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Gain of function p.E138A alteration in *Card14* leads to psoriasiform skin inflammation and implicates genetic modifiers in disease severity

John P. Sundberg[a,*], **C. Herbert Pratt**[a], **Kathleen A. Silva**[a], **Victoria E. Kennedy**[a], **Wenning Qin**[b], **Timothy M. Stearns**[a], **Jacqueline Frost**[c], **Beth A. Sundberg**[a], **Anne M. Bowcock**[c,d,**]

[a]The Jackson Laboratory, Bar Harbor, ME, USA

[b]eGenesis, Cambridge, MA, USA

[c]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[d]Departments of Dermatology, Genetics & Genome Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Abstract

Psoriasis (PS) is a common inflammatory and incurable skin disease affecting 2–3% of the human population. Although genome-wide association studies implicate more than 60 loci, the full complement of genetic factors leading to disease is not known. Rare, highly penetrant, gain-of-function, dominantly acting mutations within the human caspase recruitment domain family, member 14 (*CARD14*) gene lead to the development of PS and psoriatic arthritis (PSA) (a familial p.G117S and *de-novo* p.E138A alteration). These residues are conserved in mouse and orthologous Knock-In (KI) mutations within *Card14* were created. The *Card14*$^{tm.1.1Sun}$ allele (G117S) resulted in no clinically or histologically evident phenotype of the skin or joints in young adult or old mice. However, mice carrying the *Card14*$^{tm2.1Sun}$ mutant allele (E138A) were runted and developed thick, white, scaly skin soon after birth, dying within two weeks or less. The skin hyperplasia and inflammation was remarkable similarity to human PS at the clinical, histological, and transcriptomic levels. For example, the skin was markedly acanthotic and exhibited orthokeratotic hyperkeratosis with minimal inflammation and no pustules and transcripts affecting critical pathways of epidermal differentiation and components of the IL17 axis (IL23, IL17A, IL17C, TNF and IL22) were altered. Similar changes were seen in a set of orthologous microRNAs previously associated with PS suggesting conservation across species. Crossing the *Card14*$^{tm2.1Sun}$/WT mice to C57BL/6NJ, FVB/NJ, CBA/J, C3H/HeJ, and 129S1/SvImJ generated

[*]Correspondence to: J. P. Sundberg, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609-1500, USA. john.sundberg@jax.org (J.P. Sundberg). [**]Correspondence to: A. M. Bowcock, Departments of Oncological Sciences, Dermatology, Genetics & Genome Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10024, USA. anne.bowcock@mssm.edu (A.M. Bowcock).

Declaration of Competing Interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104286.

progeny with epidermal acanthosis and marked orthokeratotic hyperkeratosis regardless of the hybrid strain. Of these hybrid lines, only the FVB;B6N (129S4) mice survived to 250 days of age or older and has led to recombinant inbred lines homozygous for *Card14*^{E138A} that are fecund and have scaly skin disease. This implicates that modifiers of PS severity exist in mice, as in the familial forms of the disease in humans.

Keywords

Psoriatic arthritis; Psoriasis; Epidermal hyperplasia; Mouse model; CARD14

1. Introduction

Psoriasis (PS) is an incurable skin disease affecting 120 million people worldwide (Lowes et al., 2007). Up to 30% of PS patients develop psoriatic arthritis (PSA) 5–10 years after the onset of their skin disease (Nograles et al., 2009). Psoriasis is a complex genetic disease and genome-wide association studies have revealed associations at over 60 loci (Capon, 2017). Nevertheless, the full complement of genetic factors leading to disease is not known. It has been hypothesized for PS, and other complex human diseases that contributing variants can range from highly penetrant rare to low penetrant common variants although they have not all been identified, and their joint contribution to common disease is poorly understood.

