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RESEARCH ARTICLE

Modulation of scratching behavior by silencing an endogenous cyclooxygenase-1 gene in the skin through the administration of siRNA

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Abstract

Background RNA interference (RNAi) is rapidly becoming a major tool that is revolutionizing research in the bioscience and biomedical fields. To apply the RNAi technique *in vivo*, it is crucial to develop appropriate methods of guiding the short interfering RNA (siRNA) molecules to the right tissues and cells. Here, we demonstrate an efficient method for performing gene knockdown in the body skin using the *in vivo* electro-transduction of siRNA. Using this method, we examined whether the targeted silencing of the cyclooxygenase (COX) gene in the skin could modulate the scratching behavior of an atopic dermatitis mouse model.

Methods NC/Nga mice were used as the atopic dermatitis model. Using our optimized *in vivo* electroporation conditions, siRNAs were introduced into the skin; the silencing efficiency was then analyzed by Western blotting, measuring the levels of prostaglandins, and immunohistochemistry. The scratching behaviors of the mice were measured using an automatic system.

Results Targeted silencing of the COX-1 gene using our *in vivo* siRNA technique significantly accelerated the scratching behavior of NC/Nga mice, whereas the COX-2 siRNA showed no effect. In addition, the effect of COX-1 siRNA was mimicked by treatment with a COX-1-selective inhibitor (SC-560).

Conclusions We have demonstrated the successful silencing of endogenous gene expression in the skin using the intradermal transfection of unmodified siRNA via electroporation. Using this method, we revealed that COX-1-mediated prostaglandins may act as endogenous inhibitors of scratching behavior. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords siRNA; in vivo; electroporation; COX-1; scratching; atopic dermatitis

Introduction

RNA interference (RNAi) is a process in which short (21- to 23-nucleotide) double-stranded RNA, called small interfering RNA (siRNA), species are utilized to inhibit the expression of genes in a highly sequence-specific manner [1,2]. The siRNA is taken up by an RNA-inducing silencing complex (RISC) in the cytoplasm and silences the expression of mRNA based on a complementary sequence. RNAi has currently become an almost-standard method for *in vitro* experiments. A major focus of research is to explore its potential use *in vivo*, since siRNAs show promise as novel therapeutic tools. In addition, siRNAs may also be useful for deciphering the functions of

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disease-controlling genes. A number of studies have already shown promising results using various methods of administration and various siRNA formulations.

Electroporation has been shown to be a feasible approach for delivering plasmid genes directly into living tissues and to produce functional genes. The skin is a relatively attractive target for gene delivery because of its easy accessibility; thus, skin-targeted gene transfer methods using *in vivo* electroporation have become increasingly sophisticated [3]. Recently, an efficient electro-transfection method using combinations of high-voltage and low-voltage pulses has been reported [4]. A repeated-pulse technique using a novel electrode to produce optimized transgene expression in the skin with minimal tissue damage has also been reported [5]. The critical components of this technique are to configure the electrode, to optimize the transgene expression efficiency, and to minimize the tissue damage.

Itching (also called pruritus) is a sensation felt in the skin that elicits the desire to scratch that particular area. NC/Nga mice are a potential animal model for atopic dermatitis with chronic itching [6,7]. When raised in their conventional environments, the majority of these animals spontaneously develop skin lesions (eczema, bleeding, and alopecia) associated with excessive scratching within 2–6 months of birth. When the NC mice are housed in a specific-pathogen-free (SPF) environment, they do not develop skin lesions, severe scratching, or elevated plasma immunoglobulin levels; however, when returned to a conventional environment, they soon develop all of these features of atopic dermatitis and begin to scratch their faces and their rostral and nuchal regions all day long. When the mice are housed under 12-h light/dark conditions, the number of scratching bouts is larger during the period of darkness than during lighted conditions [8]. Recent studies have revealed that the spontaneous scratching in these animals is significantly reduced by the topical application of exogenous prostaglandins (PGs) or their precursor, arachidonic acid. Conversely, it is aggravated by treatment with indomethacin, an inhibitor of PG synthesis, suggesting that endogenous PGs might function as physiological inhibitors of itching [9]. Also, only cyclooxygenase (COX)-1-mediated PGs have been shown to play a role in inhibition of pruritus [10].

In the present study, we employed a simple electroporation method to produce sufficient transgene expression in the skin and successfully applied this technique to *in vivo* siRNA transduction. Moreover, we demonstrated that the silencing of the endogenous COX-1 gene in the skin by the local administration of COX-1 siRNA effectively aggravated scratching behavior in NC mice.

