



Aryl Hydrocarbon Receptor Directly Regulates *Artemin* Gene Expression

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ABSTRACT Transgenic mice expressing a constitutively active form of the aryl hydrocarbon receptor in keratinocytes (AhR-CA mice) develop severe dermatitis that substantially recapitulates the pathology of human atopic dermatitis. The neurotrophic factor artemin (*Artn*) is highly expressed in the epidermis of AhR-CA mice and causes hypersensitivity to itch (alloknesis) by elongating nerves into the epidermis. However, whether the *Artn* gene is regulated directly by AhR or indirectly through complex regulation associated with AhR remains unclear. To this end, we previously conducted chromatin immunoprecipitation-sequencing analyses of the *Artn* locus and found a xenobiotic response element (XRE) motif located far upstream (52 kb) of the gene. Therefore, in this study, we addressed whether the XRE actually regulates the *Artn* gene expression by deleting the region containing the motif. We generated two lines of *Artn*^{ΔXRE} mice. In the mouse epidermis, inducible expression of the *Artn* gene by the AhR agonist 3-methylcholanthrene was substantially suppressed compared to that in wild-type mice. Importantly, in AhR-CA::*Artn*^{ΔXRE} mice, *Artn* expression was significantly suppressed, and alloknesis was improved. These results demonstrate that the *Artn* gene is indeed regulated by the distal XRE-containing enhancer, and alloknesis in AhR-CA mice is provoked by the AhR-mediated direct induction of the *Artn* gene.

KEYWORDS AhR, alloknesis, artemin, atopic dermatitis, skin

Aryl hydrocarbon receptor (AhR) is a ligand-dependent transcription factor that belongs to the basic helix-loop-helix Per-Arnt-Sim family (1). Polyaromatic hydrocarbons (PAHs), which are one of the major air pollutants generated by the incomplete combustion of organic materials, such as fossil fuels, are typical ligands for AhR (2, 3). Following ligand binding, AhR undergoes a conformational change, resulting in its translocation into the nucleus. Nuclear AhR binds to the aryl hydrocarbon receptor nuclear translocator (Arnt), and the AhR-Arnt heterodimer directly binds to the xenobiotic response element (XRE), which enhances target gene transcription. The minimum core consensus sequence of XRE is 5'-CACGC-3' (4), and the expression of *Cyp1a1*, a prominent AhR target gene, has been used as an elaborate indicator of AhR activation (5, 6).

Atopic dermatitis (AD) is a chronic inflammatory skin condition that includes severe pruritus and eczema. Exposure to air pollutants has been reported as one of the risk factors associated with the development of AD (7–11). To analyze the effects of chronic exposure to air pollutants on the skin, we generated transgenic mouse lines expressing a constitutively active form of AhR (AhR-CA) under the control of the promoter of the gene *Keratin 14*, which is expressed exclusively in keratinocytes (12). Importantly, the AhR-CA mouse exhibits a phenotype similar to that of human AD patients with severe eczema and frequent scratching, implying that the constitutive activation of the AhR regulatory pathway may provoke pathological conditions similar to those frequently observed in AD patients. We previously found the marked transactivation of the

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neurotrophic factor artemin (*Artn*) in AhR-CA mouse skin, which caused allodynia by elongating nerves into the epidermis (13). Allodynia, a touch-evoked itch in which even mild mechanical stimulation triggers pruritus, is a characteristic of the skin of AD patients (14). Innocuous mechanical stimuli to the skin of AD patients, such as clothes that contact the skin, easily evoke scratching. Scratching further damages the skin barrier, worsening symptoms and significantly impairing the quality of life of patients (15).

Artn is a member of the glial cell-derived neurotrophic factor (GDNF)-related family that promotes the survival, proliferation, and neurite outgrowth of neurons by binding to GDNF family receptor $\alpha 3$ (*Gfr α 3*) (16). To date, little is known about the molecular mechanisms that regulate *Artn* gene and protein expression. In the skin, the overexpression of *Artn* in the epidermis induces the expression of transient receptor potential cation channel subfamilies V1 (TRPV1) and A1 (TRPA1) in cutaneous sensory neurons, leading to hypersensitivity to cold and warm stimuli (17). We demonstrated that *Artn* is an important regulatory factor for extending TRPV1-positive peripheral nerves in the skin, since the subcutaneous administration of an *Artn*-neutralizing antibody suppresses peripheral nerve elongation into the epidermis and blocks allodynia in AhR-CA mice (13).

Based on a few observations, we hypothesized that the *Artn* gene may be controlled by the AhR regulatory pathway. First, we previously reported that 7,12-dimethylbenz[*a*]anthracene (DMBA), which is one of the PAHs that activate AhR, upregulated the *Artn* gene expression *in vivo* (13). Second, human ARTN expression is positively correlated with CYP1A1 expression in the epidermis of AD patients (13). Third, in chromatin immunoprecipitation-sequencing (ChIP-seq) analyses, AhR-CA binds to a region that may retain regulatory influence for the *Artn* gene, and this region contains one copy of XRE (13). However, the XRE-containing region resides approximately 52 kb from the transcription start site (TSS) of the *Artn* gene and is located in an intron of another gene, ST3 β -galactoside α -2,3-sialyltransferase 3 (*St3gal3*). Therefore, this hypothesis needs to be tested rigorously by *in vivo* transactivation studies.

To address whether AhR directly upregulates *Artn* gene expression by binding to an XRE-containing region in AhR-CA mouse skin, which mimics prolonged exposure to air pollutants and the development of AD, we tried here a targeted knockout of the XRE-containing region. We generated two lines of mice harboring deletions in the XRE-containing region (*Artn* ^{Δ XRE} mice) by means of CRISPR/Cas genome-editing technology and examined whether AhR and the XRE-containing region directly regulate the *Artn* gene. Our results clearly demonstrate that the XRE-containing region, located more than 50 kb from the *Artn* gene, acts as an enhancer that drives *Artn* gene expression in the skin, and we refer to this region as the *Artn* distal enhancer.

RESULTS

AhR agonist induces *Artn* gene expression. To address AhR-dependent *Artn* gene expression, we first investigated whether a representative AhR agonist, 3-methylcholanthrene (3-MC), induces *Artn* gene expression *in vivo* in epidermis-specific AhR knockout (Keratin5-Cre::AhR^{fl α /fl α} ; K5Cre-AhR) mice and control K5Cre mice. Notably, 3-MC binds to AhR and stimulates the transcription of target genes via XRE (18). We applied 3-MC or acetone (vehicle) to the skin of control K5Cre mice and K5Cre-AhR mice at the postnatal age of 4 days (P4). In the epidermis of control mice, the expression of *Artn* and *Cyp1a1*, a typical AhR target gene, was upregulated approximately 7- and 30-fold, respectively, at 24 h after the topical application of 3-MC (Fig. 1A). In contrast, in the epidermis of 3-MC-treated K5Cre-AhR mice, the expression of *Artn* and *Cyp1a1* was not induced because of the lack of AhR.