Highly penetrant, gain-of-function, dominantly acting mutations within the human caspase recruitment domain family, member14 (*CARD14*) gene lead to the development of PS and PSA (Jordan et al., 2012). *CARD14* mutations include a *de-novo* c.413A > C; p.E138A mutation that leads to a severe form of generalized pustular psoriasis and a familial c.349G > A; p.G117S mutation that leads to familial PS, and to the development of psoriatic arthritis as well in some family members with the mutation (PSA). Both alterations lead to enhanced nuclear factor of kappa B (NF-kB) signaling in keratinocytes (Jordan et al., 2012). Some NF-kB targets are genes that are upregulated in psoriatic lesions such as *CCL20, IL6* and *SOD2* (Jordan et al., 2012).

The development of mouse models for PS and PSA has historically been a challenge (Hawkes et al., 2018). Although this partly reflects differences between human and mouse skin, it was also because no *bona-fide* targets had been identified for genetic manipulation. The altered bases of psoriasis causing mutations in *CARD14* are conserved in evolution such that homology between mouse and man are very high, and suggested that these alterations in mice might lead to similar phenotypes. Hence, we developed mouse knock-in models of both CARD14 p.G117S and p.E138A mutations and asked if the mouse developed a phenotype resembling PS. The *Card14* p.E138A alteration led to many features of human PS and exhibited variable severity on different strain backgrounds. Interestingly we did not observe an altered phenotype following introduction of the *Card14* p.G117A alteration into mice. Histologically, the skin of the *Card14* p.E138A mouse was very similar to that of human psoriatic skin and we identified similar transcriptomic alterations in *Card14* p.E138A mouse skin to those seen in human psoriatic skin. This included transcripts affecting critical pathways of epidermal differentiation and immune cell activation. We also identified similar

trends in a set of microRNAs known to be altered in psoriatic skin, suggesting similarities in microRNA regulation across species.

2. Materials and methods

2.1. Generation of Card14 knock-in mutant mice

Standard recombineering techniques (Liu et al., 2003) were used to create a gene-targeting vector for the purpose of knocking in (KI) a human *CARD14* single base mutation into the mouse *Card14* gene by homologous recombination. The gene targeting vector was generated by first retrieving a 13,410 bps of the mouse *Card14* gene encompassing exons 3–11 from a bacterial artificial chromosome clone (RP23_466K23) into a cloning vector and subsequently incorporating the G117S mutation into exon 5 or the E138A mutation into exon 6, along with the PGK-NEO cassette assembled by gene synthesis, by recombineering in *E. coli*. The recombineered gene targeting plasmid containing the *Card14* gene sequence and the intended mutation was confirmed by restriction analysis and sequencing (Fig. S1). The gene targeting vector was linearized by digestion with the *Asc1* enzyme and electroporated into mouse embryonic stem (ES) cells (JM8A1, C57BL/ 6N) and drug resistant clones were screened by Southern Blot analysis (Fig. S1). Confirmed ES clones were injected into mouse blastocysts, the chimeric blastocysts were implanted into pseudo-pregnant females, and chimeric pups were bred for germline transmission. Two chimeric KI lines were generated. The *tm1* and *2* chimeras were bred to B6N.·129S4-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J (JR# 16226, The Jackson Laboratory) to excise the neo cassette which produced the *tm1Sun and tm2Sun* mice with the genetic background being mostly C57BL/6N but having some 129S4. These two lines were bred to C57BL/6NJ-*Tyr*^{em7J}/GrsrJ (JR# 22003) and the *Gt(ROSA)26Sor*$^{tm1(FLP)Dym}$ gene excluded from the progeny resulting in creation of B6N(129S4)-Tyr^{em7} *Card14*$^{tm1.1Sun}$ (JR# 24676, homozygous mice, hereafter referred to as G117S) and B6N(129S4)-Tyr^{em7} *Card14*$^{tm2.1Sun}$ (JR# 024878, heterozygous mice, hereafter referred to as E138A).

2.2. Genotyping

Genotyping protocols for B6N(129*S*4)-*Tyr*^{em7} *Card14*$^{tm1.1Sun}$/Sun and B6N(129*S*4)-*Tyr*^{em7} *Card14*$^{tm2.1Sun}$/Sun mice are detailed on The Jackson Laboratory's Genotyping website (https://www.jax.org/jax-mice-and-services/customer-support/technical-support/genotyping-resources/genotyping-protocol-database)

2.3. Generation of Card14 knockout mutant mice

Card14$^{tm1a(EUCOMM)Hmgu}$ ES cells were obtained from the Helmboltz Zentrum (Munich, Germany) by The Jackson Laboratory's Genetic Resources Department to create a conventional knockout and a conditional knockout through our KOMP program using standardized protocols (Dickinson et al., 2016). *Card14*$^{tm1a(EUCOMM)Hmgu}$ ES cells were used unsuccessfully to create a complete null mutation of the mouse *Card14* gene.