Materials and methods

Animals and chemicals

Male NC/Nga mice were used (SLC Japan, Shizuoka, Japan). The animals were housed under conditions of controlled temperature ($23 \pm 3\,^{\circ}C$), humidity ($55 \pm 20\%$) and lighting (lights on from 7:00 am to 7:00 pm). Specific-pathogen-free (SPF) and conventional NC/Nga mice were used at 7–8 weeks and 15–20 weeks of age, respectively. All the experiments conformed to the Japanese Experimental Animal Research Association Standards, as defined in the Guidelines for Animal Experiments. SC-560 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

Plasmid reporter gene and siRNA transfection *in vivo*

Keratin cream (Fukuda Denshi, Tokyo, Japan) was applied to the shaved upper back skin of anesthetized mice, where a pair of electrode pads (1.0 cm in diameter; NepaGene, Chiba, Japan) had been placed. The mice were subsequently given an intradermal injection of 50 μg of CMV-β-gal plasmid (Invitrogen, Carlsbad, CA, USA) or SV40-Luc2 plasmid (pGL4.13, Promega, Madison, WI, USA) in 50 μL of TE buffer, followed by electroporation using a CUY21 electric pulse generator (NepaGene) and a 4 square-wave pulse setting with a 100-ms pulse length/pulse at 67–200 V/cm, followed by another pulse of opposite polarity. Twenty-four hours after electroporation, β-gal activity in whole-skin lysates (40 mg tissue/0.5 mL lysis buffer) was assayed using chlorophenol red-β-D-galactopyranoside (Stratagene) as a substrate at A590 nm; the resulting values were represented as the normalized value of β-gal activity (A590 nm)/40 mg tissue. The skin was also dissected, stained overnight with X-gal substrate (Stratagene), and histologically examined for β-gal expression under a microscope (3-μm sections). Luciferase activity in whole lysates from the brain, heart, lungs, liver, spleen, kidney, skeletal muscle, upper back skin of the injected site, and lower back skin of the non-injected site were assayed using a luciferase assay kit (Promega) and a Wallac 1420 Multilabel counter (PerkinElmer, Waltham, MA, USA). Each measurement was normalized by the weight of the tissue and represented as luciferase activity/mg tissue. For the *in vivo* silencing experiment, 50 μL of siPORT Amine (Ambion, Austin, TX) containing 12.5 μg (1.0 nmoL) of siRNA (siGENOME SMARTpool and ON-TARGETplus, Dharnacon, Chicago, IL, USA) was administered into the skin, and electroporation was performed using the optimized conditions. Non-silence scrambled-control RNA (Dharmacon) was used as a negative control. When applying the electroporation, the skin was clamped between the two electrode pads during all the experiments.

Western blot analysis

To analyze COX-1 and COX-2 expression, 100 μg of the skin tissue lysates/250 μL of lysis buffer were incubated with 2 μg of anti-COX-1 monoclonal (Cayman) or anti-COX-2 monoclonal antibody (Santa Cruz Biotechnology,

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Santa Cruz, CA, USA) for 1.0 h, followed by additional overnight incubation with protein A–agarose. The immunoprecipitates were subjected to the following analysis. anti-COX-1 polyclonal (Santa Cruz Biotechnology) and anti-COX-2 polyclonal (Cayman) antibody were used as the primary antibodies. Anti- β-actin monoclonal antibody (Abcam, Cambridge, UK) was used as the control. The blots were incubated with peroxidase-conjugated secondary antibody, and the immunoreactive proteins were visualized using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) and a Lumi-Imager (Roche Applied Science, Indianapolis, IN, USA).

Immunohistochemical analysis

The skin specimens were incubated with anti-COX-1 polyclonal antibody (Cayman Chemical) and were detected using the avidin-biotin peroxidase complex method. Both the epidermal and the dermal sides of the skins were observed under a light microscope (Axioplan 2 microscope; Carl Zeiss, Jena, Germany). The intensity of COX-1 staining was evaluated at five areas of the epidermis per mouse (n = 4) at a magnification of 200× in a random, blinded manner. The areas of COX-1 immunoreactivity were analyzed using an IPLab Spectrum (Scanalytics, Fairfax, VA, USA).

Measurements of skin prostaglandins

On the day of analysis, the mice were given an intravenous (i.v.) injection of indomethacin (10 mg/kg) to prevent the further production of prostaglandins (PGs), and the skin in the area where the electropads were placed was removed from each mouse. The skin was homogenized in ice-cold phosphate-buffered saline (PBS) containing indomethacin (10 μM), de-proteinized, and the amounts of PGD2, PGE2, PGI2 and PGF2α were measured using EIA kits (Cayman Chemical), in accordance with the manufacturer's instructions. The concentrations of the PGs were expressed per gram of tissue weight.