We then examined *Artn* protein expression in the skin via immunohistochemistry. The 3-MC treatment increased the expression of both *Artn* and *Cyp1a1* proteins in the epidermal layer of control mouse skin, but the vehicle treatment did not induce the expression of either protein (Fig. 1B). Importantly, both *Artn* and *Cyp1a1* proteins were not detected in the K5Cre-AhR mouse skin, even after 3-MC treatment. The vehicle

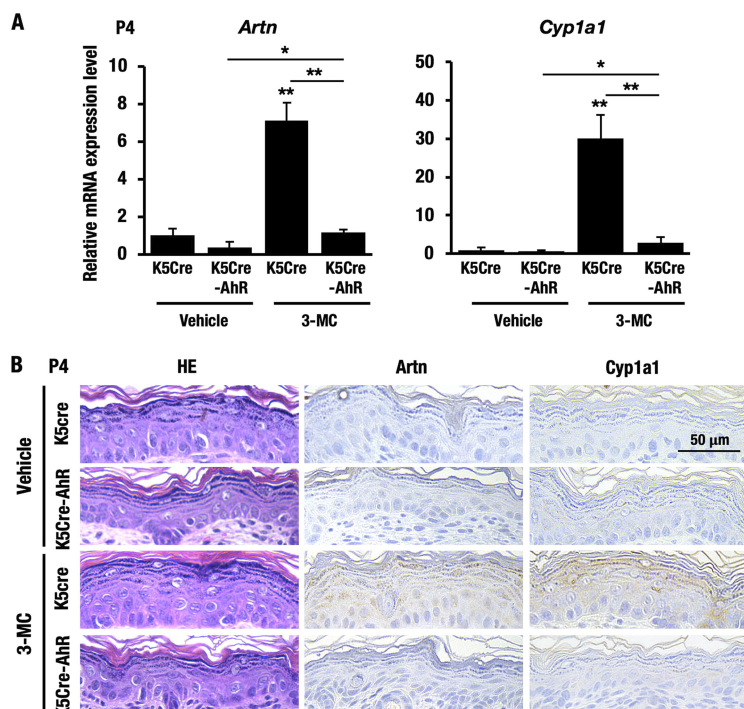


FIG 1 AhR agonist 3-MC induces *Artn* gene expression via AhR activation. (A) Expression of *Artn* and *Cyp1a1* in the epidermis of K5Cre and K5Cre-AhR (Keratin5-Cre::AhR^{lox/lox}; epidermis-specific AhR knockout) mice 24 h after topical application of the vehicle (K5Cre or K5Cre-AhR, $n = 3$ each) or 3-MC (K5Cre or K5Cre-AhR, $n = 5$ each) at P4. Note that the AhR knockout substantially abolished *Artn* and *Cyp1a1* gene expression. The results are presented relative to those of vehicle-treated K5Cre mice. Data represent the means \pm the SD. *, $P < 0.05$; **, $P < 0.01$ (asterisks without a bar indicate a comparison to vehicle-treated K5Cre mice). (B) Representative images of HE staining and *Artn* and *Cyp1a1* immunostaining in control and K5Cre-AhR mice 24 h after the topical application of vehicle or 3-MC.

treatment did not show any changes in either control or K5Cre-AhR mice. These results thus suggest that the *Artn* gene expression in the mouse skin was transcriptionally upregulated by 3-MC-induced AhR.

Identification of an XRE-containing enhancer in the *Artn* gene and the generation of *Artn* ^{Δ XRE} transgenic mice. To determine whether *Artn* gene expression is under the direct regulation of AhR or under the indirect regulation of complexes associated with AhR, we previously conducted comprehensive ChIP-seq analysis using a hemagglutinin (HA) antibody, which detects HA tag-fused AhR-CA protein (13). The analysis exploiting the epidermis of AhR-CA mice led to the identification of an AhR-CA binding site located far upstream of the *Artn* gene. Through closer inspection of the binding region, we indeed found an XRE sequence, suggesting that AhR might regulate *Artn* gene expression through binding to the XRE site in the far upstream enhancer. However, the XRE is located approximately 52 kb from the TSS of the *Artn* gene. Moreover, the enhancer region containing the XRE site is located in an intron of the *St3gal3* gene. Therefore, we realized that the potential direct regulation of the *Artn* gene by AhR needs to be verified through an experimental approach.

To examine the direct control of *Artn* gene expression by AhR through the XRE *in vivo*, we generated two lines of mice harboring an XRE deletion by using the CRISPR/Cas genome-editing approach (Fig. 2A). We designed a pair of guide RNAs (gRNAs) to delete the region, including the XRE (Fig. 2B), and obtained two mutant lines. Genomic DNA sequencing identified deletions of 128-bp (line 1) and 126-bp (line 2) genomic regions, including the XRE (Fig. 2B). The PCR products for the wild-type (WT) and *Artn* ^{Δ XRE/ Δ XRE} (*Artn* ^{Δ XRE}) mice were 483 bp (WT), 355 bp (line 1), and 357 bp (line 2) in size as determined by electrophoresis (Fig. 2C). These two lines of *Artn* ^{Δ XRE} mice showed normal growth rates and appeared to be healthy, indicating that we succeeded in generating two lines of *Artn* ^{Δ XRE} mice.

