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Integrative miRNA–mRNA profiling of human epidermis: unique signature of SCN9A painful neuropathy

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Personalized management of neuropathic pain is an unmet clinical need due to heterogeneity of the underlying aetiologies, incompletely understood pathophysiological mechanisms and limited efficacy of existing treatments. Recent studies on microRNA in pain preclinical models have begun to yield insights into pain-related mechanisms, identifying nociception-related species differences and pinpointing potential drug candidates.

With the aim of bridging the translational gap towards the clinic, we generated a human pain-related integrative miRNA and mRNA molecular profile of the epidermis, the tissue hosting small nerve fibres, in a deeply phenotyped cohort of patients with sodium channel-related painful neuropathy not responding to currently available therapies. We identified four miRNAs strongly discriminating patients from healthy individuals, confirming their effect on differentially expressed gene targets driving peripheral sensory transduction, transmission, modulation and post-transcriptional modifications, with strong effects on gene targets including *NEDD4*. We identified a complex epidermal miRNA–mRNA network based on tissue-specific experimental data suggesting a cross-talk between epidermal cells and axons in neuropathy pain. Using immunofluorescence assay and confocal microscopy, we observed that Nav1.7 signal intensity in keratinocytes strongly inversely correlated with *NEDD4* expression that was downregulated by miR-30 family, suggesting post-transcriptional fine tuning of pain-related protein expression. Our targeted molecular profiling advances the understanding of specific neuropathic pain fine signatures and may accelerate process towards personalized medicine in patients with neuropathic pain.

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Introduction

Chronic neuropathic pain is defined as pain that arises as a direct consequence of a lesion or disease affecting the somatosensory system lasting more than 3 months. It is considered a distinct clinical entity despite a large variety of aetiologies[1] and is among the most common non-communicable disorders, affecting up to 10% of the global population and 40% of patients with neurological disorders.[2-4] Guidelines for the use of first- and second-line drugs are available.[5,6] However, they do not provide healthcare professionals with any recommendations for the personalized treatment of patients, which remains an unmet clinical need. Moreover, the response to analgesic compounds is highly variable among individuals and currently there are no evidence-based criteria for determining response and therapy failure. Better understanding of pathophysiological mechanisms and modulating variables underpinning peripheral mechanisms of pain signalling would contribute to achieving effective personalized prevention strategies and treatments.

Although caused by different aetiologies and characterized by various clinical symptoms, one trademark shared by painful neuropathies is the involvement of small nerve fibres innervating the epidermis, the forefront of the somatosensory system involved in nociception and pain initiation mechanisms.[7-10] Keratinocytes account for the most represented cell type in human epidermis, providing physical and chemical barrier at the interface between the body and ecosystem and also potentially contributing to peripheral sensitization via synapses with intraepidermal nerve endings[9,11] and directly initiating nociceptive responses through neuroactive molecule release.[7,12-15] These findings suggest that a cross-talk between epidermal cells and axons could actively modulate nociception and possibly neuropathic pain.

In the past two decades, the pleiotropic nature of microRNA (miRNA) molecules has made them attractive drug candidates, especially in multifactorial diseases.[16,17] miRNA profiling studies have begun to demonstrate the important mediating role of tissue-specific miRNAs due to their ability to locally activate regulatory mechanisms.[18,19] miRNA-based discoveries are beginning to enter the clinical setting as powerful disease biomarkers[20] and highly specific therapeutic targets with minimal toxicity.[16,21]

Several studies have investigated the association between altered miRNA expression and different pain conditions, including trigeminal neuralgia,[22] migraine,[23] painful neuropathies of various aetiologies,[24] complex regional pain syndrome[25,26] and fibromyalgia.[27-30] Most of them were targeted and single assay based, and those based on an unbiased design were restricted to circulating miRNAs.[31] Moreover, data on local miRNA regulation of pain mechanisms are restricted to animal model studies where pain has been induced experimentally,[32-34] while naturally occurring epigenetic mechanisms in humans are the result of spontaneous behavioural or environmental exposure affecting gene expression and phenotype.[35] This, together with the high variability in pain signalling mechanisms and gene expression diversity across different species,[36] could hinder translation into the clinical realm.