2.4. Characterization of altered tissues in the genetically modified mice

Detailed histopathology was done on representative mice to define anatomical changes. A complete set of tissues as defined elsewhere (Sundberg et al., 2016) was reviewed by an

experienced, board certified pathologist (JPS) who provided a broad overview of all lesions in all organ systems using standardized procedures. As many of the mice died within the first week of life, longitudinal sections were made of intact mice as a preliminary screen. Mice were euthanized by CO_2 asphyxiation followed by secondary euthanasia methods using approved protocols. Tissues were fixed in Fekete's acid-alcohol-formalin solution, trimmed, processed routinely, sectioned, stained with hematoxylin and eosin (H&E) and serial sections prepared for immunohistochemistry. All work was approved by The Jackson Laboratory Animal Care and Use Committee (approval number 07005).

2.5. Immunohistochemistry

Serial sections of skin from a normal 290 day old female A/J and a 93 day old female and male C57BL/6J wildtype mice, fixed in Fekete's solution, neutral buffered formalin, 4% paraformaldehyde, Bouin's solution, and 10% zinc formalin were tested using two antibodies directed against the human CARD14 protein that were expected to work on mouse tissues (LS-B11378, Lifespan Biosciences Seattle, WA; SAB4503140, Sigma-Aldrich, St. Louis, MO). Slides were processed on a Ventura Discovery XT (Ventana Medical Systems, Inc., Tucson, Arizona) using their panel of antigen retrieval methods. All were negative (all tissues were brown using DAB as a chromogen).

2.6. Transcriptome profiling of skin

Ten, 8-day old, female mice were used. Five were heterozygous (+/B6N(129S4)-*Tyr*^{em7} *Card14*$^{tm2.1Sun}$ (E138A)) and 5 were wildtype (+/+) by genotyping. All had dorsal skin examined histologically to confirm the presence or absence of lesions and those with lesions had similar degrees of severity. Epidermal thickness measurements confirmed these histological observations. RNA was isolated from dorsal skin at the time of necropsy, stored in RNAlater (Sigma-Aldrich, St. Louis, MO) and libraries prepared for RNA-seq that was performed with routine approaches (Harbour et al., 2010) by The Jackson Laboratory's Genome Technology Service. Individually bar coded, strand-specific poly(A)-selected Illumina mRNA sequencing libraries were prepared using KAPA Biosystems mRNA stranded HyperPrep kit (Roche Sequencing, Pleasanton, CA). Libraries were multiplexed and 101 bp paired-end reads were generated with an average of 56.3 million read pairs per sample on an Illumina HiSeq4000 at The Jackson Laboratory for Genomic Medicine (Farmington, CT) following manufacturer's protocols. Sequence quality diagnostics were initially performed for each sample using FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastq) that were used to detect systematic error and/or bias in the sequence data by examining features such as quality scores and sequence composition. Reads were then aligned to the mouse transcriptome annotated by Ensembl (version 88) using the GRCm38 genome assembly using RSEM (Li and Dewey, 2011). Gene expression differences between sample groups were determined using R/edgeR (version 3.20.9) (Robinson et al., 2010). Results were analyzed using Ingenuity Pathway Analysis® software (QIAGEN, Redwood City, CA) to generate major molecular pathways (Figs. S3, S4).

For qRT-PCR, RNA was reverse transcribed with the QuantiTect Reverse Transcription kit (Qiagen) and the TaqMan Advanced miRNA cDNA synthesis kit (Life Technologies).

Differential expression was calculated with the $2^{-\Delta\Delta Ct}$ method using *Gapdh* as the housekeeping gene. Pathway analysis was performed with Ingenuity software.