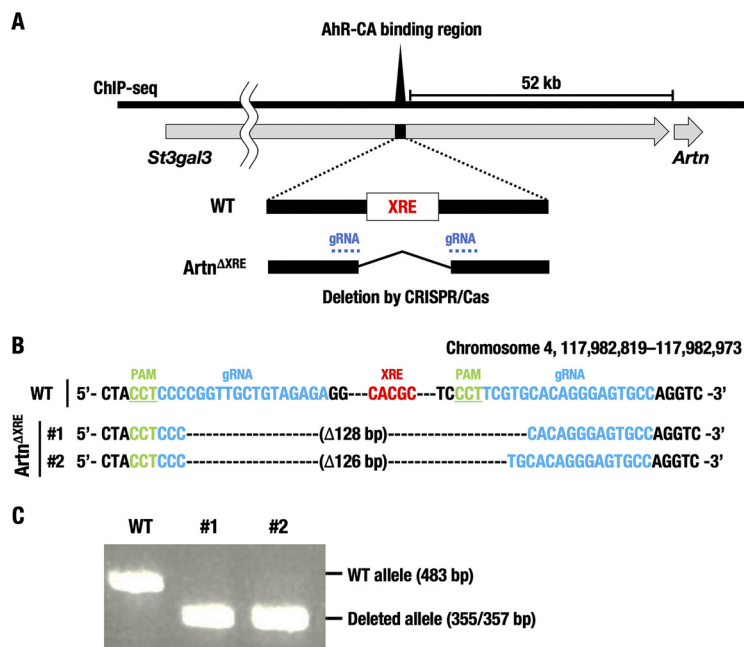


FIG 2 Generation of *Artn*^{ΔXRE} transgenic mice using the CRISPR/Cas system. (A) Schematic illustration of the AhR-CA binding region in the *Artn* distal enhancer located in an intron of the *St3gal3* gene, which is 52 kb upstream of the TSS of the *Artn* gene, including the XRE. These findings are based on ChIP-seq data using AhR-CA mouse keratinocytes (13). (B) DNA sequence around the XRE core sequence, 5'-CACGC-3' in the AhR-CA binding region in chromosome 4 (117982819 to 117982973). Positions of targeting gRNAs to generate the XRE-deleted mice (*Artn*^{ΔXRE} mice) via the CRISPR/Cas system are indicated in blue letters. Protospacer adjacent motif (PAM) sequences, 5'-CCT-3', are marked in green letters. The sequences of cleaved fragments from *Artn*^{ΔXRE} mouse lines 1 and 2 are indicated. PCR products from mouse DNA samples were sequenced. In *Artn*^{ΔXRE} mouse lines 1 and 2, 128 and 126 bp, respectively, were deleted. The black lines indicate the deleted sequence. (C) Genotyping analysis for WT and two lines of homogeneous *Artn*^{ΔXRE} mice. The genomic DNA from WT and *Artn*^{ΔXRE} mice was amplified by PCR.

Induction of *Artn* gene expression by 3-MC via the AhR-XRE axis. To clarify XRE-dependent *Artn* gene expression, we investigated whether 3-MC could induce *Artn* expression in *Artn*^{ΔXRE} mice (line 1). We applied 3-MC or acetone (vehicle) to the skin of control WT and *Artn*^{ΔXRE} mice at P4, P14, and P24 (Fig. 3, top panel). Gene expression levels were compared to vehicle-treated WT mice at P4. The expression of the *Artn* gene in the epidermis of WT mice was upregulated approximately 13-, 26-, and 37-fold at P4, P14, and P24, respectively, 24 h after the topical application of 3-MC. In contrast, the induction of gene expression by 3-MC in the epidermis of *Artn*^{ΔXRE} mice at P4, P14, and P24 was reduced approximately 5-, 7-, and 12-fold, respectively, values markedly lower than those in the epidermis of WT mice. The gene expression levels in vehicle-treated WT mice at P4 were comparable to those at P14 and P24.

The expression of *Cyp1a1* was upregulated 49-, 24-, and 37-fold in WT mice at P4, P14, and P24, respectively, after 3-MC application (Fig. 3, middle panel). The induction of *Cyp1a1* expression by 3-MC in the epidermis of *Artn*^{ΔXRE} mice at P4, P14, and P24 was upregulated approximately 56-, 27-, and 44-fold, respectively. The expression levels of *Cyp1a1* did not change substantially between WT and *Artn*^{ΔXRE} mice at three time points. We also examined the expression levels of *St3gal3* and found that there was no alteration in the deletion of XRE or the application of 3-MC in both WT and *Artn*^{ΔXRE} mice at P4, P14, and P24 (Fig. 3, bottom panel). This result indicates that the enhancer resides in the intron of the *St3gal3* gene and that the XRE sequence does not regulate *St3gal3* gene expression but strongly induces the expression of the *Artn* gene *in vivo*. These results thus demonstrate that the contribution of the *Artn* distal enhancer to *Artn* gene expression is quite reproducible in the epidermis of P4, P14, and P24 mice, while the expression levels of the *Artn* gene progressively increase with age.

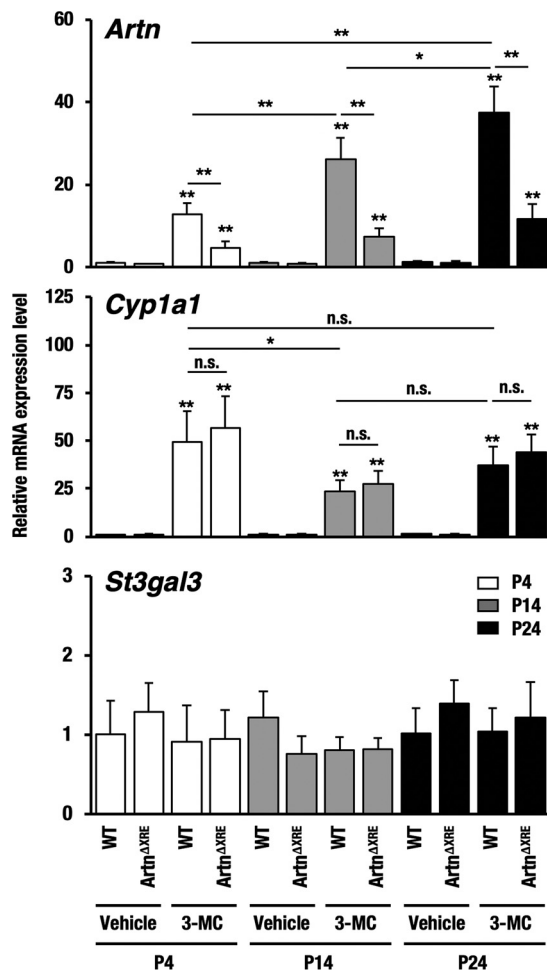


FIG 3 AhR agonist 3-MC induces the *Artn* gene via the AhR-XRE axis. The expression of *Artn*, *Cyp1a1*, and *St3gal3* in the epidermis of WT and *Artn* Δ XRE mice 24 h after the topical application of 3-MC at P4, P14, and P24 was assessed. Note that deletion of the XRE substantially reduced *Artn* gene expression. Gene expression levels were compared to vehicle-treated WT mice at P4. The data represent the means \pm the SD ($n = 3$ to 5). *, $P < 0.05$, and **, $P < 0.01$ (asterisks without a bar indicate a comparison to vehicle-treated WT mice at P4); n.s., not significant.

An important observation here is that the contribution of the *Artn* distal enhancer with an XRE is predominant; a loss-of-the-enhancer-function mutation elicits the loss of almost two-thirds of *Artn* gene expression, and this level of contribution from the enhancer is maintained at a constant level throughout the developmental stage of juvenile mice. These results suggest that there may also be independent but minor regulatory pathways for the expression of the *Artn* gene. The progressive increase in *Artn* gene expression with age further suggests the existence of additional regulatory pathways.

