miR-146a regulates TLR1/2 and 4 induced inflammation and links it with proliferation in human SZ95 sebocytes

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Abstract

Activation of Toll-like receptors (TLR) 1/2 and 4 are central in inducing inflammation in sebocytes and in the pathogenesis of acne by regulating the expression of protein coding mRNAs, however the microRNA (miRNA) profile in response to TLR activation and thus the possible role of miRNAs in regulating sebocyte functions has not been elucidated. In this work therefore, we aimed to identify the miRNA with the most abundant induction and to reveal its role in TLR1/2- and 4-activated SZ95 sebocytes. We found that miR-146a, detected with increased expression also in sebaceous glands of acne samples, showed the highest induction levels in the activated sebocytes. When exploring its role, we found that the increased levels of miR-146a led to the down-regulation of IL-8 secretion, decreased the chemoattractant potential and stimulated the proliferation of sebocytes, whereas the latter may be affected by *GNG7* down-regulation. According to our results miR-146a may be a potential player in acne pathogenesis by regulating inflammation and by providing a link between inflammation and sebocyte proliferation.

Introduction

Toll-like receptors (TLRs) belong to the family of pattern-recognition receptors and are key players in the innate immune system to selectively sense the presence of various microorganisms ^{1–3}. Importantly, elevated expression of TLR2 and TLR4 were reported in acne-involved skin ^{4,5}, suggesting that these pathways may also be involved in acne pathogenesis. While TLR2 recognizes peptidoglycans, lipoproteins, lipoarabinomannans and short-chain fatty acids from Gram-positive bacteria, TLR4 is activated by the Gram-negative bacterial component lipopolysaccharide (LPS) ⁶. Interestingly lipids, such as saturated fatty acids, are also able to activate both TLR2 and TLR4 ^{7–9}. Indeed, products of *Propionibacterium acnes* (*P. acnes*), a Gram-positive, anaerobic bacterium, which behaves both as commensal and pathogen in acne skin, and several sebaceous lipids, among them palmitic acid, whose altered ratios were detected in acne patients, are possible TLR activators in acne lesions ^{10–17}.

Sebaceous glands are known for their primary role to secrete and metabolize lipids leading to the production of sebum to moisturize the hair and the skin, which feature is regulated by a wide repertoire of stimuli such as hormones, lipids and pathogens both in physiological as well as in disease settings ¹⁸. However, with a great number of proteins and lipids that exert inflammatory properties, sebocytes are also actively involved in shaping the inflammatory environment ^{19–21}, in which their activation through TLRs ^{14,22,23} might play a central role. Supporting this postulate, our previous genome wide gene expression study has shown that sebocytes are able to rapidly gain and prioritize an immune-competent status in response to TLR1/2 and/or TLR4 activation at the level of mRNA expression ²².

MicroRNAs (miRNA) are small non-coding RNAs also selectively transcribed from the genome under various conditions. In contrast to mRNAs they do not encode proteins but control gene expression by binding and destabilizing their target mRNA ²⁴. Therefore, they can regulate transcriptional and post-

transcriptional gene expression. In human sebocytes, miRNA presence was confirmed and was proven essential for lipogenesis²⁵, but without providing any disease-specific conclusions on their possible role.

In the present study, we aimed to extend our knowledge on the gene expression regulation and profile of TLR-activated sebocytes and investigated the role of miRNAs in it. We found that in TLR1/2- and 4-activated sebocytes, miRNAs had altered expression levels, with miR-146a showing the most prominent upregulation. Confirming that sebaceous glands of acne samples also exhibited high expression levels of miR-146a, we aimed to define a possible pathophysiological role for miR-146a in sebocytes. Our results suggest that miR-146a may not only regulate TLR-induced inflammation in sebocytes but could be a missing link in connecting it with hyperproliferation, which may have both pathological and therapeutic implications in sebaceous gland-associated diseases, such as acne.

