

Identification of novel miRNA-mRNA regulatory networks in contact dermatitis by integrated microarray analysis

To the Editor,

Contact dermatitis (CD) is a major occupational disorder, characterized by pruritus and inflammation following direct topical exposure to an allergen or irritant.¹ The role of micro RNAs (miRNAs) in skin diseases, including CD, has been investigated due to their compelling potential applicability.¹ miRNAs are short single-stranded RNA

molecules, which take part in the post-transcriptional regulation.¹ Most previously conducted studies have focused on changes in miRNA expression profiles¹⁻³; however, their regulatory effects as well as changes in the miRNAome in response to contact sensitizers and irritants with different physiochemical properties needs elucidation.

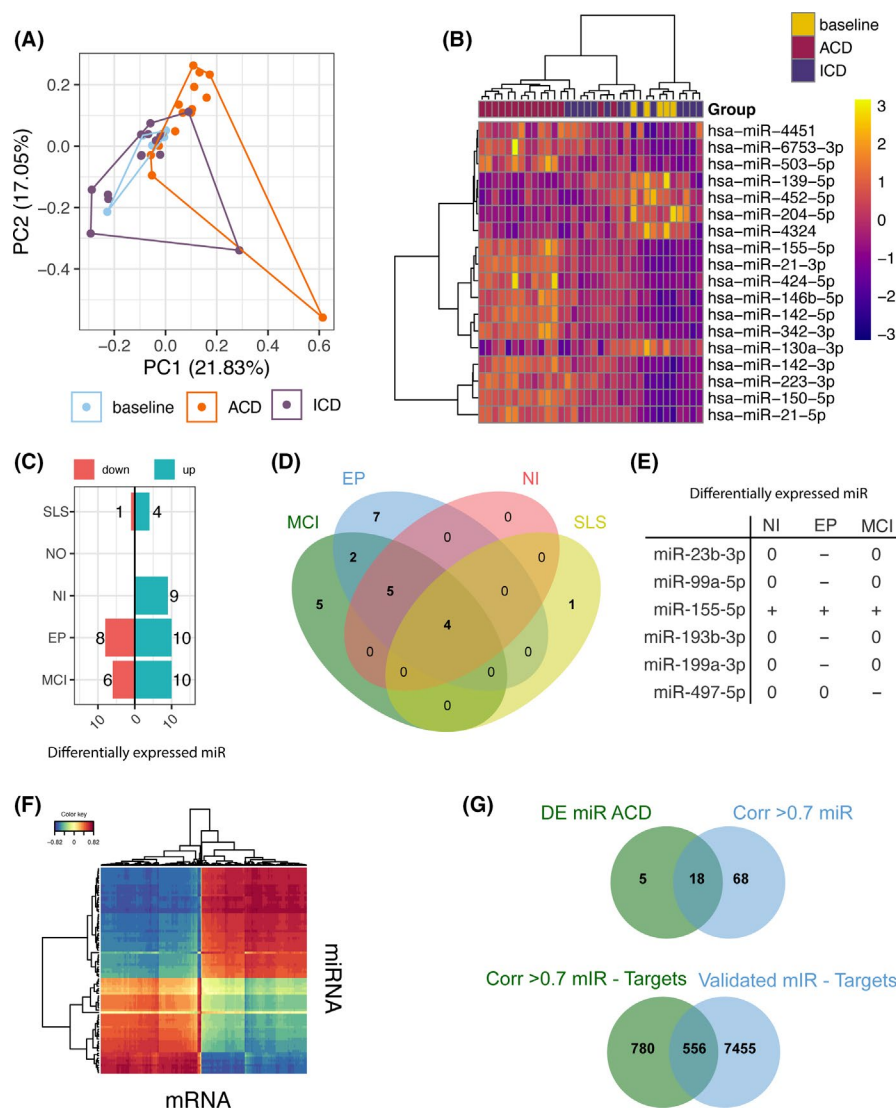


FIGURE 1 Allergic contact dermatitis and ICD induce different miRNA patterns. Principal component analysis (A) and hierarchical clustering (B) of miRNAome data. (C) Up- and down-regulated differentially expressed (DE) miRNAs. (D) Venn diagram of shared and unique miRNAs among the groups. (E) Highly relevant ACD-related miRNAs; up- (+), or down-regulated (-), non-DE (0). (F) Heat map displaying highly (>0.7) correlated miRNA-mRNA pairs. (G) Venn comparison demonstrating overlap between highly correlated and DE miRNAs (top), and highly correlated and previously validated miRNA-targets (bottom), respectively