Spontaneous scratching behavior analysis

The scratching behavior of the mice was measured as previously described [11]. Briefly, a small magnet (1 mm in diameter, 3 mm long) was implanted subcutaneously into both hind paws of each mouse, and the animals were placed individually in an observation chamber surrounded by a circular coil, through which the electric current induced by the movement of the magnets attached to the hind paws was amplified and recorded. The number of scratching bouts was automatically measured using a MicroAct (Neuroscience, Tokyo, Japan).

Statistical analysis

Data were expressed as the mean ± standard error of the mean (SE) (n = 3, 4 or 6). Comparisons between two groups were performed using Student's *t*-test. A p value of less than 0.05 was considered significant.

Results

β-Galactosidase expression in the skin

First, we attempted to optimize a method for delivering plasmid DNA (pDNA) into the skin tissue. Electrical pulses were applied in various numbers and voltages to the shaved upper back skin following the injection of a plasmid vector harboring the β-galactosidase (β-gal) gene as a marker. Figure 1a shows the β-gal activity in the targeted skin tissue 24 h after the transfection. Pulse-number- and voltage-dependent increases in enzyme activity were detected, and the maximum expression level of the marker gene with minimal tissue damage was achieved with the application of double or triple set pulses of 200 V/cm, whereas the injection of naked DNA alone (0 V) did not produce any marked increase in enzyme activity. We determined that the resistance value should be carefully adjusted to around 0.7–0.8Ω at the time of the application of each pulse by coating the skin with moderate amounts of keratin cream, which yielded an optimum current value of 0.10–0.15 A at 200 V/cm (a 7.6-fold increase in gene expression with double set pulses at 200 V/cm, compared to that with no pulse). The use of voltages greater than 200 V/cm or currents greater than 0.20 A resulted in visible tissue damage.

To localize the expression of the marker gene in the skin tissue after the electro-introduction of the pDNA, X-gal staining followed by histological analysis were performed. The normal epidermis and the plasmid-injected epidermis that was not treated with electroporation showed no colorimetric reaction, whereas the dotted β-gal expression became evident in the epidermis, but not the dermis, following electroporation using the optimal electrical pulse (double set pulses at 200 V/cm, at 0.10–0.15 A), as shown in Figure 1b. The X-gal colorimetric reaction was also detectable in some parts of the hair follicle (Figure 1b) and parts of the subcutaneous muscle layer (data not shown). Thus, the constituent cells of the epidermis, such as keratinocytes and Langerhans cells, appear to be ideal targets for skin transfection.

Using the luciferase assay system, we next tested whether the gene expression area affected by the intradermally administered plasmid was limited to the targeted part or systemic. The luciferase system was utilized instead of β-gal because of the high β-gal background activity in some organs. As shown in Figure 1c, the skin-targeted luciferase reporter gene

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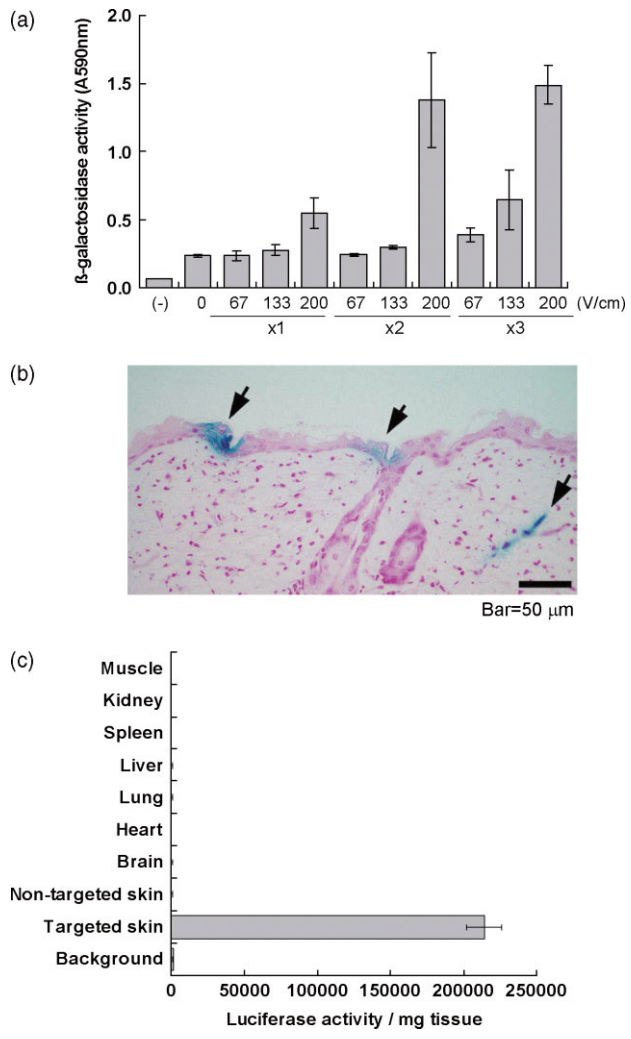