MicroRNA expression analysis was performed with predesigned miRNA TaqMan assays (ThermoFisher) for orthologous microRNAS to those known to be differentially expressed in psoriatic skin (miR-10b, miR-21–5p, miR-30e-5p, miR-31–3p, miR-155–5p, miR-203a-3p and miR-378b). Differential expression was calculated with the $2^{-\Delta\Delta Ct}$ method using snoRNA202 as the endogenous control as recommended for mouse miRNA expression by ThermoFisher.

3. Results

Orthologous Knock-In (KI) mutations for *CARD14* p.G117S (*Card14*$^{tm1.1Sun}$) and p.E138A (*Card14*$^{tm2.1Sun}$) were created with standard gene targeting technologies (Supplemental Materials and Methods, Fig. S1). The *Card14*$^{tm.1.1Sun}$ allele (G117S) resulted in no clinically or histologically evident phenotype of the skin or joints in young adult or old mice (70–330 days of age). Mild to moderate chronic inflammation was noted in a variety of organs, such as the pancreas, in old mice, typical of inbred strains. By contrast, mice carrying the *Card14*$^{tm2.1Sun}$ mutant allele (E138A) were runted and developed thick, white, scaly skin soon after birth, dying within two weeks or less. The skin was markedly acanthotic and exhibited orthokeratotic hyperkeratosis with minimal inflammation and no pustules (Fig. 1 a–f). Serial longitudinal sections of one of these pups revealed an atrial septal defect in the heart (Fig. S2).

To salvage the line, mutant male mice on the B6N(129S4) congenic background were crossed with C57BL/6NJ, FVB/NJ, CBA/J, C3H/HeJ, and 129S1/SvImJ females in an attempt alter modifier genes that may affect the survival of this line. Dorsal skin from 5 to 9 day old mice (B6, 5f/4 m; FVB, 2f; CBA, 3f; C3H, 2f; 129S1, 2 m) all had epidermal acanthosis and marked orthokeratotic hyperkeratosis regardless of the hybrid strain. The degree of hypergranulosis varied based on strain background. Of these hybrid lines, only the FVB;B6N(129S4) mice survived to 250 days of age or older. FVB/NJ has repeatedly been a strain with modifier genes that down regulate severity of genetically based skin diseases (Sproule et al., 2014). FVBB6NF1 progeny consistently yielded homozygous p.E138A mutant mice that were fecund and had scaly skin disease. These mice were maintained by brother X sister mating to create recombinant inbred lines homozygous for Card14^{E138A}. A B6N(129S4) subline, that remains difficult to maintain, was established that did have a subset of mice that survived to up to a year of age with psoriasiform skin disease. Generation of *Card14* null mice using *Card14*$^{tm1a(EUCOMM)Hmgu}$ ES cells was unsuccessful (supple mental data).

The altered transcriptome of *Card14*^{E138}/WT (*Card14*$^{tm2.1Sun}$/WT) mouse skin was analyzed with RNA sequencing. (Fig. 1g–h, Table S1). Transcriptome data on skin of the child with the *CARD14*^{E138A} mutation (before and after treatment with ustekinumab) (Jordan et al., 2012), data on involved and uninvolved skin from individuals with the *CARD14*^{G117S} mutation and individuals with classic PS (Harden et al., 2014; Jordan et al.,

2012; Swindell et al., 2011) versus skin of healthy controls were compared with the skin transcriptome from the *Card14*^{E138A}/WT mice vs homozygous WT mice.