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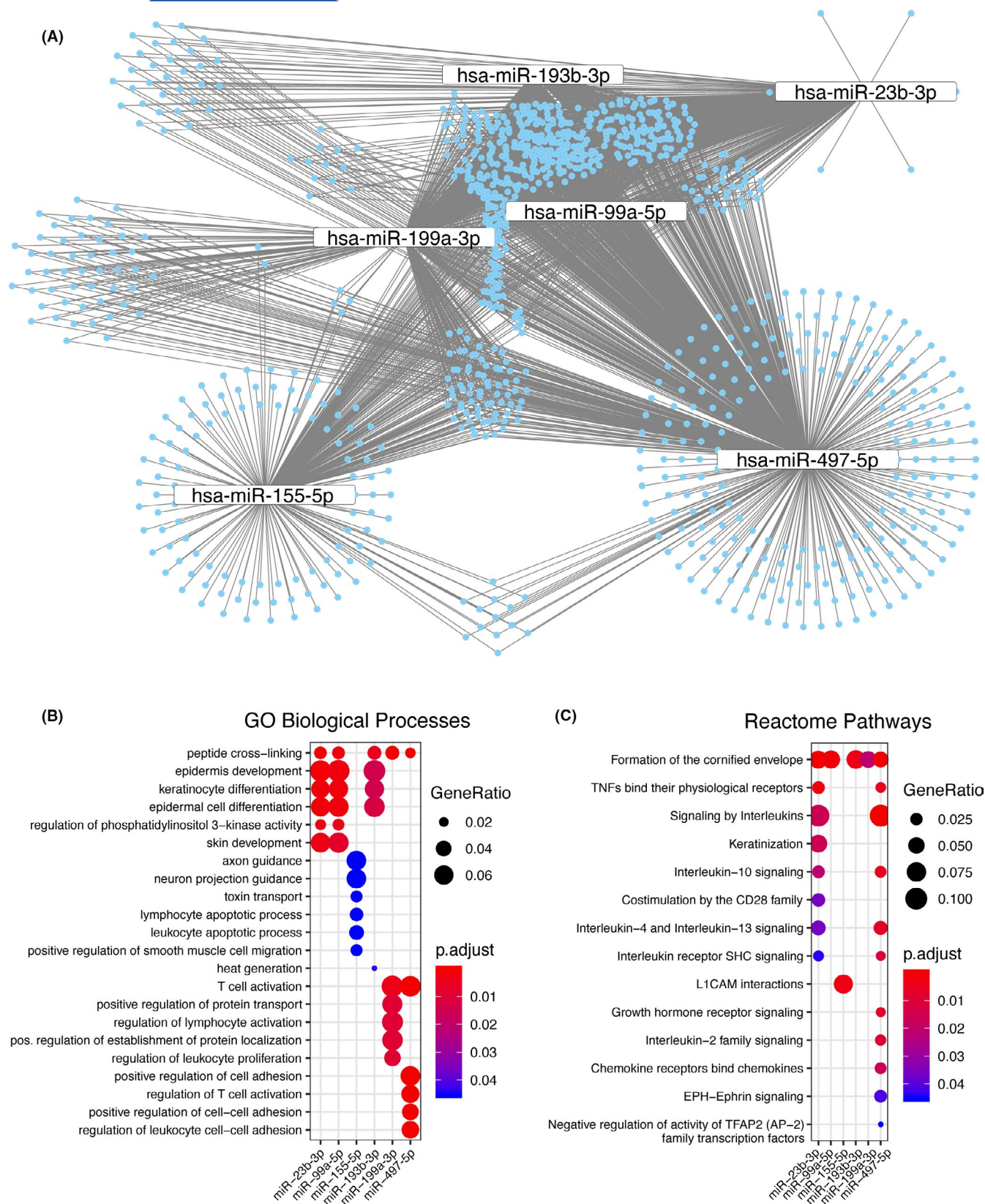


FIGURE 2 miRNA networks regulating mRNA during ACD. (A) Network of differentially expressed and top correlated miRNA-mRNA pairs in ACD. The network was visualized using Cytoscape. Gray lines illustrate correlation between the miRNA (white) and mRNA (blue dots). Enrichment analysis for GO biological processes (B) and reactome database (C) of highly correlated miRNA-mRNA target genes of ACD samples

Herein, we conducted an integrative analysis of miRNA/mRNA expression profiles in skin using same sample miRNA and mRNA expression data from positive patch test reactions from patients exposed to allergens (nickel sulfate (NI), epoxy resin (EP) and methylochloisothiazolinone (MCI); each $n = 5$), irritants (sodium lauryl sulfate [SLS, $n = 9$] and nonanoic acid [NO, $n = 5$]) and from non-affected skin (baseline, $n = 5$). Allergens, compared to irritants, induced distinct miRNA signatures in human skin (Figure 1A,B), which is mirrored in the differentially expressed miRNAs in the different exposures (Figure 1C). Overall, allergen encounter induced similar "core" miRNAs. Contrary, for the irritants, only SLS induced differentially expressed miRNAs, majorly overlapping with allergen-induced differentially expressed miRNAs (Figure 1D). All differentially expressed miRNAs are listed in Figure S1 and highly relevant miRNAs are highlighted in Figure 1E.