Figure 1. Electroporation-assisted transfection of an exogenous gene into normal skin. (a) Pulse-number- and voltage-dependent expression of the β-galactosidase (β-gal) gene in the skin after the intradermal injection of a plasmid coding for β-gal. Whole upper back skin tissues at the injected site were collected 24 h after the *in vivo* plasmid electro-transfection and assayed for β-galactosidase activity using chlorophenol red-β-D-galactopyranoside (CPRG) as the substrate. Optimum activity was obtained with double set pulses at 200 V/cm. Data are shown as the mean ± SE (n = 3). (b) Localization of β-gal expression in the skin tissue after the injection of CMV-β-gal pDNA. Skin tissues at the injected site were collected 24 h after electroporation, and X-gal staining followed by histological analysis were performed. When the optimized pulse conditions (double set pulses at 200 V/cm and 0.1–0.15 A) were applied, dotted β-gal expression became evident in the epidermis and some parts of the hair follicles (arrows). (c) Luciferase expression in major organs after the intradermal injection of pDNA via electroporation. For the luciferase enzyme assay, brain, heart, lungs, liver, spleen, kidney, the muscle under the injected site, the upper back skin of the injected site, and the lower back skin of a non-injected site were harvested 24 h after injection from mice treated with SV40-Luc2. Data are shown as the mean ± SE (n = 3)

expression was localized and expressed only in the injected skin (upper back site), but not in any other tissues, including the non-injected part of the skin (lower back).

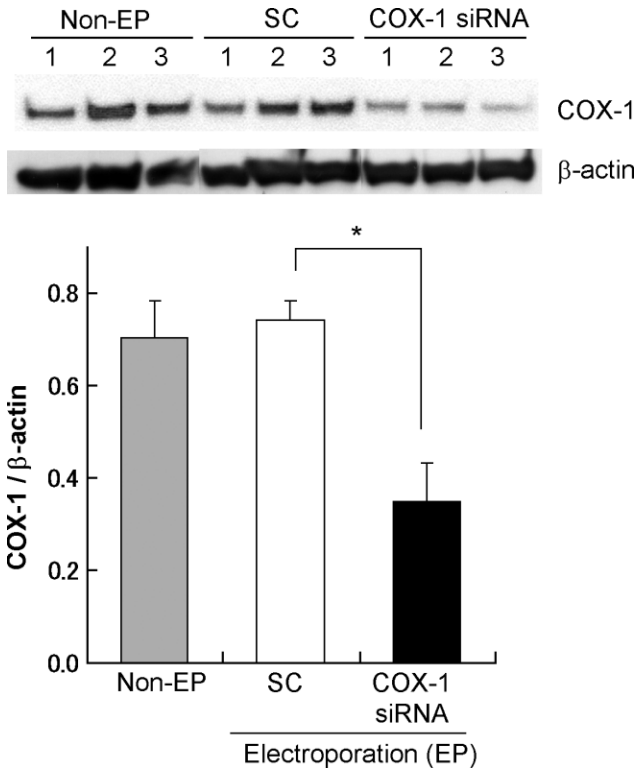

Figure 2. siRNA-mediated silencing of COX-1 expression in the skin. COX-1 expression was suppressed in the skin of NC/Nga mice. COX-1-specific siRNA was transfected under optimized conditions of electroporation. Total homogenates were extracted from the skin tissue on day 4 after the electroporation and subjected to Western blotting. The COX-1/β-actin density ratio was quantitatively evaluated and represented as the mean ± SE (n = 3). *$p < 0.05$, compared with the non-silencing scrambled-control (SC) RNA-transfected skin (Student's *t*-test)