Many molecules altered in human psoriatic involved skin were altered in the skin of *Card14*$^{tm2.1Sun}$/WT mice. This included upregulation of *Krt6b*, *Lce3b*, *Il1f6* and *Lcn2*, and downregulation of *Ccl24*, *Col26a1* and *Lef1* (Fig. 1g–h). Top canonical pathways from Ingenuity Pathway Analyses (Table 1) were granulocyte adhesion and diapedesis ($P = 7.7 \times 10^{-9}$), the role of IL17A in psoriasis ($P = 4 \times 10^{-6}$), eicosanoid signaling (2.9×10^{-5}) and IL10 signaling ($P = 4.4 \times 10^{-5}$). Figs. S3 and S4 illustrate some of the altered inflammatory and epidermal differentiation pathways found the *Card14*^{E138A} mice that are common to altered pathways found in PS. This includes upregulation of transcripts involved in the IL2/IL17 axis (Harden et al., 2015) and altered keratinocyte differentiation (Fig. S3 and S4). qRT-PCR was performed for critical cytokines operating in psoriatic skin. Analysis of *Il23*, *Il17* and *Il22,* which encode critical cytokines upregulated in PS with qRT-PCR confirmed upregulation of these transcripts in the *Card14*$^{tm2.1Sun}$/WT mice (Fig. 2A). Specifically, *Il23* and *Il17a* were the most highly upregulated, followed by *Il17c*, *Tnf* and *Il22.*

We also used qRT-PCR to investigate alterations in a set of orthologs of microRNAs known to be altered in human PS (miR10b, miR21–5p, miR30e-5p, miR31–3p, miR155–5p, miR203a-3p and miR378b). This includes miR21, which is highly upregulated in the human disease, and where its knockdown in the imiquimod mouse model of human PS has led to amelioration of symptoms (Guinea-Viniegra et al., 2014). Results are shown in Fig. 2B, where the differential expression of the microRNAs in the skin of *Card14*$^{tm2.1Sun}$/WT mice was compared to that of WT mice. This was compared to the reported fold change of microRNA levels in human psoriatic versus healthy skin (Joyce et al., 2011; Sonkoly et al., 2007; Zibert et al., 2010). All microRNAs tested were upregulated in human PS and *Card14*$^{tm2.1Sun}$/WT mouse skin. Some (Mir10b, Mir155–5p and Mir21–5p) had a higher magnitude of expression in the mouse model than has been reported in human PS.

4. Discussion

Mice with the *Card14* E138A allele develop skin hyperplasia and inflammation with remarkable similarity to human PS at the clinical, histological, and transcriptomic levels. Failure to see inflammation in mice with the familial *Card14* p.G117S allele could be due to the level to which the two mutants activate the NF-kB signaling pathway.

In human studies the p.E138A alteration activated this pathway eight fold over that seen with wild type *CARD14*. The p.G117S alteration activated NF-kB signaling four-fold over that seen with wild type *CARD14* (Jordan et al., 2012). If it was having a similar effect in mouse, it may be that the level of NF-kB activation triggered by the p.G117S alteration was insufficient to lead to inflammation. A *Card14*^{tm1Lex} null mouse was produced using ES cells obtained from Lexicon Pharmaceuticals. These mice were clinically normal and failed to develop psoriasiform dermatitis when treated with imiquimod cream (Tanaka et al., 2018). This places altered CARD14 signaling firmly in a pathway leading to the development of psoriasiform lesions. Two other heterozygous mice with alterations at *Card14* E138A (*Card14ΔE138* and *Card14*$^{E138A/+}$) resulted in gross and histologic features similar to the

mice in this study (Mellett et al., 2018; Wang et al., 2018). A third mutation (*Card14*$^{\Delta Q136/+}$) (Wang et al., 2018) had a similar phenotype. These studies confirm that specific mutations within *Card14* can generate a psoriasiform response and highlight an important role for E138 within this peptide.

We observed marked variation in clinical severity when this allelic mutation was moved to hybrid stocks on a variety of genetic backgrounds suggests that modifier genes are likely to affect the expressivity of *Card14* mutations. This is consistent with our earlier observations of variability in expression or penetrance of PS or PSA in a large multiply affected families where gain-of-function mutations in *CARD14* were segregating in a family of European origin (p.G117S) or PS skin disease severity in the case of a c.349+5G > A splicing mutation at the same splice donor in a Taiwanese family (Bhalerao and Bowcock, 1998; Hwu et al., 2005; Jordan et al., 2012). A search for genetic modifiers affecting expressivity and penetrance of *CARD14* mutations in psoriasiform disease in humans and mouse is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

JPS, WQ, and AMB designed the study. KAS, VEK, WQ, TMS, JF and JPS performed the experiments. JPS did the histopathological analysis. JPS, CHP, BAS, JF and AMB created the figures. JPS, JF and AMB wrote the initial manuscript. All authors edited and approved the manuscript. We also wish to acknowledge a generous gift from the family of the late Dr. Norman Orentreich.