Loss of the AhR binding to the *Artn* distal enhancer in *Artn* Δ XRE mouse skin. We previously revealed that AhR-CA bound to the distal XRE located ~ 52 kb from the TSS of the *Artn* gene by ChIP-seq and ChIP-qPCR analyses (13). However, it remains unclear whether endogenous AhR also binds to the region. To verify the binding of AhR, we performed a ChIP-qPCR analysis using 3-MC-treated epidermis of WT and *Artn* Δ XRE mice (Fig. 4). In the epidermis of WT mice, the binding signal of AhR to the distal enhancer region was significantly higher than that of IgG, a negative-control antibody. In contrast, the AhR signal did not increase in *Artn* Δ XRE mice. These results demonstrate that endogenous AhR also activated *Artn* gene transcription via binding to the distal

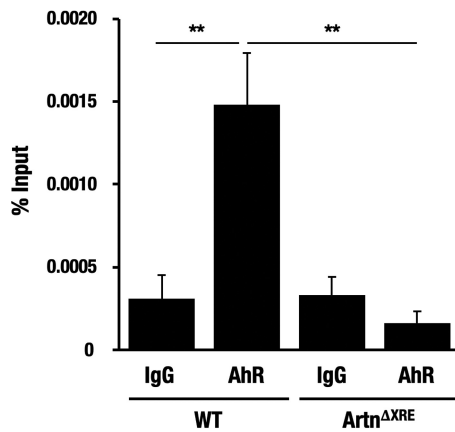


FIG 4 Endogenous AhR binds to the *Artn* distal enhancer. ChIP-qPCR analysis of the AhR binding at 52 kb upstream of the *Artn* gene in 3-MC-treated epidermis of WT or *Artn*^{ΔXRE} mice was performed. Note that the signal of AhR binding shows no increase in *Artn*^{ΔXRE} mice. The percentage of the input was calculated. The data represent the means ± the SD ($n = 4$). **, $P < 0.01$.

enhancer. Together with the results in Fig. 3, the data clearly show that AhR bound to the *Artn* distal enhancer regulates *Artn* gene expression.

Decrease in *Artn* protein expression by 3-MC in *Artn*^{ΔXRE} mouse skin. After examination of *Artn* mRNA expression, we also analyzed *Artn* protein expression in the skin via immunohistochemistry. Treatment with 3-MC increased both *Artn* and *Cyp1a1* protein levels in the epidermal layer of the skin in control mice at P4 (Fig. 5). While the *Artn* level was decreased and marginally detected in 3-MC-treated *Artn*^{ΔXRE} mouse skin, *Cyp1a1* was clearly detected in 3-MC-treated *Artn*^{ΔXRE} mouse skin at levels almost comparable to those in WT mouse skin. The application of the vehicle did not show any changes in either control or *Artn*^{ΔXRE} mice. These results clearly demonstrate that AhR directly upregulates *Artn* gene expression via XRE in the *Artn* distal enhancer and increases the expression of the gene product in the epidermis.

Induction of *Artn* via the AhR-XRE axis in AhR-CA mice. We previously generated two lines of AhR-CA mice expressing the constitutively active form of AhR in keratinocytes (12). In the epidermis of these AhR-CA mice, AhR activity was constitutively enhanced in the absence of ligands, and the *Artn* gene was substantially activated in the epidermis of AhR-CA mice (13). To address how the loss of the distal enhancer affects the expression of the *Artn* gene in AhR-CA mice, we crossed *Artn*^{ΔXRE} mice with AhR-CA mice to generate AhR-CA::*Artn*^{ΔXRE} mice. This cross-breeding strategy is shown in Fig. 6A.

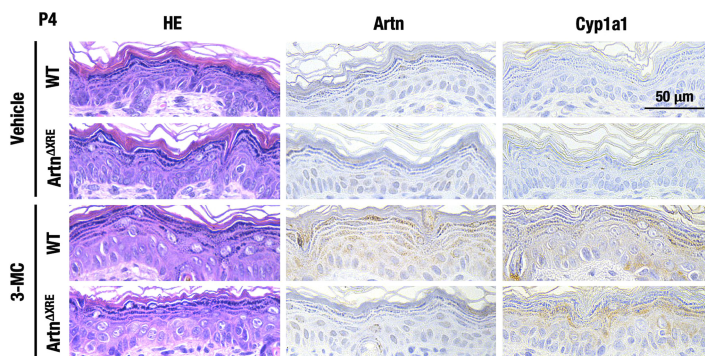


FIG 5 3-MC induces *Artn* protein expression in the epidermis via the AhR-XRE axis. Representative images of HE staining and *Artn* and *Cyp1a1* immunostaining of the epidermis of WT and *Artn*^{ΔXRE} mice at P4 24 h after the topical application of vehicle or 3-MC are shown. Note that 3-MC induced *Artn* expression in the WT epidermis but not in the *Artn*^{ΔXRE} mouse epidermis, whereas *Cyp1a1* was induced in the epidermis of both genotypes of mice.

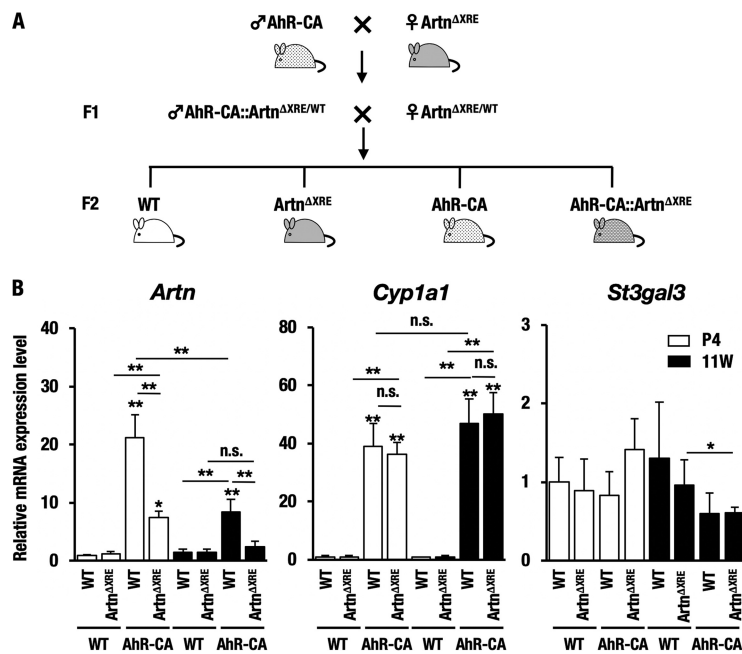


FIG 6 *Artn* gene expression is induced via the AhR-XRE axis in the epidermis of AhR-CA mice. (A) Breeding scheme to generate AhR-CA::Artn^{ΔXRE} mice. Male AhR-CA mice were crossed with female Artn^{ΔXRE} mice to obtain F₁ mice. In F₁, double heterozygous male mice (AhR-CA::Artn^{ΔXRE/WT}) were bred with female Artn^{ΔXRE/WT} mice to generate the four genotypes on mixed background. (B) Expression of *Artn*, *Cyp1a1*, and *St3gal3* in the epidermis of WT (P4 or 11W; *n* = 3 each), Artn^{ΔXRE} (P4; *n* = 5, 11W; *n* = 7), AhR-CA (P4; *n* = 4, 11W; *n* = 6), and AhR-CA::Artn^{ΔXRE} (P4 or 11W; *n* = 3 each) mice. Note that deletion of the XRE substantially reduces *Artn* gene expression. Gene expression levels were compared to WT mice at P4. The data represent the means ± the SD. *, *P* < 0.05, and **, *P* < 0.01 (asterisks without a bar indicate a comparison to WT mice at P4); n.s., not significant.