Silencing of cyclooxygenase-1 gene in the skin

Because siRNA is relatively smaller in size than the pDNA and its site of localization is the cytoplasm (whereas pDNA needs to be delivered into the nuclei), theoretically, the transduction of siRNA may be easier than that of pDNA. Therefore, we applied the electroporation technique established by us as described above to *in vivo* gene silencing in an siRNA experiment. To test our hypothesis that COX-1-derived endogenous PGs in the skin might have a physiological role in the inhibition of itching [9,10], we attempted to introduce COX-1 siRNA into the skin. As shown in Figure 2, endogenous COX-1 expression was found to be significantly inhibited at the protein level 4 days after COX-1 siRNA transfection by electroporation ($51.4 \pm 13.4\%$ inhibition; $n = 3$; $p < 0.05$, as compared with the non-silencing scrambled-control (SC) RNA treated group). The electroporation of SC RNA had no effect on endogenous COX-1 expression after 4 days, compared with normal skin (non-EP). COX-2 was hardly detectable in the normal and the skin transfected with the siRNA using the optimal electrical pulses for electroporation (not shown). The skin prostaglandin (PGD2, PGE2, PGI2, and PGF2a) levels were also

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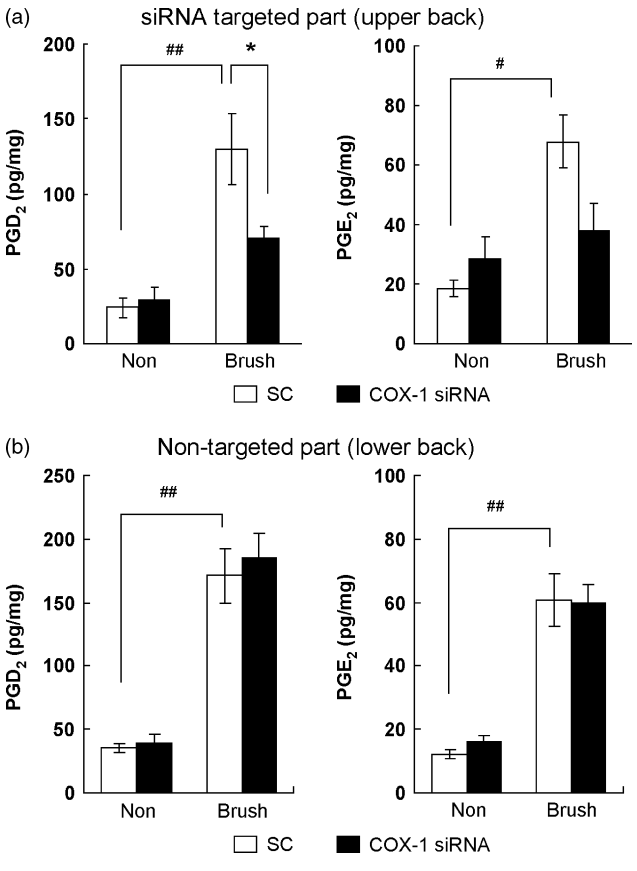

Figure 3. siRNA-mediated silencing of prostaglandin production in the skin. The levels of prostaglandin (PG) D2 and E2 in the skin tissue homogenate were measured using an enzyme-linked immunosorbent assay (ELISA). (a) The PGD2 and PGE2 levels were elevated after mechanical scratching, and the enhanced PG levels were reduced by COX-1 siRNA transfection at the targeted part of electroporation. (b) No reductions were detected in non-targeted skin. The data are represented as the mean ± SE (n = 6). ##$p < 0.01$; #$p < 0.05$, compared with the non-brush control (Student's *t*-test). *$p < 0.05$, compared with brush control (Student's *t*-test). SC = non-silencing scrambled-control RNA transfected