Since allergens and irritants exert different molecular effects on human skin, we analyzed both entities separately. To integrate the miRNA and mRNA datasets, we employed canonical correlation analysis as a multivariate exploratory approach. Correlation network analysis of the allergic contact dermatitis (ACD) reactions identified 86 miRNAs as highly positively or negatively correlated with 1336 mRNAs (Figure 1F). In addition, 18 of the 86 highly correlated miRNAs were found to be differentially expressed, indicating that highly differentially expressed miRNAs contribute to transcriptional changes during ACD. Moreover, identified mRNA targets were validated using the miRecords, miRTarBase and TarBase database, showing an overlap of 556 genes supporting the relevance of these targets (Figure 1G). Based on the 1336 mRNAs correlating with miRNA in ACD, enrichment analyses of the biological processes and pathway enrichments were performed and identified significant enrichment in processes of high relevance to ACD including T cell activation, cytokine signaling, and skin development (Figure S2).

Assuming that differentially expressed as well as highly correlated miRNAs are of biological relevance, we focused on the 18 ACD-related miRNAs (Figure S1B). Generating a miRNA-mRNA network with these miRNAs, we explored six of them with relevant functional enrichment (Figure 2A). Of these, miR-142-3p, miR-142-5p, miR-146b-5p and miR-155-5p were differentially expressed across all investigated allergens. Interestingly, only mRNA targets of miR-155-5p exhibit GO biological processes enrichment for axon guidance, smooth muscle cell migration and leukocyte/T cell apoptotic process (Figure 2B). Similarly, Reactome pathway analysis for the same mRNA targets revealed L1CAM interaction involvement (Figure 2C). miR-142-3p/5p have been described to be involved in ACD in both humans and mice,³ as well as in other inflammatory skin disorders¹ and our data suggest that miR-142-3p/5p and miR-155-5p share some mRNA targets. Additionally, miR-155-5p appears to be a key regulator of the inflammatory skin response via the regulation of tight junction expression⁴ as well as inflammation.⁵

Furthermore, we identified miRNAs which were uniquely regulated in MCI and EP encounter (Figure S1B). miR-497-5p was differentially expressed in MCI only, showing a high correlation with genes associated with T cell activation, cell-cell adhesion (Figure 2B)

as well as cytokine and chemokine regulation pathways (Figure 2C). The function of miRNA-497-5p in human skin needs further elucidation; however, previous studies suggest a putative role in TGF- β -pathways via the regulation of SMAD3.⁶ We identified SMAD3/SMAD4/SMAD5/SMAD7 as targets for miR-497-5p in our correlation experiments, underlining the importance of this miRNA during ACD.

Epoxy resin -induced allergic reactions regulated miR-23b-3p, miR-99a-5p, miR-193b-3p, and miR-199a-3p expression (Figure S1), which was not previously described in ACD. miR23b-3p and miR-99a-5p, appear to affect skin homeostasis and development in-vitro via TGIF1 and IGFR1, respectively.⁷ Our findings underline the importance of those miRNAs since enrichment and pathway analysis revealed involvement in skin and epidermis development (Figure 2B,C). Lastly, miR-193b-3p and miR-199a-3p were described during oncogenic processes such as epidermal squamous cell carcinoma.⁸ Both seems to interact with cell cycle genes controlling cellular proliferation. Our data pinpoint these proposed functions since both miRNAs show targets enriched for leukocyte proliferation and keratinocyte/epidermis differentiation. Taken together, our data suggest that the functional impact of the allergen-induced changes in the miRNAome on a cellular level may arise as a result of the interaction between several miRNAs regulating the same pathways. The fact that each miRNA targets hundreds of genes, and each gene is targeted by several miRNAs,¹ adds complexity to the interactions.

Contrary, initial correlation analysis of the irritant contact dermatitis (ICD) reactions revealed multiple correlations of miRNAs with lncRNAs (Figure S3). Since lncRNAs can act as mRNA regulators as well as "miRNA-sponges",⁹ we created a miRNA-lncRNA-mRNA network using the same approach. We identified lnc-TRIM27-15 and lnc-TAF13-1 as central lncRNAs with their associated mRNA targets displaying enrichment for keratinocyte/epidermal differentiation and skin development (Figure S4). However, this computational model needs to be verified in further in-vitro studies.