To bridge the translational gap and provide an unbiased analysis of human tissue–specific miRNA targets, we performed a comprehensive molecular profiling of epidermis using skin biopsy specimens collected routinely for diagnosing painful small fibre neuropathy.[37] To overcome the high variability in pain signalling mechanisms and aetiologies, we investigated a homogeneous cohort of deeply phenotyped patients, not responding to first- and second-line neuropathic pain treatment[5,6] and harbouring gain-of-function (GoF) variants in the voltage-gated sodium channel (VGSC) *SCN9A* gene, whose pathogenic effect on membrane excitability and gate functioning has been demonstrated by cell electrophysiological assay.[38-41]

We identified four altered tissue-specific miRNAs which strongly distinguish patients from healthy individuals. *In silico* miRNA-associated gene target analyses allowed us to identify their synergistic interplay in human epidermal pain-related pathways *via* gene expression regulation. Moreover, by utilizing microfluidic gene expression array, we validated the effect of dysregulated miRNAs on predicted gene targets and key players in identified pathways including *NEDD4* and *TRPV1*, further strengthening the concept of non-neuronal epidermal cell involvement in pain signalling.[8,10]

Overall, our findings shed light on miRNA-driven epidermal regulatory mechanisms in *SCN9A*-related painful neuropathy, providing unique molecular signatures of targeted tissue, and may contribute to accelerating progress toward personalized medicine for neuropathic pain.

Materials and methods

Study design

The primary goal of this study was to investigate the role of epidermal microenvironment, the forefront of pain initiation processes,[8,10,42] in sodium channel–related neuropathy (NavNP) patients and to identify new molecular signatures demonstrable by skin biopsy. To this aim, patients harbouring GoF *SCN9A* pathogenic variants[38,40] and age- and sex-matched healthy controls (HC) were recruited.

miRNA and mRNA profiling was assayed on RNA extracted from the epidermis of 11 NavNP patients (nos. 1–11) and 7 HC (nos. 1–7) (Table 1 and Supplementary Table 1). Immunofluorescence assay (IFA) experiments were performed on skin biopsy sections from 5 of the 11 patients (nos. 1–5) and 6 of the 7 HC (nos. 1–6). To improve the power of the IFA, we included six additional NavNP patients (nos. 12–17) and 12 HC (nos. 8–19) (Supplementary Table 7).

The aim of this work was to provide a novel investigative tool on skin biopsy tissue routinely collected in outpatients' neuropathic pain clinics. Therefore, for each subject all histological and molecular analyses were performed using the single skin biopsy sample, collected and processed according to the standard diagnostic procedure.[43] All subjects underwent clinical examination, skin biopsy and blood sampling, and gave informed consent to participate in the study. Clinical features were collected using an established protocol previously described.[44] Pain intensity was measured as

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Table 1 Clinical characterization of the recruited cohort

Patient	Sex	Age	PI-NRS	Pain features	Additional sensory symptoms	Positive sensory signs	Negative sensory signs	Distribution	Response to analgesic drugs
P1	M	64	7	Burning, stinging	Paraesthesia	Allodynia and hyperalgesia	None	Length dependent	No response
P2	M	41	5	Burning, stinging, electric shock-like	Paraesthesia, itch	Allodynia and hyperalgesia	None	Length dependent	No response
P3	F	61	9	Burning, stinging	Paraesthesia, itch	Allodynia and hyperalgesia	None	Length dependent	No response
P4	M	21	N/A	Joint pain	None	None	None	Diffuse	No response
P5	F	34	7	Electric shock-like	Paraesthesia	Allodynia and hyperalgesia	Reduced thermal sensation	Length dependent	No response
P6	M	55	N/A	Hot pain	None	None	None	Length dependent	No response
P7	F	69	8	Pins and needles	None	Allodynia	Reduced thermal sensation	Length dependent	No response
P8	M	47	8	Burning, stinging	Paraesthesia	Allodynia	Reduced thermal sensation	Length dependent	No response
P9	F	65	9	Burning, stinging	None	Allodynia	Reduced thermal sensation, numbness	Length dependent	No response
P10	F	54	7	Burning, electric shock-like	None	Allodynia	Reduced thermal sensation	Length dependent	No response
P11	F	73	8	Burning	None	Allodynia	Reduced thermal sensation	Length dependent	No response