Results

miR-146a shows the most prominent induction in TLR1/2 and 4-stimulated SZ95 sebocytes

Sebocytes are able to sense and respond to different TLR stimuli²², making them an active player in a pathogen-associated inflammatory environment. To investigate the change in their miRNA profile in such a response, we applied two different TLR activators, PAM3CSK4 (TLR1/2 activator) and LPS (TLR4 activator), to treat human SZ95 sebocytes. Performing genome wide expression studies in samples treated for 24 h, 23 microRNA responded to PAM3CSK treatment, while 54 miRNA were significantly upregulated after LPS treatment (Supplementary Table 1). Both TLR activators induced common significant elevation of 14 miRNA expression levels (Fig. 1a), of which miR-146a showed the most abundant values (Fig. 1b). This significant increase in the levels of miR-146a could also be detected by *in situ* hybridization in TLR1/2- and 4-activated SZ95 sebocytes (Fig. 1c).

miR-146a is elevated in sebaceous glands of human acne tissue samples

To provide a biological relevance for our finding, we performed *in situ* hybridization against miR-146a in 5 acne vulgaris and 5 normal skin samples from the back of young male adults. While in normal skin, miR-146a was detectable with low-intensity homogeneous staining, a more intense, mainly granular staining was observed in sebaceous glands of acne tissue samples (Figure 2.).

miR-146a decreases IL-8 secretion and negatively regulates the chemoattractant potential of SZ95 sebocytes

To assess the possible role of miR-146a, we focused on the functional analysis of SZ95 sebocytes transfected with the hsa-miR-146a inhibitor, hsa-miR-146a mimic, or negative controls for 72 h.

First, we measured the secreted interleukin 8 (IL-8) levels from supernatants, which are known to be negatively regulated by miR-146a²⁶, 72 h after transfection by ELISA. Cells transfected with mimic sequence secreted significantly lower amount of IL-8 (481.3 ± 32.7 versus control 1178.4 ± 57.2 pg/ml, p-value: 0.002), while the inhibition of the microRNA resulted in elevated IL-8 secretion (1056.1 ± 36 versus 813.5 ± 55.5 pg/ml) (Fig. 3a), confirming its negative regulatory role in the inflammatory response also of sebocytes.

As IL-8 is an important chemokine in immune cell migration, we examined the migratory capacity of peripheral blood monocytes towards supernatants of transfected SZ95 sebocytes. We observed a lower migration to mimic-transfected and an increased migration towards miR-146a inhibitor treated SZ95 sebocyte supernatants, showing that miR-146a may have a negative regulatory role on monocyte chemoattraction under inflammatory conditions (Fig. 3b).

miR-146a regulates cell proliferation but not lipid production of SZ95 sebocytes

To examine the role of miR-146a in sebocyte proliferation and lipid production of cells treated with hsa-miR-146a inhibitor, hsa-miR-146a mimic, or negative controls for 72 h, we analyzed the cell numbers in different cell cycle phases using DNA content histograms and applied Oil Red O staining to detect lipids.

When incubated with miR-146a mimic sequence, the proportion of cells in G2 and M phases increased with a significant decrease in S phase ($P < 0.05$) and a slight decrease in G0 and G1 phases in comparison with control (Fig. 3c), showing that cell proliferation is stimulated by higher miR-146a levels. Transfecting SZ95 sebocytes with miR-146a inhibitor, the cell proportion in the S phase increased and the G0/G1 population decreased, suggesting that the mitotic machinery itself was activated but might be blocked in S phase. According to the DNA histogram, a peak of apoptotic cells was also observed following miRNA inhibitor treatment.

Lipid measurements found a slight, but statistically not significant decrease in the lipid content of miRNA inhibitor-treated cells and no change in cells treated with the mimic sequence (Fig. 3d).

Whole transcriptome analysis with clustering revealed changes in genes related to inflammation and cell proliferation in miR-146a inhibitor-treated SZ95 sebocytes

To detect how levels of miR-146a could impact changes at the level of gene expression in sebocytes, we performed whole transcriptome analysis of inhibitor-treated cells at 72 h and found that 330 genes were upregulated, while 311 genes were downregulated. Functional gene clustering confirmed that the altered levels of miR-146a in sebocytes may lead to changes in immune response, in apoptosis and cell proliferation-related pathways. Importantly, genes could not be clustered into lipid production, which was in line with our lipid detection studies (Fig. 4).

miR-146a levels in sebocytes lead to changes in the expression of GNG7

According to the transcriptome analysis, G protein gamma 7 (*GNG7*), found also to contribute to a great number of the functional clusters, had one of the most robust upregulation following miR-146a inhibition (Fig. 5a). The miR-146a mediated expression changes of *GNG7* could be further confirmed by qPCR and *in situ* hybridization in SZ95 sebocyte cultures transfected with mimic, inhibitor and control sequences (Fig. 5b-c), suggesting a central role for *GNG7* in miR-146a-induced signaling.