In conclusion, we analyzed changes in the miRNA-mRNA expression in ACD and ICD and integrated our findings from the miRNAome with mRNA expression profiles from the same samples. Using this holistic approach, we found functional miRNA-mRNA networks linked to allergic and irritant responses in CD. Our study reveals a unique miRNA signature induced by ACD, with network construction of miRNA-mRNA pairs with enrichment of immunological responses including T cell activation and cytokine signaling.

To the best of our knowledge, this is the first study to integrate the miRNA and mRNA expression profiles in skin from sensitized subjects exposed to allergens and irritants, identifying known and novel miRNA-mRNA networks during CD (summarized in Figure S5). However, more studies are warranted for verification and further elucidation of the underlying regulatory mechanisms of ACD for the purpose of unraveling new therapeutic and diagnostic strategies.

KEYWORDS

allergic contact dermatitis, irritant contact dermatitis, miRNA, miRNA-mRNA integration

ACKNOWLEDGEMENTS

We thank all patients that participated in our study.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

FUNDING INFORMATION

This research has received funding from The Finnish Work Environment Fund (project nr 113314) and Forskningsrådet för hälsa, arbetsliv och välfärd (FORTE, diariennr. 2018-00601).

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SUPPORTING INFORMATION

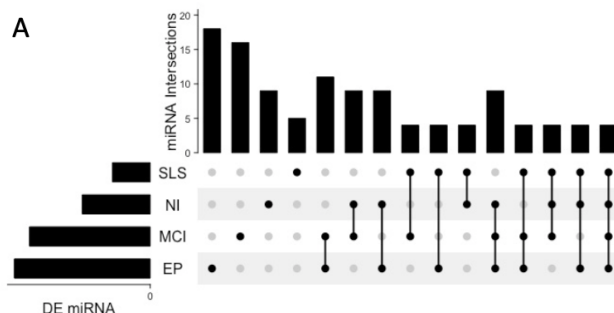
Additional supporting information may be found online in the Supporting Information section.

Appendix S1

Supplementary information

Supplementary Table 1. Metadata of the study participants. Reactions were rated +, ++ and +++ for the allergic reactions and IR0 (slight irritant reaction), IR (normal irritant reaction), IR1 (strong irritant reaction), IR2 (severe irritant reaction) for the irritant reactions. Abbreviations for the exposures were used as follows: baseline (BL), sodium lauryl sulphate (SLS), nonanoic acid (NO), nickel sulphate (NI), methylchloroisothiazolinone (MCI) and epoxy resin (EP).

