lack of proper processes in the first hair cycles from the early anagen phase. The mechanisms potentially causing the hairless phenotype might be simple, namely that the ectopic expression of the human DT receptor (HB-EGF) is driven by the *Krt71* promoter in the IRS. HB-EGF is the major molecular regulator for hair cell cycles (Mak and Chan 2003). Transgenic mice with over-expressed HB-EGF driven by a ubiquitous expression vector showed hair abnormalities, including a bare-patch phenotype. This phenotype was attributed to the ectopic and irregular over-expression of HB-EGF in the IRS of Tg mice. Similarly, a spontaneous mutation that occurred at the *Krt71* locus also caused a bare-patch phenotype

(Poirier et al. 2002). Therefore, the most likely mechanism causing the hairless phenotype found in our Tg mice would be the ectopic over-expression of HB-EGF, which disturbed the start of the normal hair cell cycle. Specifically, the *Krt71* promoter in the transgene guaranteed IRS-specific expression of HB-EGF, which would augment the ectopic expression of HB-EGF in the IRS. Several mechanisms have previously been reported in which molecular signaling via the ErbB family, located downstream of HB-EGF signaling, is critical for skin and hair development during the neonatal period. HB-EGF is an essential molecule for the initiation of hair growth and entry into the appropriate phase of the hair cycle (Mak and Chan 2003), although it is not clear how the human HB-EGF transcript might have affected the development of hair follicles in the neonatal period of NCN24 mice.

An alternative possibility is that the phenotype we have described partially resembles several phenotypic features reported in studies of the hairless mouse harboring a *hr/hr* mutation (Brooke 1926). The hairless (*hr*) mutant mice have been well characterized, showing a hairless phenotype due to a failure of the follicular papilla to ascend to the permanent portion of the hair follicle during the first catagenic phase (Panteleyev et al. 1999). Therefore, we considered that a functional defect of the follicular papilla early in the anagen phase of NCN24 mice might cause an irregular sorting of melanin granules and the weak and degraded features of the hair shaft (Fig. 2A-e, f). Hair degradation was observed in new-born animals of the NCN24 line, different to the *hr* phenotype, showing that in these hairless mice development in hair follicles progressed normally while the first cycle of the anagen phase was impaired.

We undertook experiments to compare the atopic dermatitis-like predisposition in NCN24 and its parental NC/Nga strain. To evaluate immune cell populations as indicators of lymphoid development in NCN24 mice we analyzed the cellular composition of the bone marrow, spleen, and thymus by flow cytometry. No differences in any lymphoid cell populations were found when compared to wildtype littermates (Supplemental Table S1).

Atopic dermatitis-like skin lesions in NCN24 mice appeared and progressed under conventional housing conditions (Fig. 2B). The lesions appeared first but not so obviously around the ear and neck of the NCN24 mice, where the skin became dry and scaly,



whereas AD symptoms at this stage in wildtype NC/Nga mice were very hard to observe (Fig. 2B-a, b). In particular, NCN24 mice tended to exhibit dermatitis at flexural skin regions, especially at the pinnae and scapula areas (data not shown). Thereafter, lesions were

clearly observed in NCN24 mice around these two areas. Later, the lesions progressed and spread to the face, upper limb and middle of the back, even though at the same age the lesions remained hidden in the hair of wildtype littermates (Fig. 2B-c, d). The lesions became

Fig. 2 (A) Hematoxylin-Eosin (HE)-stained sections of dorsal skin from NCN24 (a, c, e) and wildtype littermates (b, d, g) at P1 (a, b), P7 (c, d), and P12 (e, g) and enlarged inset boxes (f, h) show hair follicles at anagen phase of the first hair cycle. Hair shaft labels: irs, inner root sheath; fp, follicular papilla; m, melanocytes; hs, hair shaft. Scale bar: 200 μ m. (B) Severity and histological features of the atopic dermatitis-prone skin lesion in wildtype (a) and NCN24 mice (b). The atopic dermatitis-like skin lesions were observed in pinnae and scapula of the dorsal area with congestion and scaly symptoms, and advanced dermatitis was seen in both wildtype (c) and NCN24 mice (d). Histological analysis of dermal skin lesions observed in wildtype (e, g) and NCN24 mice (f, h). The sections were stained by HE (e–h) and Giemsa (i, j). Normal dermal skin was resected from an unaffected control (e) and NCN24 mouse (f). Strong staining with Giemsa indicates mast cells in the wildtype (i) and NCN24 (j). (C) Serum IgE levels in NCN24 mice housed in conventional conditions compared with wildtype littermates. The boxplots illustrate the 25th and the 75th percentile with the median line, while the whiskers and dots show the minimum and maximum outliers. The serum level of IgE was detected after 8 weeks of age and gradually increased with aging. A number of animals show significantly higher levels of IgE at 27 weeks of age

severe and were associated with edema, hemorrhaging, erosion and dryness, typically localized to the ears, back and neck and finally in the facial region. Histopathological examination was performed on the dorsal skin (Fig. 2B–e to h). Affected animals showed hyperplasia of the epidermis and increased numbers of mast cells with mild degradation (Fig. 2B–i, j). Drastic histological changes were detected in the mice with severe skin lesions. At this stage, a skin scratching behavior was frequently observed in severely affected mice (data not shown). Spontaneous and sporadic serum IgE elevation was observed both in NCN24 mice and wildtype littermates during aging under conventional conditions. As shown in Fig. 2C, some animals had significantly higher levels of IgE at 27 weeks. IgE levels in NC/Nga mice are known to be influenced by environmental and complex genetic factors (Gutermuth et al. 2004).

Importantly, our use of the NC/Nga AD-prone genetic background, e.g., the generation of the Tg NCN24 hairless strain of NC/Nga mice, now enables easy access to the skin without any manipulation, e.g., shaving of hair. It is therefore realistic to study test mice without physical damage caused by preparation procedures. High throughput studies of combinations of allergens, reactions to various air qualities, humidity, temperature and other factors are also now possible. Our novel strain will also allow high throughput screens for ointment therapies.

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