

Allergic contact dermatitis induces upregulation of identical microRNAs in humans and mice

Marie T. Vennegaard¹, Charlotte M. Bonefeld¹, Peter H. Hagedorn³, Nannie Bangsgaard², Marianne B. Løvendørff^{2,3}, Niels Ødum¹, Anders Woetmann¹, Carsten Geisler¹ and Lone Skov²

¹Department of International Health, Immunology and Microbiology, University of Copenhagen, 2200 Copenhagen, Denmark, ²Department of Dermato-Allergy, Gentofte University Hospital, 2900 Hellerup, Denmark, and ³LEO Pharma A/S, Ballerup, Denmark

doi:10.1111/j.1600-0536.2012.02083.x

Summary

Background. MicroRNAs are short, endogenous RNA molecules that can bind to parts of target mRNAs, thus inhibiting their translation and causing accelerated turnover or degradation of transcripts, thereby regulating gene expression. Several microRNAs have been found to be upregulated in atopic dermatitis and psoriasis, indicating a role in inflammatory skin diseases. However, there have been no studies on the expression of microRNAs in allergic contact dermatitis.

Objectives. To investigate expression of microRNAs in allergic contact dermatitis.

Methods. Lesional and non-lesional skin biopsies were collected from subjects with allergic responses to diphenylcyclopropanone (DPCP). Additional samples for profiling were collected from an experimental mouse model by use of the strong allergen dinitrofluorobenzene. RNA was purified from all samples, and locked nucleic acid microarray analysis was performed, followed by validation with quantitative polymerase chain reaction (PCR).

Results. In humans sensitized with DPCP, we found significant upregulation of miR-21, miR-142-3p, miR-142-5p and miR-223 in challenged skin. The same microRNAs were significantly upregulated in the skin of mice in a mouse model of contact allergy. The upregulation of microRNA was confirmed by quantitative PCR.

Conclusion. These are the first results indicating that microRNAs may be involved in the pathogenesis of allergic contact dermatitis, and they show that mouse models are valuable tools for further study of the involvement of microRNAs in allergic contact dermatitis.

Key words: allergic contact dermatitis; inflammation; microRNA; skin.

MicroRNAs are small non-coding RNAs that regulate gene expression by translational repression, or in some cases by degradation of their target genes (1). Primary transcripts of microRNAs (pri-microRNAs) are generated by RNA polymerase II, after which they are sequentially processed by RNase III class enzymes, Drosha and

Dicer, to first produce ~70-nucleotide intermediate hairpin structures (pre-microRNAs), and finally the ~22-nucleotide mature microRNAs. The single-stranded, mature microRNAs bind to their target sequences, which most often are located in the 3'-untranslated region of the mRNA, usually showing only partial base pair complementarity with the microRNA sequence. According to the current view, imperfect complementarity leads to repression of translation of the target mRNA, and is the main mechanism of microRNA regulation in animals. It is estimated that microRNAs comprise 1–5% of genes in the human genome, making them the most abundant class of regulators of protein expression (2, 3). Their discovery has revealed yet another layer in the complexity of cellular signalling, and they play a fundamental role in

Correspondence: Lone Skov, Department of Dermato-Allergy, Gentofte University Hospital, Niels Andersens Vej 65, 2900 Hellerup, Germany. Tel: +45 39 77 32 04; Fax: +45-3977-7615. E-mail: LOSK@geh.regionh.dk

Conflicts of interest: The authors state no conflict of interest. This work was supported in part by research funding from the Danish Foundation for Advanced Technology (Højteknologifonden), Lægevidenskabens Fremme, LEO Pharma A/S, and Exiqon A/S.

Accepted for publication 18 February 2012

biological processes such as proliferation, differentiation, and apoptosis (4). MicroRNAs have been implicated in several inflammatory diseases, as well as cancer (1). In relation to skin, studies have elucidated and characterized the roles of different miRNAs in differentiation, morphogenesis, wound healing, cancer, atopic dermatitis, and psoriasis (5–10). Regarding keratinocytes, miR-203 and miR-205 appear to be crucial for their development and differentiation (5–7, 9). In psoriasis and atopic dermatitis, it has been shown that there is altered expression of several microRNAs, suggesting that microRNAs have a role in the development of these diseases (5, 10–12). Furthermore, we have recently shown that the microRNA profile can be used to distinguish cutaneous T cell lymphomas from benign skin diseases (13). Thus, microRNAs appear to have an important role in both healthy and diseased skin.