To follow up with broad-range phenotypes, we utilized in this analysis P4 and 11-week-old (11W) mice. At both P4 and 11W, expression of the *Artn* gene was increased in the epidermis of AhR-CA mice (Fig. 6B, left panel) but reduced in the epidermis of AhR-CA::Artn^{ΔXRE} mice. An intriguing observation is that the expression of the *Artn* gene was induced >21-fold by the constitutive activation of AhR in the epidermis of P4 mice, and this induction could not be completely canceled by the deletion of the distal enhancer. There was a significant difference in the expression of *Artn* mRNA between WT and AhR-CA::Artn^{ΔXRE} mice; almost 7-fold induction remained. In contrast, while there was an induction of *Artn* gene expression by the constitutively active expression of AhR at 11W, the magnitude of the induction was ~8-fold, which is much lower than that in the mouse epidermis at P4, and the level of *Artn* mRNA returned to the WT level by the deletion of the distal enhancer in 11W mouse epidermis. These results imply that there are additional regulatory influences on the expression of the *Artn* gene that may be prevalent in the epidermis of P4-stage mice.

We confirmed that *Cyp1a1* gene upregulation was comparable between P4 and 11W AhR-CA mice (Fig. 6B, middle panel). The upregulation of *Cyp1a1* gene expression was similar in AhR-CA and AhR-CA::Artn^{ΔXRE} mice, indicating that *Cyp1a1* gene expression was not affected by the deletion of the *Artn* distal enhancer. The expression of *St3gal3* was also not altered by deleting the XRE (Fig. 6B, right panel). These results thus demonstrate that *Artn* gene induction in the AhR-CA mouse epidermis was significantly reduced by the targeted deletion of the enhancer, supporting the contention that *Artn* gene expression in the AhR-CA mouse epidermis is indeed under the regulation of the *Artn* distal enhancer.

Loss of the *Artn* distal enhancer improves allknesis in AhR-CA mice. AhR-CA mice spontaneously develop AD-like skin lesions and frequent scratching with allknesis (12, 13). Available lines of evidence suggest that the high level of *Artn* expression is

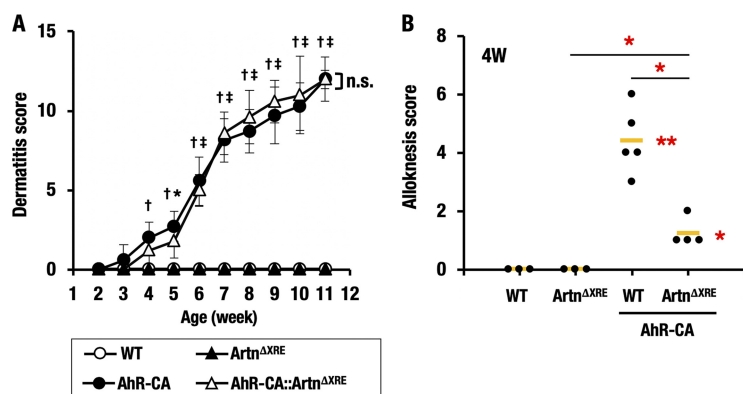


FIG 7 Loss of the *Artn* distal enhancer reduces touch-evoked scratching behavior in AhR-CA mice. (A) Dermatitis scores of WT ($n = 5$), Artn^{ΔXRE} ($n = 5$), AhR-CA ($n = 7$), and AhR-CA::Artn^{ΔXRE} ($n = 5$) mice. *, $P < 0.05$, and ‡, $P < 0.01$ (comparisons of WT mice to AhR-CA::Artn^{ΔXRE} mice); †, $P < 0.01$ (comparison of WT mice to AhR-CA mice); n.s., not significant (AhR-CA mice versus AhR-CA::Artn^{ΔXRE} mice). (B) Alloknesis scores of WT ($n = 3$), Artn^{ΔXRE} ($n = 3$), AhR-CA ($n = 5$), and AhR-CA::Artn^{ΔXRE} ($n = 4$) mice. Note that the alloknesis of AhR-CA::Artn^{ΔXRE} mice is improved significantly compared to that of AhR-CA mice. *, $P < 0.05$, and **, $P < 0.01$ (asterisks without a bar indicate a comparison to WT mice).

responsible, at least in part, for the pathogenesis of AD-like skin lesions (13). To address how the loss of the *Artn* distal enhancer improves the AD-like phenotype in AhR-CA mice, we assessed the clinical severity of dermatitis. Skin lesions first appeared at 2 to 4 weeks after birth in AhR-CA and AhR-CA::Artn^{ΔXRE} mice and were gradually aggravated with age. There was no significant difference in the severity of dermatitis between these two groups of mice by 11W (Fig. 7A). In contrast, no skin lesions developed in WT and Artn^{ΔXRE} mice.

To address how the loss of *Artn* gene expression by the deletion of the *Artn* distal enhancer improved alloknesis in AhR-CA mice, we attempted the von Frey test, which mechanically irritated the mouse skin and induced itch. To avoid the obvious influence of dermatitis, we conducted this analysis using 4-week-old (4W) mice in which dermatitis was not very apparent. Innocuous tactile stimulation with a von Frey filament induced scratching in neither WT nor Artn^{ΔXRE} mice at 4W (Fig. 7B). In contrast, light stimulation of the skin elicited severe scratching in the AhR-CA mice. Notably, this stimulation-induced scratching was significantly diminished in AhR-CA::Artn^{ΔXRE} mice, indicating that the *Artn* distal enhancer contributes to the establishment of alloknesis in AhR-CA mice.