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Abbreviations:

***Card14*/CARD14**
caspase recruitment, dominant family, member 14 mouse gene/protein

***CARD14*/CARD14**
human gene/protein

E138A, Card14tm2.1Sun
mutant allele

***G117S, Card14*$^{tm.1.1Sun}$**
mutant allele

PS
psoriasis

PSA

psoriatic arthritis

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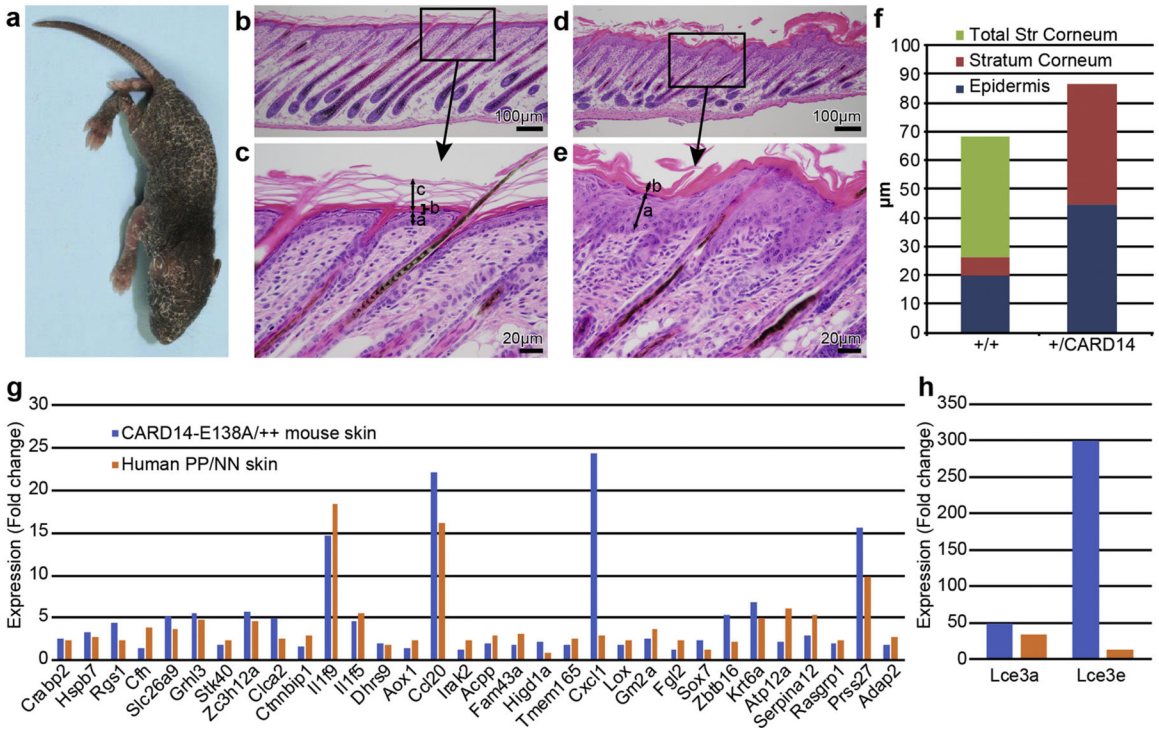

Fig. 1.

B6N(129S4)-*Tyr*^{em7} *Card14*$^{tm2.1Sun}$/Sun 8 day old female with scaly skin (a). Mutants were runted compared to wildtype mice (insert). The skin phenotype (b–e) looked remarkably similar to the human patient with this mutation (Jordan et al., 2012). The thick scaly skin in the heterozygous mutant mouse was confirmed by epidermal thickness measurements (bar graph) (f); malphigian layer and str. corneum (red is very compact str. corneum while green is the lose “basket weave” pattern in normal epidermal str. corneum). Fold changes of psoriasis-specific transcripts where those from skin of *Card14*$^{tm2.1Sun}$ (E138A) heterozygous mice are compared with +/+ controls versus human psoriatic involved (PP) compared with normal skin (NN) (g). *Lce3a* and *Lce3e* are shown separately because their fold changes are so high (h).