Although allergic contact dermatitis has been studied extensively, and many mechanisms in allergic contact dermatitis have been discovered, no studies have been conducted to investigate microRNA expression in allergic contact dermatitis. In this study, we examined the expression of microRNAs in skin from humans and mice with allergic contact dermatitis.

Materials and Methods

Sensitization of human subjects

Six subjects (mean age 44 years, range 35–58 years) were sensitized with diphenylcyclopropanone (DPCP) in acetone on the skin of the buttocks. Petrolatum-backed 11 mm filter discs were soaked in 50 µl of 0.0625% DPCP in acetone (25 µg/50 µl). Each filter disc was mounted inside a 12 mm aluminium Finn Chamber®, and taped to the skin (Scanpor®; Epitest Oy, Tuusula, Finland). The disks were left on for 48 hr. Challenge was carried out on the upper inner arm 3 weeks after sensitization, with DPCP in acetone (3.125 µg/15 µl) and one acetone control on 7-mm filter discs in 8-mm Finn Chambers®. The discs were left on for 6 hr. The challenge sites were all marked with a surgical skin marker and evaluated 48 hr later. Both sensitization and challenge were performed on healthy skin. The study was approved by the local ethics committee (H-C-2007-0123), and all subjects included gave their written informed consent before enrolment.

Visual assessment

The elicitation responses were assessed with a visual score, as suggested by Cooper et al. (14): 1 = no reaction; 2 = mild, macular erythema; 3 = moderate erythema, occasionally with papules; 4 = strong erythematous

reaction (including vesicular changes); and 5 = extreme or spreading reaction (including bullous or ulcerative reaction).

Biopsies

Two 4 mm punch biopsies were taken from each subject, one from the area where DPCP (3.125 µg/15 µl) had been applied, and one from the area where vehicle (acetone) had been applied. The biopsies were taken 48 hr after challenge. Before the biopsies were taken, the skin was frozen with a liquid nitrogen spray to inhibit RNA degradation. The skin biopsies were immediately placed in liquid nitrogen and transferred to a – 80°C freezer until RNA extraction.

Mice

Female C57BL/6 mice (Taconic, Ry, Denmark) were housed in specific pathogen-free facilities at the Department of Experimental Medicine, Panum Institute, in accordance with national animal protection guidelines. The facility veterinarians checked the welfare of the animals, and experimental procedures were approved by the national animal ethics committee (permission no. 2007/561-1357). The mice were 8 weeks old at the time when the experiments were started.

Sensitization of mice

The mice were sensitized for three consecutive days with 25 µl of either 0.15% 2,4-dinitrofluorobenzene (DNFB) in olive oil/acetone(1:4) or vehicle alone, on the dorsal side of both ears. After 3 weeks of rest, the animals were challenged with the same concentration of DNFB once, and 48 hr after challenge they were killed. Ear thickness was measured with an engineer's micrometer (Mitutoyo, Tokyo, Japan). Ears from one side of the mice were snap-frozen and stored at – 80°C until RNA extraction. Draining auricular lymph nodes were taken out and used for flow cytometric analysis.

Flow cytometry

From the draining lymph nodes, cell suspensions were prepared manually, and the cells were counted. To determine the distribution of T and B cells, the cell suspensions were incubated with fluorochrome-conjugated anti-CD4 (RM4-5), anti-CD8a (53–6.7) or anti-CD19(ID3) monoclonal antibodies, all from BD Biosciences (San Jose, CA, USA). The cells were incubated for 30 min on ice with the indicated monoclonal antibody diluted in ice-cold phosphate-buffered saline containing 2% foetal bovine

Table 1. The 20 microRNAs with the most change in expression in diphenylcyclopropenone (DPCP)-sensitized subjects, and changes in their murine microRNA orthologues in the dinitrofluorobenzene (DNFB) mouse model