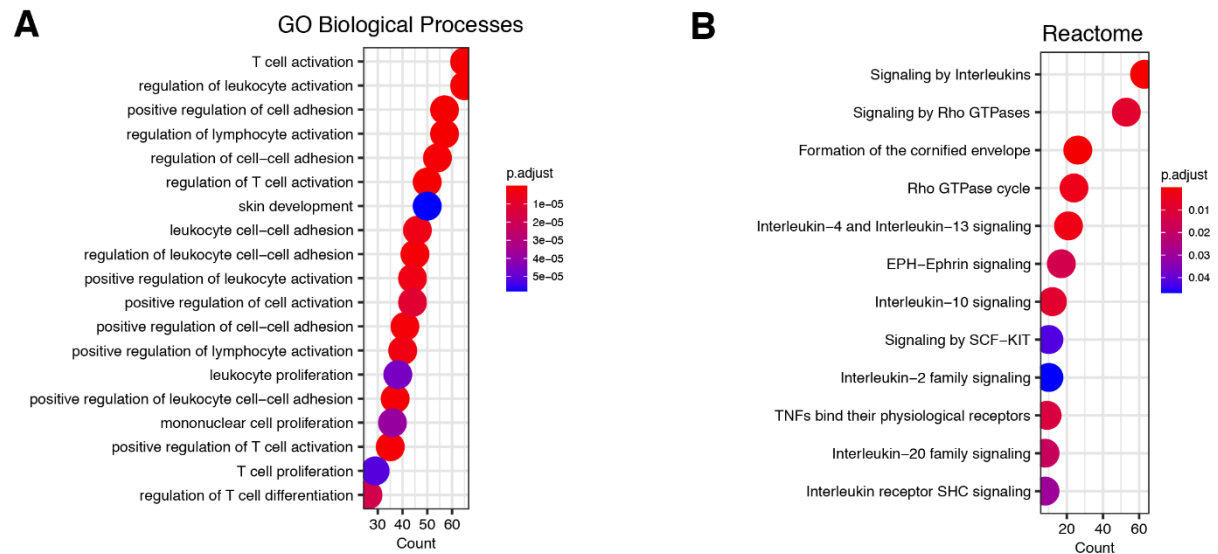
Patient	Gender	Age	Group	Reaction strength (allergen)	Reaction strength (irritant)	Occupation
1	F	39	BL, SLS	0	IR2	Stock clerk
2	F	50	SLS	-	IR	Dentist
3	F	44	NI	+++	-	Sales clerk
4	F	30	NI, SLS	+	IR	Life science technician
5	F	21	NI	+++	-	Electronic-equipment assembler
6	F	46	MCI	+	-	Nursing associate
7	M	42	EP	+++	-	Floor layer
8	M	62	SLS	-	IR	Motor vehicle mechanic
9	M	47	NO	-	IR	Assembler
10	M	60	BL, NO	0	IR	Machinery mechanic
11	M	53	BL	0	-	Intercity educational instructor
12	M	24	EP	+	-	Plumber and pipe fitter
13	M	57	BL	0	-	Machine operator and assembler
14	F	59	NO	-	IR	Beautician
15	F	58	EP, SLS	+	IR	Building electrician
16	F	51	NI	+	-	Nursing associate
17	M	36	MCI	+	-	Motor vehicle mechanic
18	M	49	MCI	++	-	Welder and flame cutter
19	F	55	SLS	-	IR2	Building cleaner
20	F	56	MCI	+	-	Dentist
21	M	27	MCI	+	-	Dairy-products worker
22	M	55	EP	+++	-	Building electrician
23	M	48	BL, NO	0	IR	Chemical-product machine operator
24	F	36	NI, SLS	+++	IR	Painter
25	F	24	NO	-	IR	n/a
26	F	51	EP	+++	IR	n/a
27	F	22	SLS	-	IR	n/a