To assess if *GNG7* mRNA could be also detected *in vivo*, *in situ* hybridization was performed on human acne and normal skin samples. The presence of *GNG7* mRNA could be visualized in sebaceous glands of normal skin, while in acne samples, in which elevated miR-146a levels were shown, it could not be detected (Fig. 5d).

Discussion

The application of a system-based approach of whole genome sequencing of SZ95 sebocytes, treated with specific and selective TLR1/2 and TLR4 activators, provided evidence that miRNAs are selectively induced in sebocytes upon TLR activation. Moreover, identifying miR-146a as the miRNA with the most abundant induction with an increased expression also in the sebaceous glands of acne samples, our work is the first to identify a miRNA, which is increased in sebaceous glands in a disease setting. Characterizing sebocytes with altered levels of miR-146a, we showed that miR-146a is not only a marker for activation, but could have a regulatory role on basic cellular functions as well.

In line with our previous results, showing that TLR1/2 and TLR4 pathways induced a similar change in the mRNA profile of sebocytes, miRNAs also changed similarly in response to the used activating agents. This finding further supports our previously raised hypothesis that these pathways and the related changes are not stimulus-/pathogen-specific in sebocytes as these receptors can be activated with a wide range of stimuli both of pathogenic and of non-pathogenic origin²². In other words, sebocytes use these receptors to sense changes in their environment, such as an altered microbiome or the presence of lipids, which activation needs to be further modulated to gain its disease specific role. Based on our results miR-146a could represent a negative regulator, just as it is observed in various cell types of lymphoid, myeloid and of non-immune origin, where miR-146a decreases the production of inflammatory cytokines²⁷.

Regarding dermatological diseases, increased levels of miR-146a was already confirmed in keratinocytes of atopic dermatitis and psoriasis samples, with a suggested role to regulate inflammation. In psoriasis, its genetic alterations even showed an association with disease severity^{28–30}. Importantly, higher levels of miR-146a was also detected in keratinocytes of acne samples, where it may down-regulate *P. acnes*-induced production of IL-6, -8, and TNF- α by inhibiting the TLR2/IRAK1/TRAF6/NF- κ B and MAPK pathways³¹. Our findings that miR-146a was also highly expressed in sebaceous glands of acne samples, confirms that miR-146a may be involved in acne also at the level of sebocytes and adds further

important details on the immune-competence of this cell type. Therefore, the most interesting finding of the induction of the TLR-miR-146a axis in sebocytes may result in a decreased production of IL-8, a cytokine characteristic in acne-related inflammation, and a decreased chemoattractant potential of sebocytes, a feature that was recently reported by our group¹⁹. Speculating on the *in vivo* relevance of this finding, it is reasonable to put forward that the increased levels of miR-146a in sebocytes could serve as a negative regulator of inflammation in acne lesions with an impact on the production of inflammatory cytokines and the number of infiltrating immune cells.

Further important result of our studies was the role of miR-146a to regulate the proliferation of sebocytes. By showing that the proliferation and apoptosis of sebocytes were dependent on the levels of miR-146a, the increased levels led to an increased proliferation while decreased ones to apoptosis, may bring us closer to understand the morphological and functional changes of sebaceous glands observed under inflammation. Although there was no evidence for an altered lipid metabolism in our results, a recent publication³² reported that the pathogenetic basis of acne is the alteration in sebocyte differentiation. The less differentiated and with that the more proliferating the sebocytes are the more they are responding to regulatory stimuli. It is intriguing to put forward that the promoted cell proliferation due to the increased levels of miR-146a may sensitize sebocytes to regulatory stimuli both of pathogenic and of therapeutic relevance that need further characterization.

Our unbiased strategy of whole transcriptome analysis performed on sebocytes treated with a specific miR-146a inhibitor, revealed that miR-146a may also influence the gene expression profile of sebocytes. Although the exact mechanisms remain to be elucidated, in inhibitor-treated sebocytes, pathways with pivotal roles in sebocyte functions, such as Wnt, EGF or insulin signalling, might be altered at the level of transcription³³. These results showed that the complex changes induced by miR-146a may go beyond altering the inflammatory properties of sebocytes. Based on the fold change values, the functional clustering of the differentially expressed genes and the *in situ* hybridization studies, *GNG7* came into the focus showing an opposite regulation with miR-146a both in sebocytes and in sebaceous glands of acne samples, in which the expression of miR-146a increased while *GNG7* had a lower expression. *GNG7*, predominantly expressed in nervous tissue³⁴, inhibits cell proliferation, promotes cell differentiation and induces cell death by inhibiting mTOR signalling^{35,36}, with so far little data on its involvement in skin. Considering that the activation of mTOR pathway is also central in the regulation of sebocyte proliferation and maturation, and its induction by various agents is central in the development of acne³⁷⁻⁴⁰, further studies are needed to identify in more details the contribution of *GNG7* to sebocyte biology and how its decreased levels may be involved in disease pathogenesis (Fig 6).