To evaluate the response on analgesic drugs, only responsiveness to first-line analgesic drugs was considered. PI-NRS, pain intensity numerical rating scale.

the average score of the last 3 weeks using the pain intensity numerical rating scale (PI-NRS). Satisfactory pain relief was defined as a reduction of pain intensity >50% on the PI-NRS.

The study was approved by the local Ethical Committee of the Fondazione IRCCS Istituto Neurologico 'Carlo Besta' of Milan (FINCB), Italy, and Maastricht University Medical Center+ (Maastricht UMC+), Maastricht, The Netherlands, under the PAIN-net project (grant agreement number 721841). The study design is illustrated in Fig. 1.

SCN9A genetic variants

Genotypes of *SCN9A* genetic variants were extracted from next-generation sequencing (NGS) coming from target sequencing of 107 pain-related genes. Whole blood samples, collected during neurological exam, were used for DNA extraction. DNA was extracted using QIAamp DNA blood Maxi Kit, Puregene® Blood Core Kit (Qiagen). NGS was performed using Illumina® TruSeq® Custom Amplicon-Next Generation Sequencing, Molecular Inversion Probes-Next Generation Sequencing or Illumina® Nextera Flex for Enrichment and run in the Miseq sequencer (Illumina), according to the manufacturers' protocol. Sequenced data were analysed by using our in-house NGS data analysis pipeline, which aligns sequenced data to the human reference sequence GRCh37 using bwa v. 0.7.17-r1188 (mem algorithm).[45] Variant calling was performed using GATK module Haplotype Caller (version 4.1.9) over the target region.[46] Variant annotation was performed using the SnpEff software.[47]

Skin biopsy

Skin biopsies taken with 3-mm diameter punch for diagnostic procedure of intraepidermal nerve fibre density (IENFD) assessment as previously described[48] were used for all immunohistological and molecular analysis. Briefly, specimens were fixed in 2% paraformaldehyde–lysine–periodate solution for 24 h at 4°C, cryoprotected overnight at 4°C, serially cut with a cryostat into 50-μm sections and stored at −20°C until further processing.

Molecular analysis of skin biopsy tissue

RNA isolation and quality assessment

Total RNA was isolated from the skin epidermis (layer enriched with keratinocytes and small nerve fibres) of two 50-μm skin biopsy sections per subject, dissected under the dissection microscope, using a TruXtract FFPE total NA kit—column (Covaris, cat.no.PN520220), according to the manufacturer's instructions. The RNA purity, concentrations and integrity were measured with NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) prior to the preparation of miRNA and mRNA array. All RNA samples that achieved adequate purity ratios (A260/A280: 1.5–2.0) were used for subsequent analysis. The same RNA sample was utilized for both miRNA and mRNA profiling.

miRNA profiling

miRNA expression quantification was performed using predesigned TaqMan™ Array Human MicroRNA A + B Cards (Thermo Fisher) containing 754 miRNAs. First, 15 ng of total RNA was reverse transcribed using Megaplex™ RT Primers, Human Pool A v2.1 and Megaplex™ and RT Primers and Human Pool B v3.0. Next, cDNAs were pre-amplified using Megaplex™ PreAmp Primers, Human Pool A v2.1 and Megaplex™ PreAmp Primers, Human Pool B v3.0, respectively, according to the manufacturers' instructions. The pre-amplification products were diluted in 75 μl of 0.1 × TE buffer, pH 8.0, and used for the reverse transcription quantitative PCR (RT-qPCR). The PCR reaction mix was prepared using 9 μl of the diluted pre-amplification product, 450 μl TaqMan™ Fast Advanced Master Mix and 441 μl nuclease-free water. Each reservoir of the card was loaded with 100 μl of the PCR mix and centrifuged. RT-qPCR experiments were performed on a ViiA™ 7 Fast Real-Time PCR System (Thermo Fisher Scientific), using the following cycling protocol: enzyme activation on 92°C for 10 min, followed by 40 cycles of denaturation at 95°C for 1 s and annealing at 60°C for 20 s. The reaction volume of each micro-well was 1 μl.