DPCP-sensitized patients			Mice sensitized and challenged with DNFB		
Human miRNA	<i>p</i> SAM	Fold change	Fold change	<i>p</i> SAM	Murine miRNA orthologue
hsa-miR-223	0.00093	2.07	1.88	0.00046	mmu-miR-223
hsa-miR-518b	0.00128	-1.26	-	-	-
hsa-miR-634	0.00263	-1.21	-	-	-
hsa-miR-1469	0.00307	-1.32	-	-	-
hsa-miR-602	0.00314	-1.25	-	-	-
hsa-miR-142-3p	0.00337	1.72	2.19	0.05041	mmu-miR-142-3p
hsa-miR-21	0.00349	1.49	2.31	0.00221	mmu-miR-21
hsa-miR-492	0.00369	-1.17	-	-	-
hsa-miR-886-3p	0.00386	1.29	-	-	-
hsa-miR-142-5p	0.00427	2.11	1.58	0.00706	mmu-miR-142-5p
hsa-miR-638	0.00455	-1.27	-	-	-
hsa-miR-149*	0.00565	-1.25	-1.36	0.10259	mmu-miR-149
hsa-miR-150	0.00754	1.29	-1.05	0.88469	mmu-miR-150
hsa-miR-1303	0.00776	1.12	-	-	-
hsa-miR-193a-3p	0.00855	-1.32	-1.47	0.06707	mmu-miR-193
hsa-miR-342-3p	0.00860	1.26	-1.14	0.43179	mmu-miR-342-3p
hsa-miR-33b	0.01017	-1.08	-1.39	0.06123	mmu-miR-33
hsa-miR-146b-5p	0.01017	1.41	1.04	0.84306	mmu-miR-146b
hsa-miR-181d	0.01169	-1.10	-1.38	0.09303	mmu-miR-181d
hsa-miR-648	0.01309	1.11	-	-	-

*miR annotation is common nomenclature for miRNA and is also used in MiRbase. Significance analysis of microarrays (SAM) was performed on microarray data from six biopsies from DPCP-challenged skin in a paired test with skin biopsies from vehicle-treated skin from the same patients. Of the 20 most changed microRNAs in the 6 subjects, 11 of them have murine orthologues. Changes in expression of these 11 murine microRNAs in ears of the DNFB model from mice sensitized and challenged with DNFB ($n = 4$) as compared with vehicle control ($n = 4$) are listed in the right section of the table.

serum and 0.1% NaN_3 . After being washed, the cells were analysed on a FACSCalibur (BD Biosciences) with CELLQUEST software.

RNA extraction

Total RNA was isolated from six 10 μm frozen tissue sections with the RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, Carlsbad, CA, USA), according to the manufacturer's guidelines. The quantity and quality of total RNA was checked by spectrophotometry (Nanodrop ND-1000).

Microarray

From each RNA sample, 100 ng of total RNA was labelled with Hy3 fluorescent dye with the miRCURY locked nucleic acid (LNA) array power labelling kit (Exiqon, Vedbeak, Denmark). Reference samples were made from 100 ng of total RNA from a human universal reference pool (AM600; Ambion, Austin, TX, USA), and similarly labelled with Hy5 dye. All Hy3/Hy5 sample pairs were labelled on the same day with the same master mix, in order to minimize technical variation, and hybridized to

miRCURY LNA arrays (version 11.0; Exiqon) containing capture probes targeting all human microRNAs registered in miRBASE (version 15.0) of the Sanger Institute. The hybridization was performed overnight at 56°C , according to the manufacturer's specifications, with a Tecan HS4800 hybridization station (Tecan, Grödig, Austria). After hybridization, the microarray slides were scanned with an Agilent G2565BA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA) at 5 μm resolution, and the resulting TIFF images were analysed with IMAGE 8.0 software with standard settings (BioDiscovery, El Segundo, CA USA).

Preprocessing of microarray data

Probe signals were corrected for background by fitting a convolution of normal and exponential distributions to the foreground intensities, with the background intensities as a covariate (15). For each array, Hy3 sample probe intensities were referenced to Hy5 reference probe intensities by locally weighted polynomial regression (16). Four technical replicate spots for each probe were combined to produce one signal by taking the logarithmic base-2 mean of reliable spot intensities.