In summary, our findings deliver novel data, that in the active role of sebocytes to shape the inflammatory environment, miRNAs are also utilized. Moreover, our results also point on the therapeutic relevance which the modulation of miR-146a levels may deliver to acne therapy by targeting inflammation and cell proliferation at the same time.

Methods

Cells, transfection and treatment

Immortalized human SZ95 sebocytes were maintained as adherent culture at 37°C in a humidified chamber containing 5% (v/v) CO₂ in Sebomed basal medium (Sigma-Aldrich, St. Louis, MO, USA) as previously described ⁴¹.

For microRNA profiling, SZ95 cells were treated with 1 µg/ml PAM3CSK4 (TLR1/2 activator; dissolved in sterile water; Cat. no.: TLRL PMS, InvivoGen, San Diego, CA, USA) and 1 µg/ml LPS (TLR4 activator; derived from Escherichia coli; dissolved in sterile water; Cat. no.: L4391, Sigma-Aldrich) for 24 h.

For studies on transfected cells, SZ95 sebocytes were transfected with 25 nM hsa-miR-146a-5p power inhibitor or negative control (Qiagen, Hilden, Germany), and 1 nM hsa-miR-146a-5p mimic (Ambion, Austin, TX, USA) or negative control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 50000 cells/well seeding density on 24 well plates. All experiments were carried out in triplicates. Cells and supernatant were harvested at 72 h post-transfection for further analysis.

Histological samples

Formalin-fixed and paraffin-embedded (FFPE) human skin tissue samples from the Department of Dermatology, University of Debrecen were used after approval of the Regional and Institutional Ethics Committee, Clinical Center, University of Debrecen (Approval ID: UD REC/IEC No. 4103-2014). The study was conducted according to the guidelines of the Declaration of Helsinki. Normal control skin samples were selected from the archive of the Department of Dermatology. Specimens from subjects with papulopustular acne were surgically resected from the back area, fixed in 10% buffered formaldehyde for 24 h, and processed routinely into paraffin blocks. Informed consent was obtained from all subjects involved in the study. Five normal and five acne vulgaris skin samples were evaluated.

ELISA

Secreted IL-8 protein levels were quantified with IL-8 DuoSet ELISA Kit (R&D Systems, Minneapolis, MS, USA) according to the manufacturer's instructions. Supernatants were collected after 72 h of transfection, centrifuged and stored at -20°C until use. 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma-Aldrich) was used as visualizing reagent, substrate reaction was stopped with 1M H₂SO₄. Optical density was measured with Epoch microplate spectrophotometer (BioTek, Winooski, VT, USA) at the wavelength of 450 nm.

RT-qPCR and RNA sequencing

Total RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH, USA) according to the provided protocol. RNA concentration and purity were measured with Nanodrop 2000 (Thermo

Scientific, Waltham, MA, USA). Before reverse transcription (RT)-quantitative polymerase chain reaction (qPCR)), total RNA was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random primers. Gene expression levels were analyzed using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) using SYBR Green master mix (Roche, Basel, Switzerland) for *GNG7* (forward primer 5'-3': GACAATGTCAGCCACTAACAACA; reverse primer 5'-3': CAGTAGCTCATGAGGTCAGACG). Expression levels were normalized against *PPIA* (forward primer 5'-3': CAGTGCTCAGAGCTCGAAAGT; reverse primer 5'-3': GTGTTCTTCGACATCACGGC) using the comparative Ct method.

RNA-Seq method

Small RNA-Seq sequencing libraries were generated from 1 µg total RNA using NEBNext Multiplex Small RNA Prep Set for Illumina (1-48) 96 rxn kit (New England BioLabs, Ipswich, MA, USA), according to the manufacturer's protocol. Fragment size distribution and molarity of libraries were checked on Agilent BioAnalyzer DNA1000 chip (Santa Clara, CA, USA). Then single read 50bp sequencing run was performed on Illumina NextSeq 500 instrument (San Diego, CA, USA).