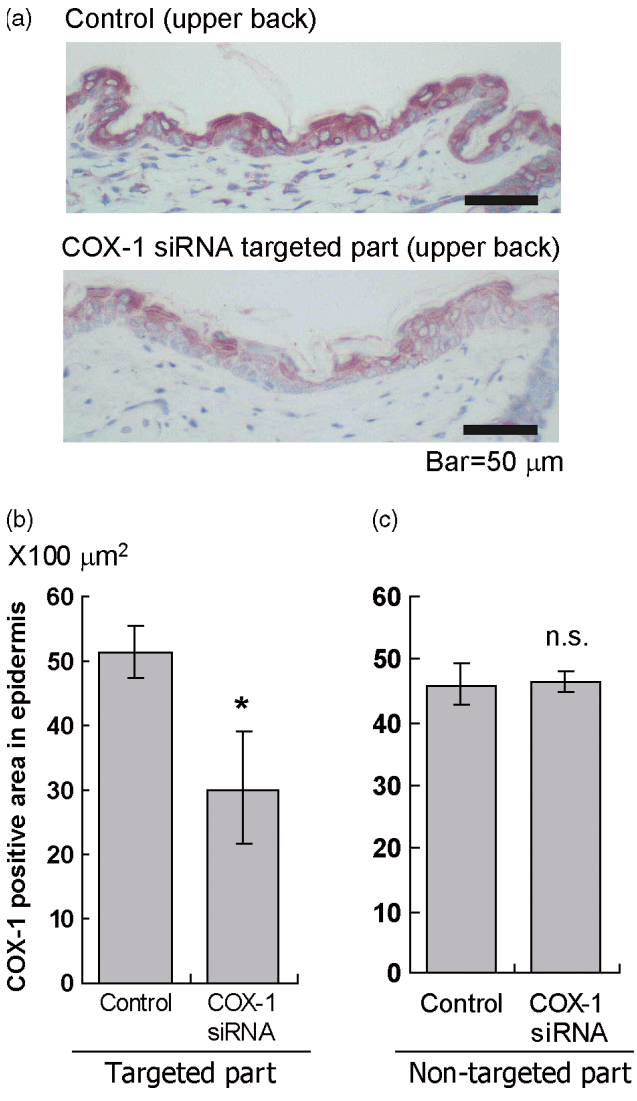

Figure 4. siRNA-mediated silencing of COX-1 expression in the skin tissue. The localization of COX-1 expression was immunohistochemically analyzed. (a) Endogenous COX-1 expression in the epidermal area was obviously silenced in the COX-1 siRNA-targeted part of the skin after 4 days of electroporation. (b, c) COX-1-positive areas in the epidermis were quantitatively evaluated and represented as the mean ± SE (n = 4). *$p < 0.05$, compared with the scrambled-control RNA-transfected skin (Student's *t*-test). No knockdown effect was detected in the skin that was not treated with siRNA

analyzed. To stimulate endogenous phospholipase A2 activity in the skin, the siRNA-targeted upper back skin of the mice was mechanically scratched 10 times using a wire brush. As shown in Figure 3a, the mechanical scratching induced significant elevations in cutaneous PGD2 and PGE2 levels. On the other hand, no significant changes in the PGI2 and PGF2a levels in the skin were observed following mechanical scratching, with the respective levels being less than 10 pg/mL (data not shown). PGD2 was the major PG in the skin (24.2 ± 6.6 pg/mL), and its synthesis was increased robustly by mechanical scratching (129.5 ± 23.7 pg/mL; $p < 0.01$, compared with the non-brush control; n = 6). Furthermore, the PGD2 level was significantly reduced by the COX-1 siRNA transfection (70.4 ± 7.8 pg/mL, $p < 0.05$, compared with the brush control; n = 6). A similar pattern was detected for the PGE2 level, while a significant reduction was barely detected (Figure 3a). For comparison, the lower back skin of the mice was also scratched mechanically in an area that was not treated with siRNA, and a coetaneous PG level analysis was performed. As shown in Figure 3b, PG production in the non-targeted skin was not reduced by COX-1 siRNA transfection, suggesting that the knockdown efficacy of the intradermally administered siRNA was limited to the targeted region.

The efficacy of COX-1 knockdown was further examined by immunohistochemical analysis. Statistically significant silencing of endogenous COX-1 expression was observed in the epidermal area after 4 days of electroporation in the COX-1 siRNA-targeted skin (Figures 4a and 4b). However, knockdown was not detected in the skin areas not targeted by siRNA (Figure 4c). These findings are consistent with the observation of a marked increase in electroporated transgene expression in the targeted skin

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(epidermis), but not in the non-targeted skin (Figures 1b and 1c). Technically, to identify the exact localization of the siRNA-targeted area, β-gal plasmid was co-transfected with the siRNA and a Western blot analysis as well as a histological analysis were performed. The co-transfected area of the skin was excised and stained with highly sensitive β-gal assay substrate (CPRG), and the β-gal-positive area was precisely excised and subjected to the following assays to demonstrate the successful knock-down of COX-1 expression.

Enhancement of scratching behavior in the NC/Nga mice following the silencing of the COX-1 gene in the skin

Subsequently, the effect of silencing COX-1 expression in the skin on the spontaneous scratching behavior of the NC/Nga mice was examined. To induce spontaneous scratching, SPF-NC/Nga mice were co-housed with skin-lesioned conventional-NC/Nga mice for 1 day. We previously showed that mite antigens are major triggers of scratching behavior in NC/Nga mice [12,13]. The co-habited (antigen-exposed) SPF mice were then electro-transfected with COX-1 siRNA or SC RNA and their scratching behavior was tracked for 6 days using an auto-analysis system. As shown in Figure 5a, the antigen-induced scratching behavior of the NC/Nga mice was strikingly aggravated by COX-1 siRNA transfection, mimicking the effect of treatment with SC-560, a COX-1-selective inhibitor (Figure 5b). Marked aggravation of the scratching behavior was detected especially during the hours of darkness, similar to the pattern observed in the SC-560-treated mice. The aggravation of the scratching behavior in the COX-1 siRNA-transfected mice began to be observed 3 days after the transfection, peaking at 5 days and persisting for at least 6 days. Therefore, we chose day 4 as a suitable time point for analyzing the knockdown efficiency (Figures 2, 3, 4). COX-1 siRNA transfection by itself, without co-habitation with conventional-NC/Nga mice, did not induce any spontaneous scratching behavior in the SPF mice (data not shown). Therefore, endogenous PGs might have a role in inhibiting yet-to-be identified scratch inducers. For comparison, as shown in Figure 5c,