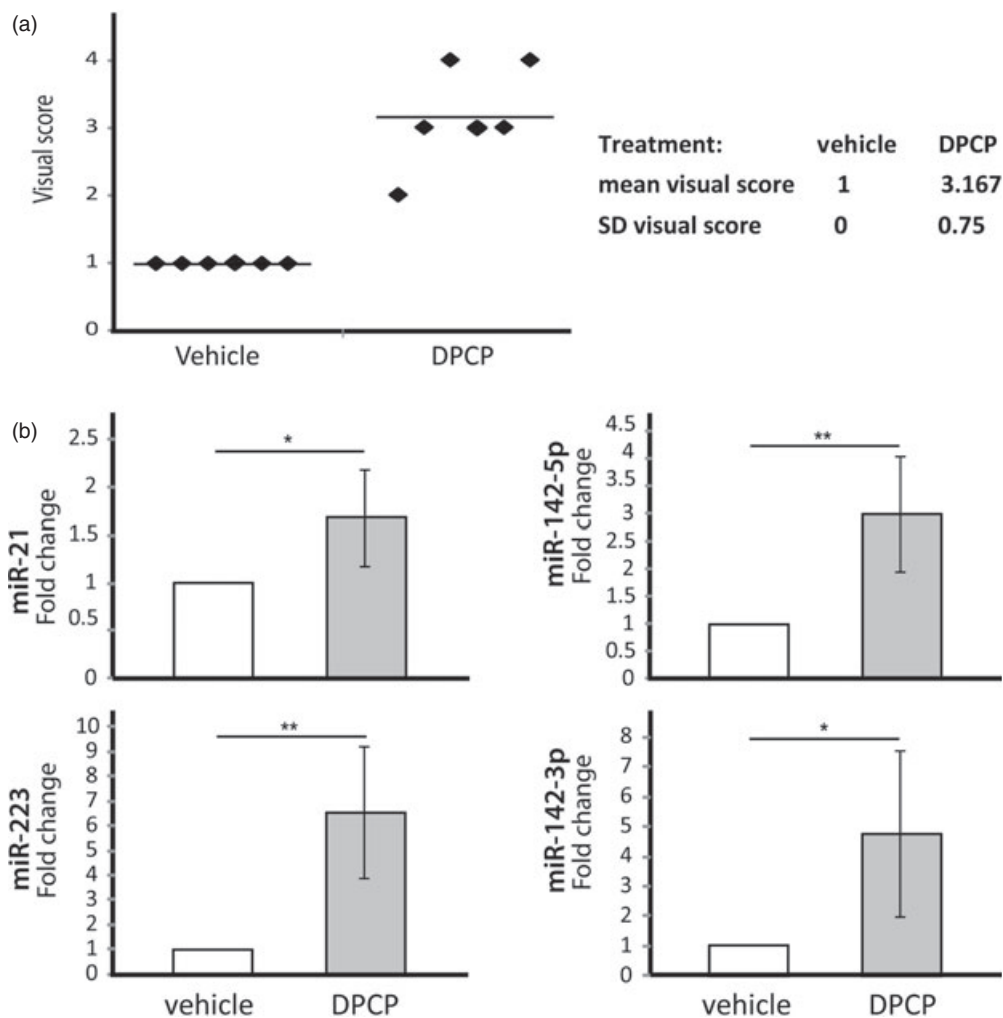


Fig. 1. Allergic response in diphenylcyclopropanone (DPCP)-sensitized subjects. (a) Visual score for the challenge reaction at the DPCP-challenged sites and vehicle-challenged sites of all subjects included. (b) miR-21, miR-223, miR-142-5p and miR-142-3p expression in skin from DPCP-challenged areas and vehicle control, measured by quantitative PCR. Expression was normalized to expression of let7d microRNA and fold changes were calculated in pairs (as compared with vehicle control) for each subject ($n = 6$). Mean fold change (for each subject) in three independent quantitative PCRs is shown. * $p < 0.05$, ** $p < 0.01$ (two-tailed t -test).

If all four replicates for a given probe were judged to be unreliable, that probe was removed from further analysis.

Analysis of microarray data

For each microRNA probe, a significance of difference in expression levels between groups was assessed by calculating a modified t -statistic called significance analysis of microarrays (SAM) (17) from the scaled mean log probe intensities measured in each group. Briefly, for each microRNA in each patient, we calculated an expression $\log_2$ fold change between involved and uninvolved skin. We then calculated an average $\log_2$ fold change for

each microRNA for all 6 patients. Using SAM for each microRNA, we evaluated whether the average $\log_2$ fold change was significantly different from 0, and sorted all of the microRNAs in order of most significant to least significant.