To obtain global transcriptome data high throughput mRNA sequencing analysis was performed on Illumina sequencing platform as described previously^{22,42,43}. Total RNA sample quality was checked on Agilent BioAnalyzer using Eukaryotic Total RNA Nano Kit according to the manufacturer's protocol. Samples with RNA integrity number value >7 were accepted for library preparation process. RNA-Seq libraries were prepared from total RNA using Ultra II RNA Sample Prep kit (New England BioLabs) according to the manufacturer's protocol. Briefly, poly-A RNAs were captured by oligo-dT conjugated magnetic beads then the mRNAs were eluted and fragmented at 94°C for 15 minutes. First strand cDNA was generated by random priming reverse transcription and after second strand synthesis step double-stranded cDNA was generated. After repairing ends and adapter ligation steps, adapter-ligated fragments were amplified in enrichment polymerase chain reaction and finally, sequencing libraries were generated. The sequencing run was executed on Illumina NextSeq500 instrument using single-end 75 cycle sequencing.

RNA-Seq data analysis

Raw sequencing data was aligned to human reference genome version GRCh37 using HISAT2 algorithm and BAM files were generated. Downstream analysis was performed using StrandNGS software (version 2.8, build 230243; Strand Life Sciences, Bangalore, India). BAM files were imported into the software, DESeq1 algorithm was used for normalization. Sequencing data have been deposited into the GEO database (PRJNA673828). mRNA levels with 1.3/-1.3 fold-change or higher/less were stated as

significantly changed. Pathway analysis was performed with the PANTHER Classification System (version 15.0)^{44,45}.

Cell proliferation assay

SZ95 cells were collected at 72 h post-transfection by trypsinization, washed in 1x phosphate buffered saline (PBS), permeabilized, and fixed with 70% cold ethanol for 30 min at 4°C. Cells were treated with 200 ng/μl RNase (Invitrogen) for 45 minutes at 37°C and stained with 5 ng/ml propidium iodide (Invitrogen). Cell cycle phases were assessed with FACS Aria flow cytometer (BD Biosciences, San Jose, CA, USA) and FlowJo software (version X.0.7 BD Biosciences) based on the DNA histogram.

Lipid content analysis

Intracellular lipid content was measured by Oil Red O staining 72 h after transfection on 96-well plates. Cells were fixed in 10% buffered formaldehyde for 15 min, washed twice with PBS, and stained with Oil Red O (Sigma-Aldrich, 3:2 parts of 0.6% Oil Red O dye dissolved in isopropanol and distilled water) for 15 min. After washed three times with distilled water, Oil red O was washed out with isopropanol, and absorbance was measured with Epoch microplate spectrophotometer (BioTek) at the wavelength of 510 nm.

Chemotaxis cell migration assay

Monocytes were isolated from whole blood of healthy donors by density centrifugation (Ficoll, Paque Plus, GE Healthcare, Chicago, IL, USA), CD14 microbeads (Miltenyi Biotech, Bisley, UK), resuspended in RPMI 1640 medium (Invitrogen) supplemented with 1 v/v% L-glutamine (Sigma-Aldrich) and 0,5 v/v% Antibiotic-Antimycotic (penicillin, amphotericin-B, streptomycin, BioSera, Nuaille, France). 1×10^5 isolated monocytes were added to the top of a 5 μm pore cell migration chamber plate (Chemicon QCM 96-well chemotaxis cell migration assay, Temecula, CA, USA). Feeder trays were loaded with supernatants of SZ95 cells transfected with miR-146a-5p mimic, inhibitor or control sequences in triplicates. After 24 h at 37°C with 5% CO₂ migrated cells were harvested, lysed and stained with CyQuant GR (Chemicon). Fluorescence was measured with Epoch microplate reader (BioTek) at the wavelength of 520 nm.