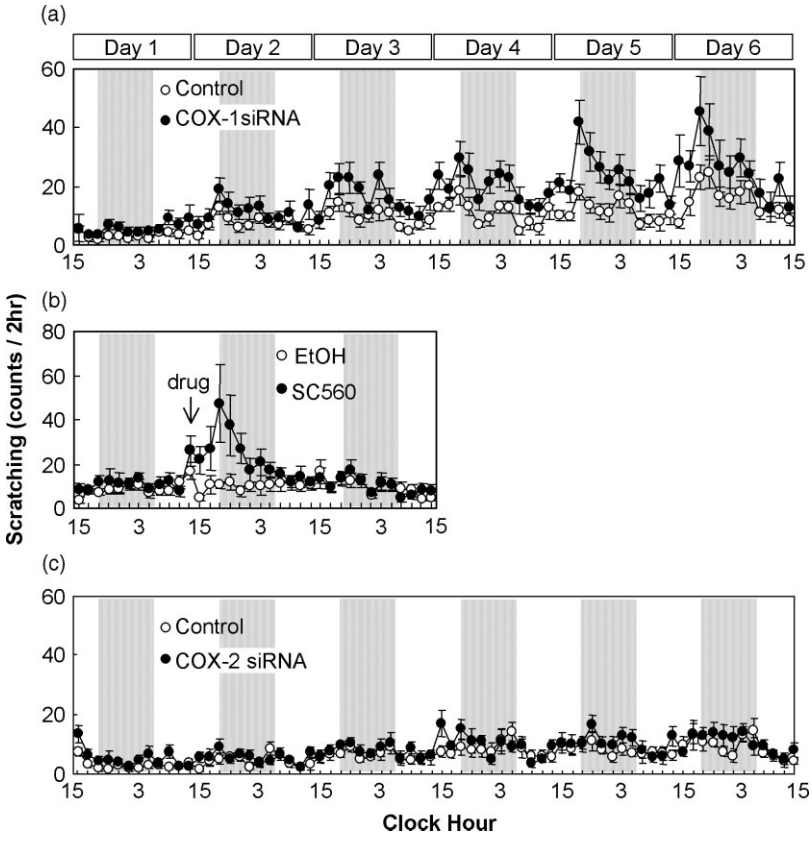

Figure 5. Phenotypic effect of siRNA-mediated silencing of the COX-1 gene in the skin. (a) Augmentation of scratching by silencing of the endogenous COX-1 gene in the skin. NC/Nga mice were injected with 12.5 μg/50 μL/site (2 sites/mouse) of COX-1 siRNA or non-silencing scrambled-control (SC) RNA, followed by electroporation. The number of scratching bouts was counted automatically for 6 days after the siRNA transfection. Representative data of four reproducible experiments are shown. (b) Augmentation of scratching behavior by inhibition of COX-1 activity in the skin. NC/Nga mice were treated with vehicle (ethanol) or the COX-1-specific inhibitor, SC-560. The arrow indicates the time of the drug treatment. The number of scratching bouts was counted for a total of 72 h. (c) Efficacy of COX-2 silencing on spontaneous scratching behavior. NC/Nga mice were injected with COX-2 siRNA or SC RNA, followed by electroporation. The number of scratching bouts was counted automatically for 6 days after the siRNA transfection. The lateral axis indicates the clock hour, and the shaded area represents the night time (7 : 00 pm to 7 : 00 am). All data are shown as the mean ± SE (n = 8)

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COX-2 siRNA electro-transfection had no effect on the scratching behavior, consistent with observations in mice treated with the COX-2-selective inhibitor (NS-398) [10], suggesting that COX-1-derived endogenous PGs in the skin may have a physiological role in the inhibition of itching.