We used the relative threshold (C_{rt}) method, suggested by the microfluidic array analysis guidelines as more robust to analyse

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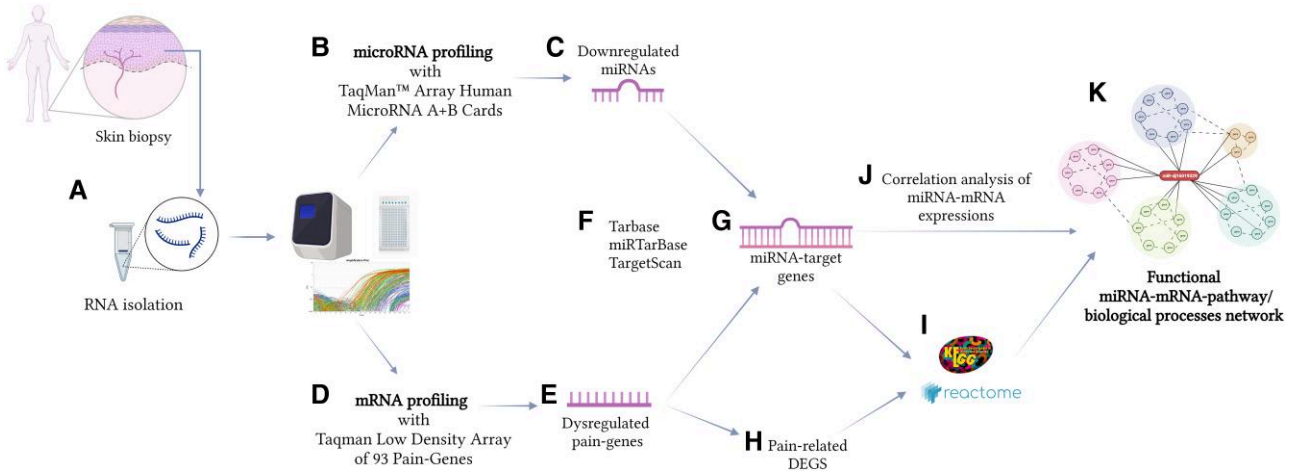

Figure 1 Skin biopsy profiling. Workflow of integrative miRNA and mRNA profiling that allowed miRNA–mRNA pathway network construction. (**A**) The epidermis was dissected from skin biopsy samples and total RNA was isolated. (**B**) The miRNA profiling was performed using Taqman Array Human cards containing 754 miRNAs. (**C**) The bioinformatic pipeline allowed the identification of four significantly downregulated miRNAs. (**D**) Next, from the same RNA sample, mRNA profiling was performed using custom-designed microfluidic TaqMan™ Array Card with 93 pain-related genes. (**E**) This allowed identification of significantly dysregulated genes in the epidermis of 11 NavNP patients compared to 7 HC. (**F**) *In silico* analysis using publicly available databases (TargetScan, miRTarBase and Tarbase) was used to identify miR-30 family, miR-181a-2-3p and miR-203a-3p putative gene targets matched with differentially expressed genes (DEGs). (**G**) Six DEGs were found to be miRNA-target genes, (**H**) while six other DEGs did not emerge as direct gene targets of miR-30 family, miR-181a-2-3p and miR-203a-3p. (**I**) KEGG and REACTOME pathways and biological processes were employed for enrichment analysis to identify pain-related epidermal terms. (**J**) Correlation analysis was used to identify functional relationships between miRNA and target mRNA expression, and was applied to each miRNA and its putative target that was significantly dysregulated in NavNP patients. (**K**) The functional network was reconstructed integrating the obtained data. The workflow scheme was designed using BioRender.com.