Real-time quantitative polymerase chain reaction (PCR)

Real-time PCR was performed with a standard protocol on an MX3000 instrument (Agilent Technologies, Santa Clara, CA). We used Taqman microarray assays (Applied Biosystems) according to the manufacturer's protocol, and analysed the results with MXPRO software. All Taqman microRNA assays were performed in triplicate. Expression

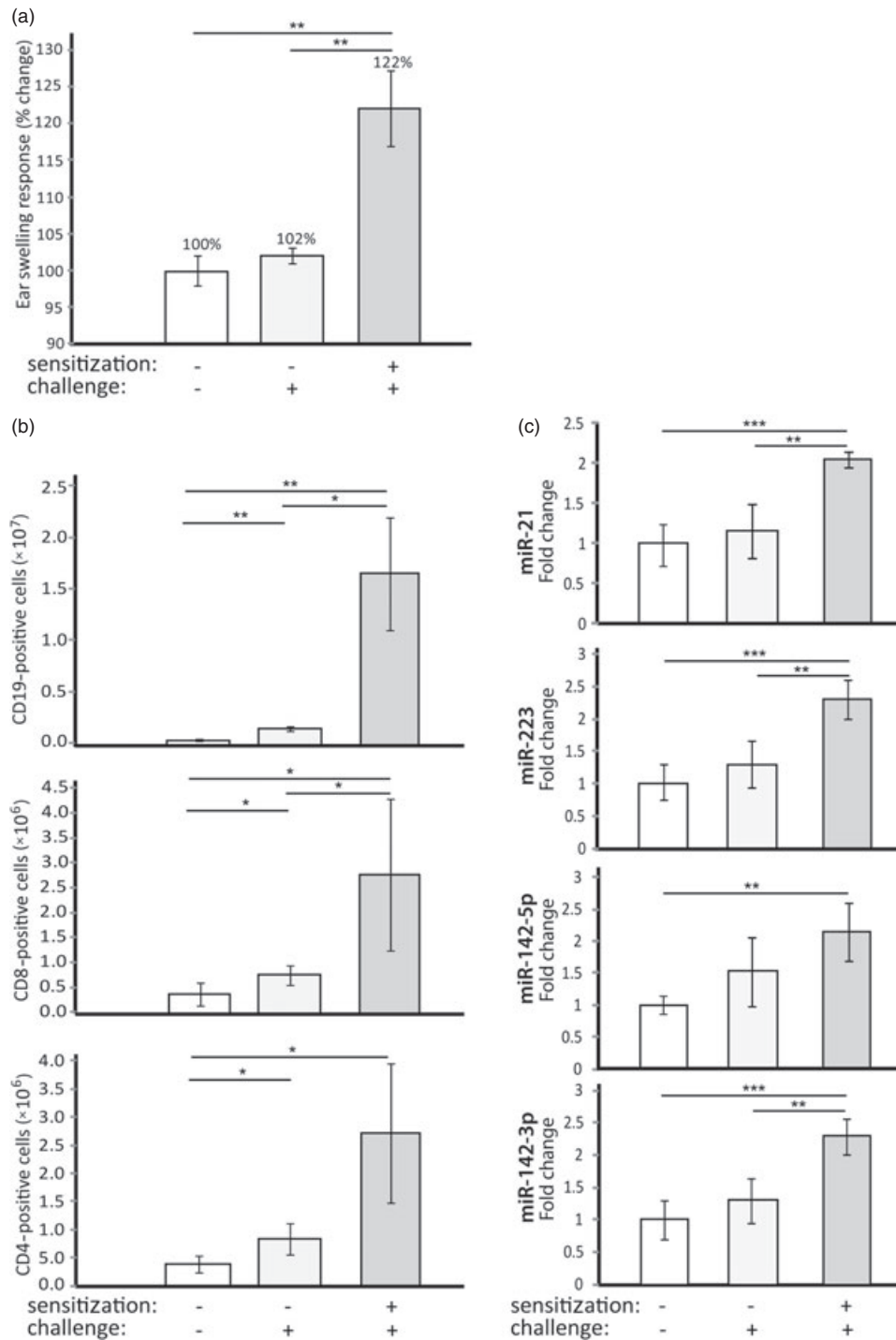


Fig. 2. Allergic response in dinitrofluorobenzene (DNFB)-sensitized mice. Mice were sensitized with either 0.15% DNFB or vehicle, and challenged after 21 days. (a) Change in ear thickness of DNFB-challenged mice. (b) Total numbers of CD4-positive, CD8-positive and CD19-positive cells from draining auricular lymph nodes of the mice, measured by flow cytometry. The results are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ (two-tailed t -test). (c) miR-21, miR-223, miR-142-5p and miR-142-3p expression in ears from mice sensitized and challenged with DNFB, mice only challenged with DNFB, and vehicle control mice, measured by quantitative PCR. Expression was normalized to expression of let7d microRNA. Mean expression of the vehicle control mice was set to 1 ($n = 4$). Mean fold change (for each mouse) in three independent quantitative PCRs is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (two-tailed t -test).

was normalized on the basis of the CT values of the Taqman probe against let7d microRNA.