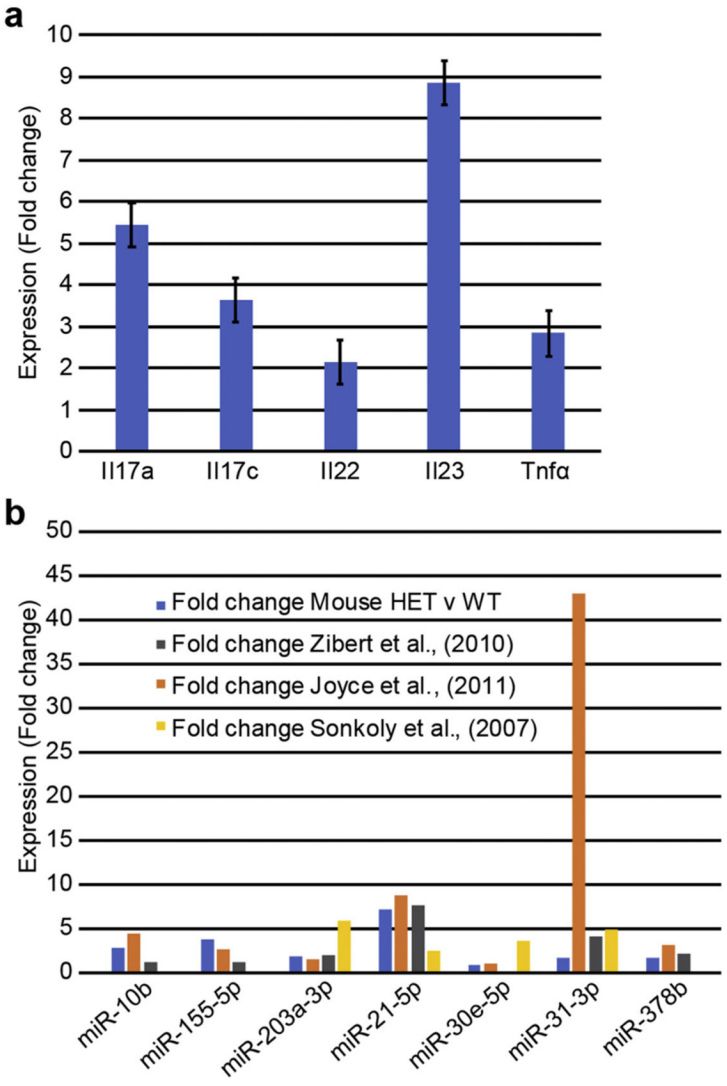

Fig. 2.

Investigation of psoriasis-specific transcripts in the skin of *Card14*$^{tm2.1Sun}$ (E138A) heterozygous mice. a). Differential expression of mRNAs for pathogenic cytokines in the skin of *Card14*$^{tm2.1Sun}$ (E138A) heterozygous versus wild type mice. b). Fold changes of a subset of microRNAs differentiating psoriatic from healthy skin in a number of different studies versus fold changes seen in the skin of orthologous microRNAs in *Card14*$^{tm2.1Sun}$ (E138A) heterozygous versus wild type mice.

Table 1

IPA® top analysis ready molecules.

Gene	Value	Gene	Value
Sprr2d	+11.343	*Mup1*	−7.835
Sprr2b	+10.822	*Mrgprb3*	−6.385
Sprr2e	+10.416	*Erdr1*	−5.915
Sprr2i	+9.761	*Serpinb6e*	−5.478
Sprr2g	+9.270	*Crisp1/Crisp3*	−3.816
Defb3	+9254	*Gng13*	−3.808
Lce3a	+9.165	*Bcan*	−3.680
Spink7	+9.043	*Gnmt*	−3.530
S100a9	+8.930	*Add2*	−3.510
Ctsj	+8.589	*Sowaha*	−3.368