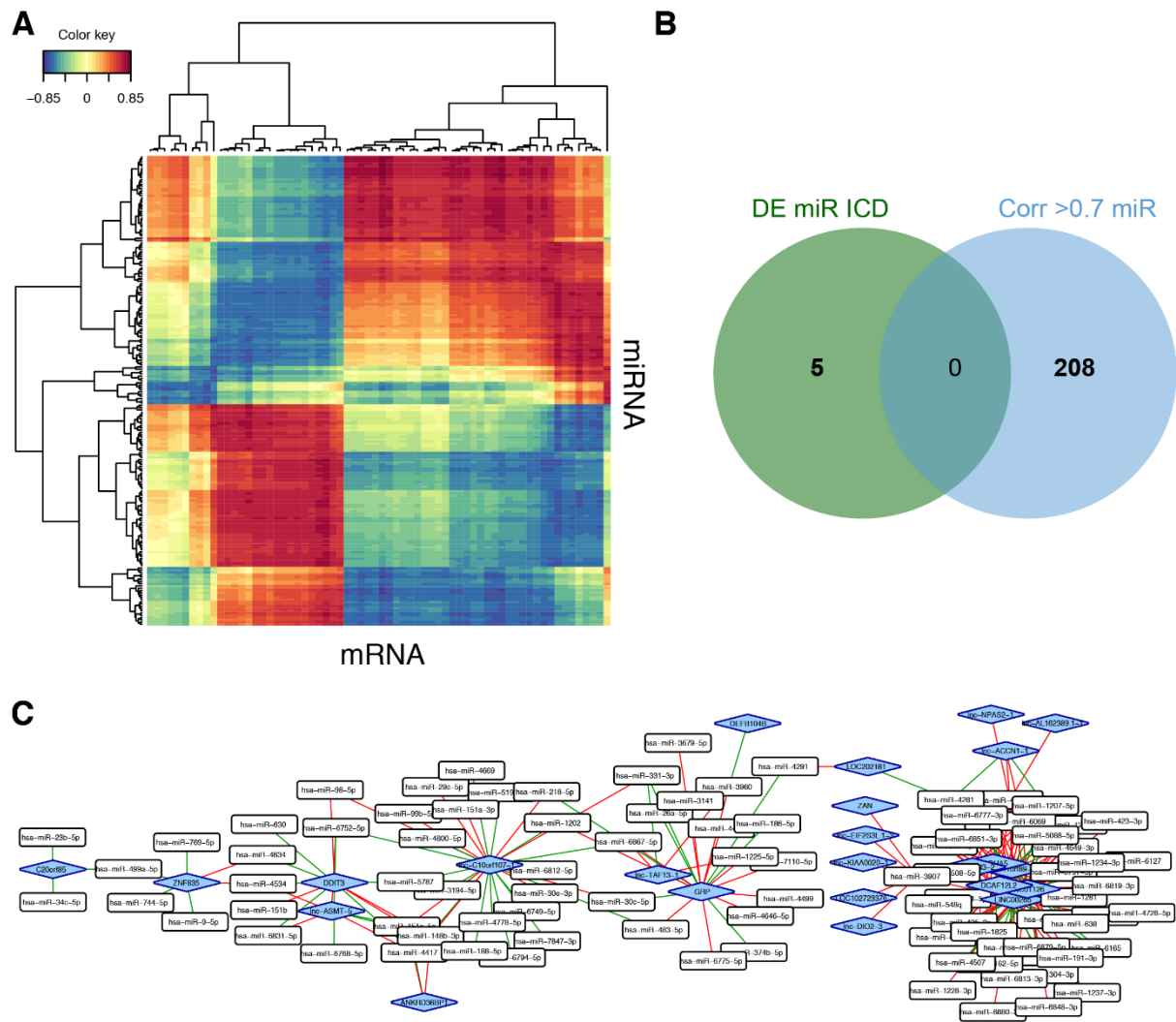
B

Group	DE miRNA
MCI	hsa-miR-365a-3p hsa-miR-130a-3p hsa-miR-126-3p hsa-miR-224-5p hsa-miR-10b-5p hsa-miR-497-5p hsa-miR-424-5p hsa-miR-146b-5p hsa-miR-342-3p hsa-miR-155-5p hsa-miR-142-5p hsa-miR-21-5p hsa-miR-142-3p hsa-miR-21-3p hsa-miR-223-3p hsa-miR-150-5p
NI	hsa-miR-142-3p hsa-miR-142-5p hsa-miR-146b-5p hsa-miR-150-5p hsa-miR-155-5p hsa-miR-21-3p hsa-miR-21-5p hsa-miR-142-3p hsa-miR-342-3p
EP	hsa-miR-23b-3p hsa-miR-193b-3p hsa-miR-365a-3p hsa-let-7c-5p hsa-miR-199a-3p hsa-miR-99a-5p hsa-miR-148a-3p hsa-let-7b-5p hsa-miR-424-5p hsa-miR-342-3p hsa-miR-146b-5p hsa-miR-155-5p hsa-miR-21-5p hsa-miR-142-5p hsa-miR-21-3p hsa-miR-223-3p hsa-miR-142-3p hsa-miR-150-5p
SLS	hsa-miR-1247-5p hsa-miR-150-5p hsa-miR-21-3p hsa-miR-21-5p hsa-miR-223-3p

Supplementary Figure 1. Differentially expressed (DE) miRNA according to allergens and irritants used. (A) Upset plot of differentially expressed miRNA in the different groups. (B) List of the differentially expressed miRNA in each group.