Artn-neutralizing antibody reduces continuous scratching behavior in AhR-CA mice. While the *Artn* gene expression in AhR-CA::Artn^{ΔXRE} mice was lower than that in AhR-CA mice, the expression level was still ~7-fold higher than that in WT mice at P4 (Fig. 6B). This observation suggested that there may be other contributions of *Artn* in the AhR-CA::Artn^{ΔXRE} mouse epidermis. To clarify how significantly *Artn* upregulation contributes to the AD-like phenotype of AhR-CA mice, we administered an *Artn*-neutralizing antibody (Artn-ab) or IgG2a antibody (control) to AhR-CA mice. As described in the Introduction section, our previous study revealed that Artn-ab ameliorates alloknesis in AhR-CA mice at 4W (13). In this study, to follow up with broad-range phenotypes, we evaluated the clinical severity of dermatitis and spontaneous scratching behavior for up to 11W. The Artn-ab blocks Artn-Gfrα3 interactions (19). Skin lesions appeared at 2 to 4 weeks after birth and gradually aggravated with age in both control and Artn-ab-injected AhR-CA mice. There was no significant difference in the severity of dermatitis between these two groups of mice by 11W (Fig. 8A). No skin lesions developed in control or Artn-ab-injected WT mice.

We monitored the scratching behaviors of the four groups of mice. Figure 8B shows the results of two representative analyses for each group of mice. We did not find any significant scratching in WT mice with or without control or Artn-ab injection. In contrast, continuous scratching behavior was observed in AhR-CA mice treated with

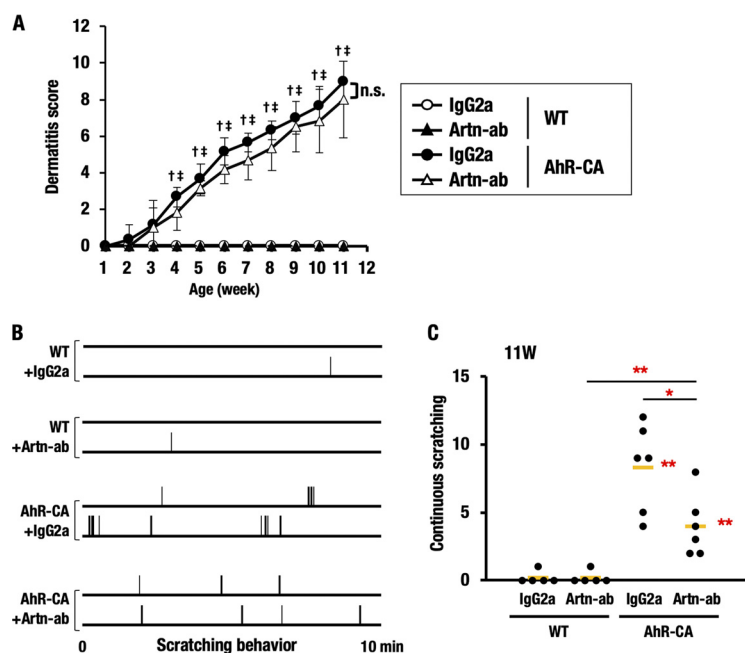


FIG 8 Application of an Artn-neutralizing antibody (Artn-ab) reduces continuous scratching behavior in AhR-CA mice. Mice on ICR background received a subcutaneous injection of an Artn-ab or IgG2a antibody (control) once a week from the first week to 11 weeks of age. (A) Dermatitis scores of WT (IgG2a or Artn-ab; $n = 5$ each) and AhR-CA mice (IgG2a or Artn-ab; $n = 6$ each) were evaluated. †, $P < 0.01$ (IgG2a-injected AhR-CA mice versus IgG2a-injected WT mice); ‡, $P < 0.01$ (Artn-ab-injected AhR-CA mice versus IgG2a-injected WT mice); n.s., not significant (IgG2a-injected AhR-CA versus Artn-ab-injected AhR-CA mice). The data represent the means \pm the SD. (B) Time of scratching behavior (vertical lines) in two WT and AhR-CA mice treated with IgG2a or Artn-ab. (C) Quantification of continuous scratching behavior (scratching without an interval of >5 s between bouts) in the WT (IgG2a or Artn-ab; $n = 5$ each) and AhR-CA mice (IgG2a or Artn-ab; $n = 6$ each). Note that continuous scratching in Artn-ab treated AhR-CA mice is improved compared to that in IgG2a-treated AhR-CA mice. *, $P < 0.05$, and **, $P < 0.01$ (asterisks without a bar indicate a comparison to IgG2a-treated WT mice).

the control antibody, which indicated the presence of allorknesis. Notably, the injection of Artn-ab markedly reduced the consecutive scratching in the AhR-CA mice.

The administration of Artn-ab significantly improved the scratching behavior of AhR-CA mice (Fig. 8C), supporting the notion that the *Artn* gene contributes to the characteristic continuous scratching behavior in AhR-CA mice. These results thus indicate that the development of AD-like dermatitis in AhR-CA mice is attributable to multiple AhR downstream pathways and that the Artn-scratching pathway is one of the important cascades leading to AD-like dermatitis.

DISCUSSION

To elucidate how *Artn* gene expression is upregulated in the AhR-CA mouse epidermis, we previously examined the mouse *Artn* gene and identified an *Artn* distal enhancer that harbors one copy of XRE (13). This enhancer resides 52 kb from the TSS of the *Artn* gene. To evaluate the contribution of the enhancer and the XRE, we deleted the XRE and some surrounding sequences and established the *Artn* ^{Δ XRE} mouse lines, which enabled us to study the contribution of the distal enhancer and XRE. As summarized in Fig. 9, our results revealed that the pharmacological and genetic activation of AhR in the epidermis upregulates *Artn* gene expression through the *Artn* distal enhancer containing XRE. The inducible expression of *Artn* via this enhancer provokes allorknesis in mice, which contributes, at least in part, to the pathogenesis of AD-like dermatitis.

Mechanisms of transcriptional enhancement through the AhR-XRE axis have been well characterized using *CYP1* genes that are representative AhR targets (4, 20–22). Both *CYP1A1* and *CYP1B1* have several XREs within ~ 1 -kb sequences upstream of each gene,

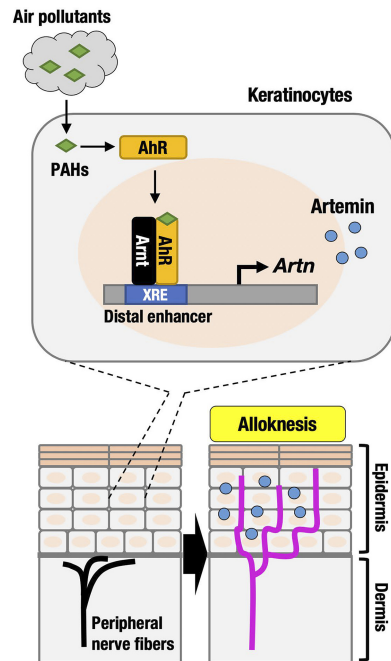


FIG 9 Schema of the AhR-mediated development of alloknesis in AD. AhR is activated by PAHs in environmental pollutants. Activated AhR directly binds to the XRE in the *Artn* distal enhancer and induces the expression of the *Artn* gene in keratinocytes. Artn enhances the growth of peripheral nerves to induce hyperinnervation of the epidermis, resulting in hypersensitivity to itch (alloknesis).