Results

MicroRNA expression in human allergic contact dermatitis

To investigate the role of microRNA in human allergic contact dermatitis, 6 subjects were sensitized with DPCP. The challenge responses were assessed with a visual scoring system (14). None of the subjects showed any sign of inflammatory response at the site challenged with vehicle, whereas all of the subjects included showed visible signs of allergic contact dermatitis at the DPCP-challenged site, ranging from mild macular erythema to strong erythematous reaction (Fig. 1a). To study the changes in microRNA expression profiles of the skin following challenge with allergen, we used microRNA array profiling of skin samples from the 6 subjects challenged with DPCP, and compared the profiles with background microRNA profiles of vehicle-challenged skin from the same subjects. Significance analysis of microarrays showed that there were significant differences in the expression of a large number of microRNAs between challenge and vehicle sites. The 20 microRNAs whose expression changed most are listed in Table 1. We confirmed the results obtained from the microRNA arrays by performing real-time quantitative PCR assays on the four most upregulated microRNAs (miR-21, miR-223, miR-142-3p, and miR-142-5p) in allergen-challenged skin from sensitized subjects. The validation by quantitative PCR was performed with Taqman assays targeting only the mature forms of these particular microRNAs. Target gene expression was normalized by basing it on the expression of let7d microRNA. Let7d was chosen as a reference because it was more stably expressed in the microarrays than the most commonly used endogenous control, U6 (data not shown). A significant degree of upregulation of miR-21, miR-223, miR-142-3p and miR-142-5p was found in RNA from allergen-challenged skin as compared with RNA from vehicle-challenged skin (Fig. 1b).

MicroRNA expression in murine allergic contact dermatitis

Next, we investigated the changes in microRNA in mouse skin, using a mouse model of allergic contact dermatitis. Mice were either exposed to DNFB for three consecutive days on both ears followed by challenge 21 days later (sensitized and challenged mice) or were only exposed to DNFB once (challenged only) (Fig. 2a). To determine the local inflammatory responses, changes in ear

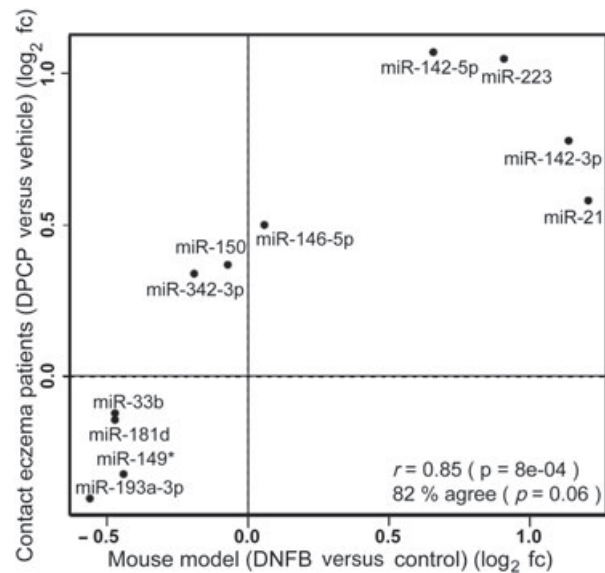


Fig. 3. Comparison of change in expression of allergic contact dermatitis-induced microRNAs in human and murine skin. In order to compare changes in expression of microRNAs in human and mice induced by repeated exposure to allergen, log₂ of the microarray-derived fold changes (fc) for 11 of the 20 most changed microRNAs and their murine orthologues are plotted against each other. DPCP, diphenylcyclopropanone.

thickness were measured. In the group of sensitized and challenged mice, we found an increase in ear thickness of approximately 22% as compared with vehicle controls, whereas the group of mice that were challenged but not sensitized showed no significant difference in ear thickness relative to vehicle-treated mice (Fig. 2a). In order to study the effect of exposure to DNFB further, we investigated the cellular response within the draining lymph nodes. Small increases in CD4 T cells, CD8 T cells and B cells were found in mice that were challenged but not sensitized, as compared with control mice (Fig. 2b). In contrast, massive infiltration/proliferation of CD4 T cells, CD8 T cells and B cells was found in mice that had been both sensitized and challenged, as compared with control mice (Fig. 2b). Collectively, these results indicate that a memory response towards DNFB was induced in our model.