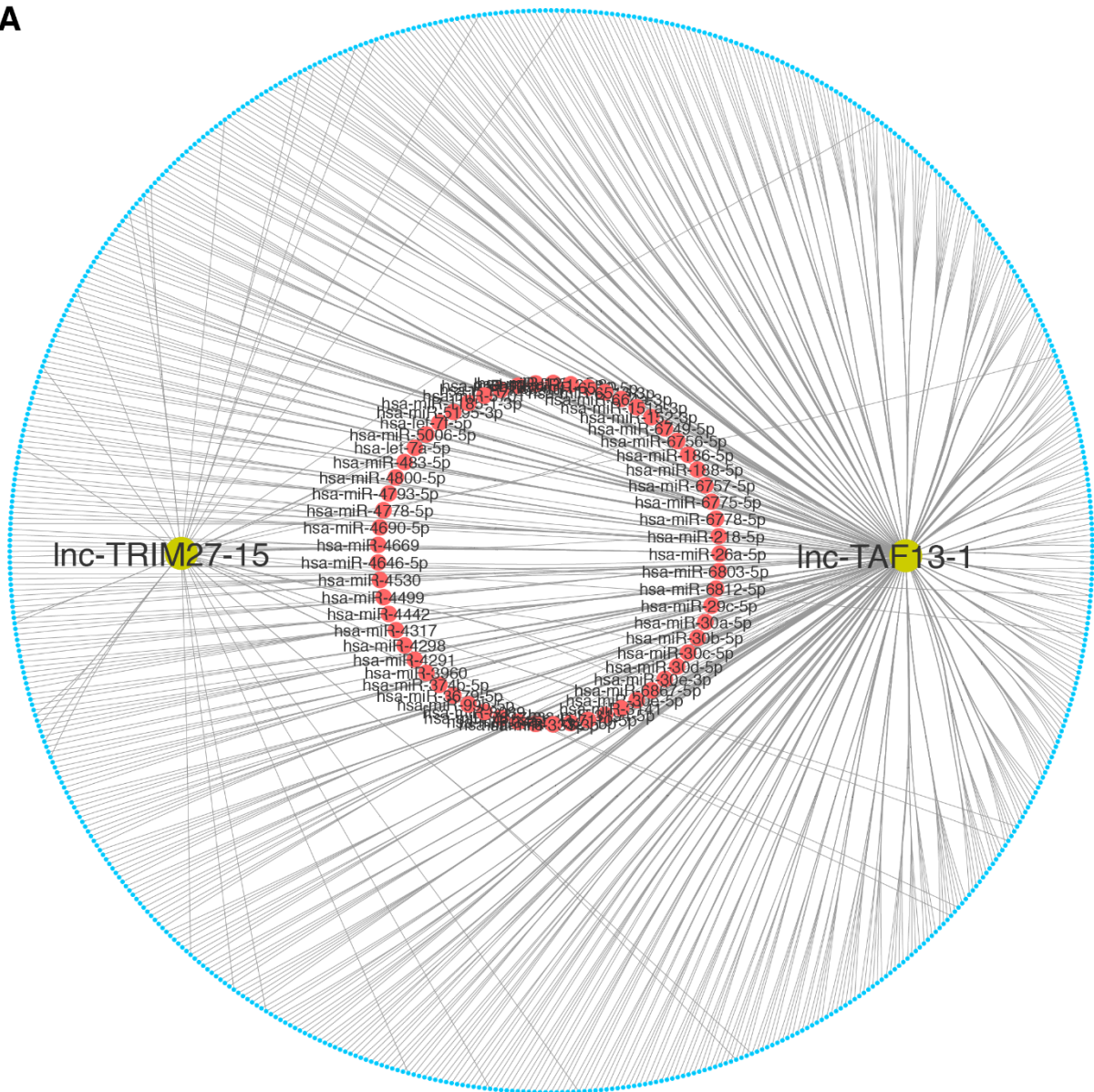


Supplementary Figure 2. Gene ontology (A) and Reactome pathway (B) enrichment of identified mRNA targets of highly correlating miRNAs in allergic contact dermatitis.



Supplementary Figure 3. Correlation analysis of ICD samples. (A) The heatmap displays highly (>0.7) correlated miRNA-mRNA pairs of ICD samples. (B) Venn comparison demonstrate an overlap between differentially expressed miRNAs and highly correlated miRNAs identified by canonical correspondence analysis. (C) Network of miRNAs and mRNA targets of ICD samples. The network was visualized using Cytoscape. Red lines indicate negative, and green lines indicate positive correlation between miRNA (white) and mRNA/lncRNA (blue).

A

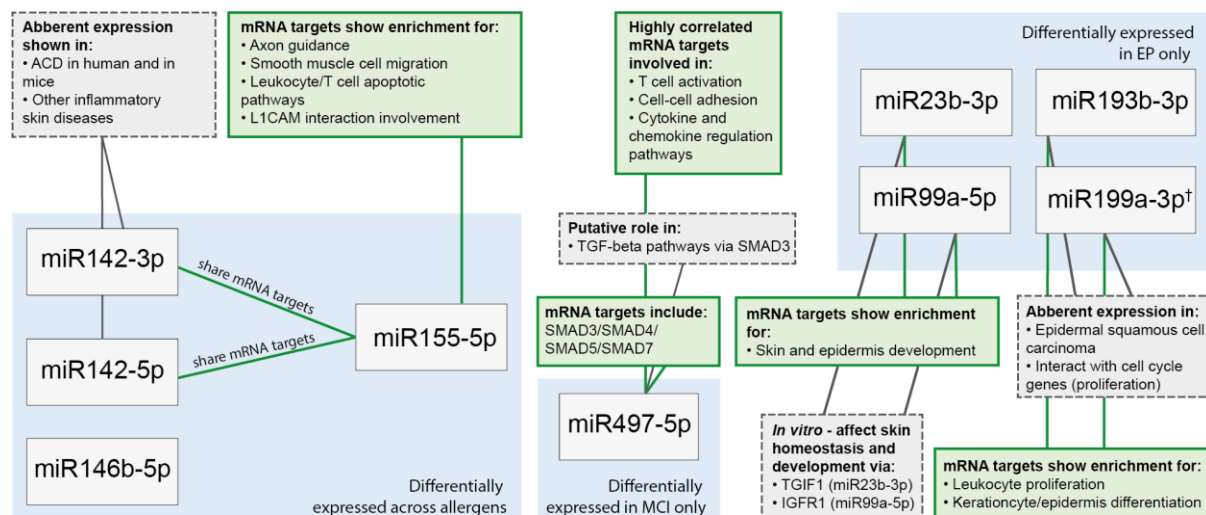


B

GO Enrichment Analysis

peptide cross-linking (GO:0018149)	2.958e-6
keratinocyte differentiation (GO:0030216)	2.968e-6
epidermal cell differentiation (GO:0009913)	1.174e-5
skin development (GO:0043588)	1.700e-5