Discussion

RNAi represents a promising gene-silencing technology and a potential therapeutic strategy for a variety of genetic diseases. The widespread use of RNAi *in vivo* depends on the establishment of an effective cellular uptake and tissue delivery system. Recently, a number of successful approaches have been reported for the targeted delivery of siRNAs *in vivo*. Several tools, including liposomal (stable nucleic acid lipid particles, SNALP) formulations [14], nanoparticles [15], polyethylene (PEI)-based cationic peptides [16], cholesterol [17], protamine/antibody fusion proteins [18], atelocollagen [19], and aptamer-siRNA chimeras [20], have been developed and refined for systemic as well as cell-type-specific delivery. *In vivo* electroporation also appears to be a feasible experimental technique to express siRNA in tissues [21]. Recently, unmodified siRNA was functionally introduced into the mouse joint cavity [22] and brain [23] using *in vivo* electroporation. Also, siRNA-harboring plasmid DNA has been electro-introduced into the ear auricle of mice [24]. The local injection of siRNAs could also inhibit co-injected reporter gene expression in the footpad skin of mice that did not receive electroporation [25]. In the present report, we show for the first time the successful silencing of endogenous gene expression in the body skin using an intradermal transfection of unmodified siRNA by electroporation and applied this procedure as a tool for performing a functional analysis.

Electroporation has come to be widely used for the introduction of small molecules and macromolecules, including DNA, into various cells and tissues and is becoming recognized as one of the most efficient and simple non-viral methods of gene transfer. The majority of *in vivo* siRNA silencing experiments using electroporation reported so far have been related to cancer research, because of the availability of a direct target and the ease of evaluation [26]. Muscles [27], kidneys [28], joints [22], brain [23], ears [24], and possibly skin are now considered as possible direct targets. Optimizing the electroporation protocol and validating the gene knockdown efficiency for each target tissue is of utmost importance. In the present study, satisfactory gene knockdown was detected in the COX-1 siRNA-transfected skin, even though only a small portion of the epidermis was stained by X-gal after reporter gene transfection using our optimized mild electroporation conditions. Importantly, we were extremely careful to minimize electric-shock-induced tissue damage. In addition to tissue damage, some recent works have also shown that some siRNAs also activate a dsRNA-dependent protein kinase, resulting in the induction of interferon-stimulated genes in mammalian cells *in vitro*, although whether such induction also occurs *in vivo* remains uncertain [29]. The appearance of a non-specific toxic phenotype by off-targeting siRNA has also been reported [30]. We examined scrambled siRNA and COX-2 siRNA as negative controls and also COX-1 siRNA with a different sequence (data not shown) and confirmed the reproducibility. Our results revealed that the effect of COX-1 siRNA is sequence-specific.

Although the knockdown efficacy of intradermally administered siRNA was limited to the area of electroporation, the question of whether the scratching behavior of the mice was localized to the area of COX-1 knockdown or increased overall, even in areas not targeted by siRNA, remained unanswered. NC/Nga mice tend to scratch the upper areas of their body, particularly their backs or faces, wherever their toenails can reach; therefore, the siRNA was applied to the upper backs of the mice. We were unable to evaluate the scratching counts in the siRNA-targeted area and the non-targeted area separately using our automated system. However, we believe that the COX-1 siRNA-mediated itchiness was localized to the injected site because the skin that was not treated with siRNA expressed and produced control levels of COX-1 and PGs, as shown in Figures 3 and 4, and these factors might play a role in inhibiting itching. Meanwhile, to emphasize the involvement of COX-1 expression on the pharmacologic profile, 'day 4' was chosen as the best time point for a knockdown analysis because the enhancement of scratching behavior began gradually from day 3 after the siRNA transfection but reached a peak at night just after 'day 4'.

Itching is a cardinal symptom of atopic dermatitis (AD), but the underlying cellular mechanism and association with the cutaneous nervous system are still not clearly understood. We have intensively analyzed the relationship between chronic itching and prostaglandins [9] and recently showed that the spontaneous scratching behavior of conventional NC/Nga mice was significantly aggravated by the topical application of SC-560 but remained unaffected following that of NS-398 (a COX-2-selective inhibitor), suggesting that COX-1-derived skin PGs might be endogenous inhibitors of itching in NC/Nga mice [10]. Among the COX-1-derived PGs, we speculated that PGD2 might play a central role in this inhibition via its DP1 receptor [31]; however, experiments using small-molecule inhibitors have generally limitations because of the inhibitors' specificities [9,31]. Thus, the knockdown technique presented in this paper fulfilled its purpose in validating the available evidence regarding which target molecules or receptors might be responsible for the inhibition of itching. Further studies are currently underway.

In conclusion, the present study demonstrated the successful silencing of endogenous gene expression in targeted areas of body skin in mice using an intradermal injection of unmodified siRNA administered via

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electroporation. This method was used to demonstrate that COX-1-derived prostaglandins might act as endogenous inhibitors of itching in NC/Nga mice, with possible applications in patients with AD.

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