Next, we investigated the microRNA expression profile in the skin of mice that had been both sensitized and challenged with DNFB. Arrays were run on RNA purified from ear biopsies from all 12 mice included in the study. We compared the microRNA expression induced by allergic contact dermatitis in humans with the microRNA expression induced by allergen in mice, focusing on the 20 most altered microRNAs in humans. Eleven of the 20 most changed microRNAs identified in human skin following allergen challenge had a mouse orthologue.

Nine of the 11 murine orthologue microRNAs were changed in the same direction as in human skin after DPCP sensitization and challenge. When we plotted log₂ of the fold changes in humans and mice against each other, we obtained 85% correlation (Fig. 3). To compare the reactions in humans and mice further, we performed quantitative PCR validation of the mouse RNA on the four most upregulated microRNAs found in human skin. Significant upregulation of the expression of miR-21, miR-223, miR-142-3p and miR-142-5p was found in skin from the sensitized and challenged group relative to the vehicle-treated group (Fig. 2c). In contrast, only a small (and not statistically significant) increase in expression of all four of these microRNAs was found in mice that were challenged but not sensitized, as compared with control mice (Fig. 2c).

Together, these results suggest that induction of allergic contact dermatitis does indeed change microRNA expression in a similar way in human and murine skin.

Discussion

In this study, we analysed the patterns of expression of microRNA in skin from subjects who were sensitized with DPCP. We found that the four most upregulated microRNAs were miR-21, miR-223, miR-142-3p, and miR-142-5p. The upregulation of these four microRNAs was confirmed by quantitative PCR. Furthermore, we found that production of these four microRNAs was also upregulated in mouse skin during the challenge reaction to DNFB.

To our knowledge, this is the first publication to address microRNA expression in contact allergy. Allergic contact dermatitis is usually described as a classic T cell-mediated disease, mediated primarily by cytotoxic CD8 T cells and Th1 cells (18, 19). The four microRNAs that were found to be upregulated most after allergen sensitization and challenge are all microRNAs that have been shown to be related to T cells and T cell activation, which is in agreement with the fact that allergic contact dermatitis is mainly a T cell-mediated skin disease (20). The significant upregulation of these four microRNAs may not be

specific for allergic contact dermatitis, and further studies on other allergens and irritants, such as sodium lauryl sulfate, are needed. MicroRNA profiles have been studied in other inflammatory skin disorders, both malignant and non-malignant (10, 12, 13). Upregulation of miR-223, miR-142-3p and miR-142-5p has been reported in atopic dermatitis (10) and in psoriasis (11). In addition, miR-21 has been found to be upregulated in psoriasis lesions (11). Thus, the four microRNAs that we found to be upregulated most in allergic contact dermatitis are also involved in other skin inflammatory diseases. As T cells play an important role in the pathogenesis of all three diseases, this might not be surprising. However, the role of the altered microRNA profile both in allergic contact dermatitis and in psoriasis and atopic dermatitis is largely unknown. We found that the upregulation of certain microRNAs was similar in humans and mice. In this study, we show that miR-223, miR-142-3p, miR-142-5p and miR-21 were upregulated upon challenge in the sensitized mice but not significantly upregulated upon challenge in the non-sensitized mice, indicating a role in the adaptive response. The mouse model of allergic contact dermatitis is easy to use, and may be a valuable tool for future studies on the role of the individual microRNAs found in both allergic contact dermatitis and other skin inflammation diseases.

In the present study, we have focused on changes in microRNA induced by the two strong allergens DNFB and DPCP. It would be of interest to investigate whether the microRNA profile found is also relevant to other allergens, such as metals. Identification of a specific microRNA profile in the skin for allergic contact dermatitis would be a major step forwards, as allergic contact dermatitis and irritant contact dermatitis are difficult to differentiate, owing to their clinical and histological resemblance.

In this article, we have thus provided the first evidence that microRNAs may be involved in the pathogenesis of allergic contact dermatitis, and we have demonstrated that mouse models are valuable tools for study of the involvement of microRNAs in allergic contact dermatitis.

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