Supplementary Figure 4. miRNA-lncRNA-mRNA network in ICD. (A) Generated network of miRNAs (red), lncRNAs (yellow) and mRNA targets (blue) during irritant-induced contact dermatitis. (B) Go biological process enrichment of identified mRNA targets.



Supplementary Figure 5. Schematic figure illustrating differentially expressed miRNAs in allergic contact dermatitis (light grey) and key findings of the study (green). Previous knowledge of these miRNAs is described in the dark grey boxes. † not previously shown to be differentially expressed in allergic contact dermatitis.

Materials and methods

Patient recruitment and sample collection

Patients over 18 years of age were recruited by dermatologists at the University of Helsinki and at the Finnish Institute of Occupational Health. The study was approved by the Ethics Committee of Helsinki University Central Hospital (approval number 43/13/03/00/14) and performed according to the Declaration of Helsinki principles. All patients gave written informed consent before participating in the study. The selection of patients was based on patch test readings and clinical history, including true positive patch test results (+, ++ and +++) against epoxy resin (EP), methylchloroisothiazolinone (MCI), or nickel sulphate (NI). Patch test reactions against corrosive irritant sodium lauryl sulfate (SLS) and noncorrosive irritant nonanoic acid (NO) were collected from the same patients. The causative agents used were chosen to represent a wide variety of agents with different physicochemical properties. Metadata of the 27 patients included in the study are depicted in the supplementary Table S1. A 3mm punch biopsy was collected from positive patch test areas 48h after application from all subjects and biopsies from non-affected skin (baseline) were collected from 5 patients as

controls (total number of samples $n = 34$). The biopsies were immersed in RNAlater solution (Thermo Fischer Scientific, Wilmington, USA) and stored at -80°C until further analysis.

RNA preparation and miRNA Microarray analysis

Total RNA, including miRNA, was extracted from the skin biopsies using the AllPrep DNA/RNA/miRNA universal kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The RNA concentration and purity was analyzed by NanoDrop ND-1000 (Thermo Fischer Scientific, Wilmington, USA), where a A_{260}/A_{280} ratio of > 1.8 was considered pure. A sample input of 100 ng of total RNA was prepared and fluorescently-labeled using the SurePrint G3 Human miRNA r21 Array Kit (Agilent, Santa Clara, USA). After hybridization of the labeled targets to the microarrays, the fluorescence image of the array was scanned using the Agilent SureScan Microarray Scanner (Agilent, Santa Clara, USA), generating fluorescence image data. The fluorescence image was converted into expression level values using the Agilent Feature Extraction program (Agilent, Santa Clara, USA). Data pre-processing and differential expression analysis of the gene expression data (matched mRNA and miRNA microarrays) were done in eUTOPIA¹. Probe profile expression data were normalized using quantile normalization and corrected for batch processing effects using ComBat². After mapping from probe sets to gene symbols, a differentially expressed gene (DEG) list was identified for each time point using limma³. DEGs were defined as a fold change ± 1.5 and a $p\text{-value} < 0.05$ after adjustment for multiple comparisons (Benjamini-Hochberg). Data can be accessed via ArrayExpress under the accession numbers: E-MTAB-9026 & E-MTAB-9051.

Canonical correlation analysis

Interdependencies between mRNA/lncRNA transcriptome and miRnome were detected via regularized canonical correlation analysis between the datasets using the mixOmics package⁴. A correlation score cut-off of $>|0.7|$ was assumed as highly correlating mRNA/lncRNA–

miRNA pairs. Further enrichment of highly correlating genes was conducted using Enrichr⁵. Correlation inferred targets were compared to experimentally validated mRNA targets using the multiMIR package⁶. Network visualization was conducted using Cytoscape 3.7.0. Data analysis and graph visualization was conducted using R software (v3.6.1, R core team 2019) with the ggplot2⁷, ggpubr⁸ and ggfortify⁹ package for R. Venn diagrams were generated using the VennDiagram¹⁰ package.

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