indicating that AhR utilizes juxtaposed XREs and enhancers. Similarly, a ChIP-seq analysis using TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin)-treated MCF-7 human breast cancer cells revealed that the peak densities of AHR and ARNT are highest within a 1 kb from the TSS, supporting the notion that many AhR target genes are regulated by AhR binding to the proximal promoters (23). One salient exception is the *AHR repressor* (*AHRR*) gene, another well-known AhR target gene. *AHRR* has a functional XRE located ~17 kb downstream of the TSS, which is distal compared to the other important AhR target genes (24). Our present results provide another salient example that the AhR-XRE axis has the potential to regulate target gene expression by binding to the distal XRE located more than 50 kb away. Importantly, in an inspection of the above-mentioned ChIP-seq data (23), we have noticed several genes that harbor distant AHR-ARNT cobinding regions within 100 kb from the TSS, but the functional significance of the peak regions awaits further characterization.

To the best of our knowledge, this result is the first demonstration that the transcription of the *Artn* gene is directly regulated by AhR through binding to XRE. In this regard, we found that deletion of the *Artn* distal enhancer abrogated approximately two-thirds of the *Artn* gene expression induced by 3-MC in *Arnt*^{ΔXRE} mice at P4, P14, and P24, but almost one-third of the *Artn* gene expression remained in these mice. This observation was reproducible in the AhR-CA::*Arnt*^{ΔXRE} mice at P4, which harbor the constitutively active expression of AhR combined with the *Artn* distal enhancer deletion. In contrast, in 11W AhR-CA::*Arnt*^{ΔXRE} mouse epidermis, the level of *Artn* mRNA was comparable to that of WT mice, and the remaining activity disappeared. While the precise regulation that maintains *Artn* gene expression in the absence of the *Artn* distal enhancer in juvenile mice remains to be clarified, we surmise that the following observations may be pertinent.

One plausible explanation for these observations is that there may be an additional XRE. However, since we did not find any obvious peaks in our ChIP-seq analyses within 50 kb upstream and downstream of TSS, we feel that this possibility is unlikely. Alternatively, there may be additional transcription activation pathways that lead to

Artn gene expression. It should be noted that the estrogen receptor (ER) has been reported to regulate the expression of the *Artn* gene (25). Estrogen stimulation induces the expression of *ARTN* in the ER-positive mammary carcinoma cell lines MCF-7 and T47D (25). This induction has been shown to be ER-dependent in an experiment exploiting the ER-negative mammary carcinoma cell line BT549 (25). ER α and ER β are ligand-activated transcription factors that belong to a nuclear receptor superfamily. Intriguingly, there are interactions between the AhR and ER signaling pathways (26). In fact, AhR ligands induce the formation of an AhR-ER complex that binds to the estrogen response element and stimulates the transcription of ER target genes (27, 28). ERs are expressed in keratinocytes (29, 30). Therefore, we surmise that ERs and related factors may be some of the candidates responsible for the remaining *Artn* gene expression.

Available lines of evidence suggest that the high-level expression of *Artn* is responsible, at least in part, for the pathogenesis of AD-like skin lesions (13). In this study, we found that the deletion of the *Artn* distal enhancer did not result in complete remission from AD symptoms. We surmise that the constitutive activation of AhR may also evoke abnormalities in addition to nerve elongation into the epidermis that depend on *Artn* overexpression. Nonetheless, we found that the suppression of *Artn* gene expression induced by the deletion of the *Artn* distal enhancer improved touch-evoked or spontaneous scratching behaviors in the AhR-CA::Artn Δ^{XRE} mice. This finding shows very good agreement with our current and previous studies (13) using an *Artn*-neutralizing antibody. Subcutaneous injection of the anti-*Artn* antibody markedly reduced the consecutive scratching in the AhR-CA mice. Our results thus demonstrate that alloknesis is suppressed at the local site in AhR-CA mice by the *Artn*-suppression-mediated blocking of peripheral nerve elongation into the epidermis.

Itch is an important trigger of AD, and innocuous mechanical stimuli to the skin of AD patients can easily evoke itching. Scratching further damages the skin barrier, leading to inflammation in the skin. Currently, the common treatment for AD-associated itch is the topical use of corticosteroids (31). The topical application of corticosteroids inhibits inflammatory cytokines or chemokines, thereby decreasing local inflammation and indirectly suppressing pruritus (32). Novel therapeutic drugs are required for AD patients for whom steroids are ineffective or in whom adverse effects from steroids after long-term use occur. To this end, we hypothesized that controlling the itch in AD patients by eliminating alloknesis is one of the important approaches in the treatment of AD.

We envisage that the development of drugs that inhibit the activation of the AhR-*Artn* axis is likely to proceed from the following two aspects. First, the blockade of the *Artn*-induced neuronal extension appears to contribute to the elimination of the molecular basis underlying the disease. Second, this approach may have good efficacy in the treatment of patients with aggravated AD symptoms, especially those aggravated by environmental pollutants.

MATERIALS AND METHODS

Mice. Transgenic mouse lines expressing the constitutively active form of AhR (AhR-CA) conjugated with a hemagglutinin (HA) tag under the control of the *Keratin 14* gene promoter were generated as described previously (12). The AhR-CA mouse line 239 was used in this study. A mouse line with loxP-flanked AhR alleles (AhR $^{fllox/fllox}$) on the C57BL6/J background was supplied from Jackson Laboratory (33). A mouse line expressing Cre recombinase from the epidermis-specific *Keratin 5* gene promoter (Keratin5Cre; K5Cre) on the C57BL6/J background was kindly provided by Junji Takeda (Osaka University) (34). We crossed AhR $^{fllox/fllox}$ mice with K5Cre mice to generate K5Cre::AhR $^{fllox/fllox}$ (K5Cre-AhR) mice on the C57BL6/J background. AhR-CA mice were maintained on a mixed or ICR background. All mice were treated according to the regulations of the Standards for the Human Care and Use of Laboratory Animals of Tohoku University and the Guidelines for the Proper Conduct of Animal Experiments of the Ministry of Education, Culture, Sports, Science, and Technology of Japan. All animal experiments were conducted with the approval of the Tohoku University Animal Care Committee.