Chromogenic in situ hybridization (CISH)

CISH was performed on 5 μm thick FFPE sections from acne, normal skin specimens and air dried, Carnoy's-fixed (methanol:acetic acid 3:1, -20°C for 5 min) SZ95 sebocyte preparations with 120 nM

double-digoxigenin labeled hsa-miR-146a-5p, *GNG7* mRNA and scramble negative control locked nucleic acid (LNA, Qiagen) detection probes according to the manufacturer's instructions. Sections were treated with 2xproteinase K solution (miRNA ISH Buffer Set, Qiagen) for 15 min, then 120 nM of previously linearized (90°C for 4 min) probe mix diluted in 1xISH buffer (miRNA ISH Buffer Set, Qiagen) was applied. Hybridization was performed at 54°C with hsa-miR-146a-5p, 54°C with *GNG7* and 57°C with scramble negative control LNA probe for 60 min in a hybridization chamber (StatSpin ThermoBrite, Abbott Molecular, Chicago, IL, USA). For visualization alkaline phosphatase conjugated anti-DIG antibody (Roche) and 4-nitro-blue tetrazolium/5-bromo-4-chloro-indolylphosphate (NBT/BCIP, Roche) AP chromogen substrate was applied for 2 h at 30°C. Slides were counterstained with liquid-stable nuclear fast red (VWR, Radnor, PA, USA), covered with Eukitt mounting medium (Sigma-Aldrich), coverglassed, and imaged using a Leica DM200LED (Leica, Wetzlar, Germany) microscope.

Statistical evaluation

Values are presented as mean $\pm$ SEM. All experiments have been performed in triplicate. P values were calculated using Mann-Whitney test, *P < 0.05; **P < 0.01; ***P < 0.001 considered as statistically significant.

Data availability

RNA sequencing data have been deposited into the GEO database under accession number PRJNA673828. Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Declarations

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Author contributions statement

K.D. and D.T. conceived the experiments, K.D., D.D., F.F., D.K. and Sz.P. performed the experiments, K.D., D.T., A.Sz. and C.C.Z. analysed the results. All authors reviewed the manuscript.

Additional information

Accession codes

RNA sequencing data have been deposited into the GEO database under accession number PRJNA673828.

Competing interests statement

The authors declare no competing interests.

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Figures

Figure 1

(a) Venn diagram showing the numbers of the upregulated miRNAs in TLR1/2 and TLR4 activated SZ95 sebocytes. 14 miRNAs were upregulated 24 hours after both LPS and PAM3CSK4 treatments. (b) Heatmap visualizing the 14 miRNAs’ fold-change values after TLR activation. miR-146a showed the most significant upregulation in both treatments. (c) Detection of miR-146a by in situ hybridization in SZ95 sebocytes. Compared to untreated SZ95 sebocytes (A), a more intense blue cytoplasmic hybridization signal was detected in TLR1/2 activated (24 hours PAM3CSK4 treatment) sebocytes (B). Chromogenic in

situ hybridization, NBT/BCIP blue chromogen reaction with nuclear Fast Red background staining. Original magnification x200. Experiments were carried out in triplicates.

Figure 2

In situ detection of miR-146a on FFPE tissue samples. Compared with normal skin samples (A), a more intense, granular blue hybridization signal was detected in sebaceous glands of acne vulgaris samples (B). Samples obtained from the back of patients. Arrows show miR-146a positive sebocytes. Chromogenic in situ hybridization, NBT/BCIP blue chromogen with nuclear Fast Red background staining. Original magnification x200, Representative microphotograph, n=5.

Figure 3

Characterization of miR-146a-transfected SZ95 sebocytes. (a) Inhibition of miR-146a (a-miR) caused a significant elevation p-value: 0.002), while transfection with mimic sequence caused a significant decrease in the secreted IL-8 levels as measured from the supernatants of SZ95 sebocytes by ELISA. IL-8 levels were normalized to the levels secreted by SZ95 sebocytes transfected with adequate control sequences. (b) Migration assay revealed that monocytes migrated more towards inhibitor-treated and less towards mimic-transfected sebocyte supernatants. Graphs show the fluorescence intensity of migrated cells normalized to control samples after staining as described in the Materials and Methods. p-value: 0.028 (c) Measurement of G0, G1, S, G2, M cell cycle phases and apoptosis, in SZ95 sebocytes treated with miR-146a mimic or inhibitor for 72 hours. Graphs show the percentage of cells in each phase based on the DNA content analysis. Cell populations treated with mimic sequence showed a significantly lower number of cells in S phase (p-value: 0.05) and larger in G2/M phase. In the sebocyte cultures

treated with inhibitor, more cells were found in S phase and less in G2/M phase. Note the sub-G0/G1 peak, representing apoptotic cells (blue arrow). (d) Oil Red O staining of SZ95 sebocytes. Treatment with mimic or with inhibitor sequence caused no significant change in the lipid content of SZ95 sebocytes when normalized to control. Graphs show the mean optical density normalized to control. RFU: relative fluorescence unit. All experiments were carried out in triplicates.