Generation of Artn Δ^{XRE} mice. Based on the ChIP-seq data of AhR in AhR-CA mouse keratinocytes, Artn Δ^{XRE} mice were designed and generated to delete the XRE located 52 kb upstream of the *Artn* gene by CRISPR/Cas genome-editing technology. *Cas9* mRNA, a pair of guide RNAs (gRNAs), and targeting oligonucleotides were injected into fertilized eggs derived from BDF1 mice. The founder mice were crossed with WT mice, and the mutations were transmitted to the germ line. The homozygotes (Artn $\Delta^{XRE/\Delta^{XRE}}$; Artn Δ^{XRE}) were obtained by crossing the heterozygous mutants. The Artn Δ^{XRE} mutant mice

were identified by PCR (30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) using the forward primer 5'-TCCCTCTCTCCATTCTCT-3' and the reverse primer 5'-TACCCAGCCAGAGAAGCAGT-3'. The ChIP-seq analysis data (GSE72449) were used in the Gene Expression Omnibus (GEO). We crossed AhR-CA mice with Artn^{AXRE} mice to generate AhR-CA::Artn^{AXRE} mice on a mixed background.

DNA sequencing. A genomic DNA region around the XRE located in the *Artn* distal enhancer was amplified by PCR (30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s) using the forward primer 5'-CAGGTCGCTGCAGATTCT-3' and the reverse primer 5'-TACCCAGCCAGAGAAGCAGT-3'. The amplified samples were then sequenced by ABI3100 (Applied Biosystems) using standard protocols.

Exposure to 3-MC. 3-MC (Sigma-Aldrich), a carcinogen in experimental animals and human cells, was dissolved in acetone at a concentration of 0.04 mg/g (body weight). At 24 h after shaving, the male mice were anesthetized, and 3-MC or vehicle was topically applied rostrally to their shaved backs. Samples of the epidermis at the application sites were harvested 24 h after the application.

cDNA synthesis and quantitative reverse transcription-PCR. To obtain murine epidermal samples, whole skin samples were incubated for 20 h at 4°C with 0.5% (wt/vol) trypsin. The samples were then separated into epidermal and dermal layers by peeling the layers apart. Total RNA was extracted from the epidermis using Sepasol-RNA I Super G (Nacalai Tesque). cDNA was synthesized from total RNA using ReverTra Ace qPCR RT master mix with gRNA remover (Toyobo). Quantitative reverse transcription-PCR (RT-qPCR) was performed by using a KAPA SYBR Fast qPCR kit (Nippon Gene) with the StepOne RT-PCR system (Applied Biosystems) or the QuantStudio 3 real-time PCR system (Life Technologies). The data were normalized to *rRNA* expression. Relative expression levels were determined using the $\Delta\Delta C_T$ method. The primer sequences used for RT-qPCR were as follows: *Artn*, 5'-AGACGGCCTCATAGCGAGT-3' (forward) and 5'-CGAGCTGATACGTTCCGCTT-3' (reverse); *Cyp1a1* (37), 5'-GGGTTTGACACAGTCACAAC-3' (forward) and 5'-GGGACGAAGGATGAATGCCG-3' (reverse); *rRNA* (38), 5'-CGGCTACCACATCCAAGGAA-3' (forward) and 5'-GCTGAATTACCGCGGCT-3' (reverse); and *St3gal3*, 5'-TGGGAAGACTCAATTCATTGCT-3' (forward) and 5'-AGTTTGCGTACTTGGTGGCT-3' (reverse).

ChIP-qPCR analyses. WT and Artn^{AXRE} mice were treated with 3-MC for 24 h. Whole skins were incubated for 20 h at 4°C with 0.5% (wt/vol) trypsin and then separated epidermal layers. The epidermis was fixed with 1% (vol/vol) formaldehyde and quenched with glycine. Lysates were sonicated with an ultrasonic homogenizer Sonifier 250 (Branson) to obtain chromatin fragments 200 to 1,000 bp in length. The protein, DNA, and antibody complexes were retrieved with protein A- and protein G-Sepharose (GE Healthcare). Chromatin lysate was precleared and incubated overnight with anti-AhR antibody (sc-5579; Santa Cruz Biotechnology) and normal rabbit IgG (sc-2027; Santa Cruz Biotechnology). DNA was purified using a phenol-chloroform extraction. To analyze protein-bound DNA, the primers for qPCR were as follows: *Artn*, forward (5'-TCCCTCTCTCCATTCTCT-3') and reverse (5'-TACCCAGCCAGAGAAGCAGT-3'). qPCR was performed by using the KAPA SYBR Fast qPCR kit (Nippon Gene) with a QuantStudio 3 real-time PCR system (Life Technologies). The percentage of the input that was bound was calculated.

Histology. Skin samples fixed in Mildform 10N (Wako Pure Chemical) were embedded in paraffin and sectioned. For histological analysis, the samples were stained with hematoxylin and eosin (HE). For immunohistochemistry, the sections were reacted with anti-Cyp1a1 antibody (sc-9828; Santa Cruz Biotechnology) or anti-Artn antibody (MAB10851; R&D Systems) overnight. Anti-Cyp1a1 antibody or anti-Artn antibody binding was detected with a horseradish peroxidase-conjugated anti-goat IgG antibody. The positive reactivity was visualized through diaminobenzidine staining.

Evaluation of dermatitis. The clinical severity of dermatitis was assessed as reported previously, with minor modifications (35). This scoring is based on the severity of four possible symptoms (scaling, erosion, hemorrhage, and erythema) and the area in which the skin lesions occurred. The degree of each symptom was scored as follows: 0 (no symptoms), 1 (mild), 2 (moderate), or 3 (severe). The area of the skin lesions was scored as 1 for each location (face, rostral back, and caudal back). The sum of the individual scores (minimum, 0; maximum, 15) was taken as the dermatitis score.

Measurement of scratching behavior. Male mice were acclimated in an acrylic cage for 10 min, and the frequency of scratching was measured for 10 min. According to a previous report (13, 36), scratching behavior was defined as movement of the hind paw up to the back or face of the mouse. The behavior was considered finished when the mouse placed its hind paw back on the floor. Continuous scratching behavior was defined as scratching occurring at intervals of <5 s.

Measurement of alloknesis. Alloknesis scores were determined using a previously reported method (13). The rostral part of the back of each male mouse was shaved on the day before analysis. After an acclimation period of approximately 10 min in an acrylic cage, the mice received three separate innocuous mechanical stimulations with a von Frey filament (0.16-g bending force) on the shaved regions of their backs. This stimulation rarely elicited scratching behavior in WT mice. The presence or absence of scratching behavior was recorded immediately after each stimulus. These three innocuous stimuli were repeated three times at 5-min intervals (nine stimulations in all), and the alloknesis score was the total number of positive responses.

Artn neutralization. Male mice received a subcutaneous injection at 50 mg/kg (body weight) of Artn-neutralizing antibody (MAB10851; R&D Systems) or IgG2a antibody as a control (MAB006; R&D Systems) in the rostral part of the back once per week from the first week to 11 weeks of age.

Statistical analysis. The average values were calculated, and the error bars indicate the standard deviations (SD). The differences were analyzed using Student *t* test. A *P* value of <0.05 was considered statistically significant.

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