Pathway	Genes
Wnt signalling	FDZ8, GNG7, ACTG2, LRP5L, FZD9, PCDHAC1, PCDH10
CCKR signalling map	HDAC7, MAPK10, SERPINB2, PRKD1, TFF2, CXCL1
Alzheimer disease-presenilin	MMP28, FZD8, ACTG2, LRP5L, FZD9, MAPK10
Cadherin signalling	FZD8, ACTG2, FZD9, PCDHAC1, PCDH10
Integrin signalling	ACTG2, MAPK10, COL1A2, COL4A2, COL4A3
Angiogenesis	FGF1, MAPK10, PRKD1, FLT4
Heterotrimeric G-protein signalling	ADORA1, GNG7, GRM8, CACNA1B, PDE6A
Inflammation mediated by chemokine and cytokine signalling	ACTG2, GNG7, PLCE1, CCL26
FGF signalling	FGF18, FGF1, MAPK10
Interleukin signalling	IRS2, IL23A, SLA2
Metabotropic glutamate receptor group II- III	GNG7, GRM8, CACNA1B
Thyrotropin-releasing hormone receptor signalling	GNG7, PLCE1, CACNA1B
5HT1, -2, -4 type receptor mediated signalling	GNG7, PLCE1
Blood coagulation	A2M, SERPINB2
EGF receptor signalling	MAPK10, PRKD1
Endogenous cannabinoid signalling	GNG7, CACNA1B
GABA-B receptor II signalling	GNG7, CACNA1B
Histamine H1, -2 receptor mediated signalling	GNG7, PLCE1
Huntington disease	ACTG2, CAPN11
Insulin/IGF	IRS2, INSRR
Nicotinic acetylcholine receptor signalling	ACTG2, MYO7A
Oxidative stress response	MAPK10, DUSP13
Oxytocin receptor mediated signalling	GNG7, PLCE1
VEGF signalling	PRKD1, FLT4

Figure 4

Whole transcriptome analysis of SZ95 sebocytes treated with miR-146a inhibitor for 72 hours (n=3). Pathway analysis of significantly regulated genes resulted in clusters such as inflammation and cell proliferation.

Figure 5

Detection of GNG7 mRNA in miR-146a inhibited sebocytes and in sebaceous glands of histological specimens. (a) Genes with the highest fold change values in their expression levels in miR-146a inhibitor treated SZ95 sebocytes as revealed by our RNAseq measurements. Note that GNG7 was contributing also to a large number of the functional clusters. (b) Changes of relative GNG7 mRNA levels measured by qRT-PCR in SZ95 sebocytes normalized to PPIA. GNG7 mRNA levels decreased in SZ95 sebocytes transfected

with mimic and increased in the inhibitor treated cells. FC: fold change value, compared to relative GNG7 expression values of sebocytes transfected with miR-146a mimic or inhibitor sequences. (c) in situ hybridization for the detection of GNG7 mRNA in SZ95 sebocytes. Note, that when compared to miR-146a mimic (a), a more intense blue hybridization signal was seen in the cytoplasm of the inhibitor treated (b) SZ95 sebocytes. Representative photomicrograph, n=3. (d) in situ hybridization for the detection of GNG7 in sebaceous glands of normal (A) and acne vulgaris (B) FFPE human tissue samples. Note that while in acne samples, GNG7 mRNA could not be detected, in healthy skin samples obtained from the back, weak hybridization signals were observed. Arrows show GNG7 mRNA-positive sebocytes. Representative photomicrograph, n=5. Chromogenic in situ hybridization, NBT/BCIP blue chromogen with nuclear Fast Red background staining. Original magnification x200.

Figure 6

miR-146a regulates inflammation and cell proliferation in TLR-activated sebocytes. Our data showed that in response to TLR1/2 and -4 activation the expression of miR-146a, a microRNA leading to the degradation of its target mRNAs, is abundantly induced in sebocytes. The increased levels of miR-146a may have a negative regulatory role on cytokine secretion and chemotaxis and induce cell proliferation while decrease apoptosis. GNG7 is down-regulated in parallel with the elevation of miR-146a levels in human SZ95 sebocytes and sebaceous glands of human acne samples which altogether may have pathological as well as therapeutical relevance.

Supplementary Files

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