microarray data. All raw real-time PCR data were imported into the DataConnect cloud and automatic C_{rt} threshold has been applied in Design and Analysis software (DA2) (Thermo Fisher Scientific, online version). Only miRNAs with good amplification quality (Amplification Score >1 and Cq confidence >0.8) were included in the analysis.

mRNA profiling of pain-related genes

We selected 93 genes that were prioritized by their involvement in neuropathic pain-related pathways (Supplementary Table 2). Gene expression was quantified by custom-designed microfluidic TaqMan™ Array Card (Thermo Fisher, card design 96a). Each card was customized equally and allowed us to analyse four subjects at the time. RNA used for miRNA analysis was reused, a new quality check was performed and all samples that passed quality and quantity standards were analysed. Reverse transcription was performed by InvitrogenTM SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) where 50 ng of total RNA was reverse-transcribed to cDNA. A pre-amplification step with custom pre-amplification pool was then performed for all samples simultaneously. Pre-amplification PCR reaction was performed under following thermal cycling conditions: enzyme activation on 95°C for 10 min, followed by 14 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 4 min. Enzyme was inactivated at 99°C for 10 min. Samples were then diluted in 1 × TE buffer (dilution factor 1:20) and the PCR mix was prepared. Card reservoirs were filled with 100 µl of the prepared mix and ran at ViiATM 7 Fast Real-Time PCR System (Thermo Fisher Scientific) at the following thermal protocol: enzyme activation on 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 s. The reaction volume of each micro-well was 1 µl.

Immunofluorescence analysis of skin biopsy tissue

Nav1.7 immunofluorescence assay

For sodium channel signal intensity examination, we used antibody against Nav1.7 by using free-floating immunofluorescence staining protocol. Briefly, two sections per subject were pre-incubated in the blocking solution [1% bovine serum albumin (BSA), 2% normal goat serum (NGS), 0.5% Triton X-100 in phosphate-buffered saline (PBS)] for 1 h on room temperature. Primary antibody against Nav1.7 (Alomone labs, cat.no. ASC-008, 1:100) to detect keratinocyte immunoreactivity was diluted in 10% of blocking solution and PBS. Sections were incubated overnight at 4°C. Incubation in secondary goat anti-rabbit IgG-Alexa488 antibody (1:1000, Invitrogen) prepared in 10% of blocking solution and PBS followed. Control experiments performed without primary antibody (not shown) and with antibody pre-incubated with corresponding immunizing peptide confirmed antibody specificity (Supplementary Fig. 1).

RNAscope *in situ* hybridization

RNAscope 2.5 Assay-RED (Advanced Cell Diagnostics, ACD) *in situ* hybridization was performed according to the manufacturer's protocol with the human-specific *SCN9A* probe (BioTechne Italy, catalogue number: 562251), utilizing 50-µm thick sections. The tissue was first permeabilized with 0.5% Triton X-100 to allow nuclear staining with DAPI (4′,6-diamidino-2-phenylindole, ThermoFisher) that was performed after RNAscope protocol.

Confocal microscopy and image analysis

Images were captured with a D-Eclipse C1 laser scanning confocal microscope (Nikon) with a 40×/1.3 NA oil immersion objective using a 488 nm Ar/Ar–Kr laser line. Laser power and photomultiplier gain were adjusted to minimize background noise rate and saturated pixel and kept constant for all the acquisitions. By using the dedicated module of Fiji software, we measured fluorescence intensity in the designed region of interest of constant area, selecting three representative regions in each image. Quantification was performed by two independent operators.

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Bioinformatic and statistical analyses

miRNA and mRNA relative expression analysis

In order to select the reference miRNA with minimal intra- and inter-group variations, we employed the NormFinder algorithm.[49] Expression stability ranking identified miR-200c-3p as the most suitable miRNA reference for pool A and miR-625-3p for pool B, respectively.

The geometric mean, calculated considering only genes expressed in 100% of the samples, was used as normalization factor[50,51] for mRNA profiling.

The differential expression of miRNAs and mRNAs was quantified as relative quantitation (RQ) via the 2–ΔΔct approach,[52] with selected endogenous controls or geometric mean for normalization and healthy control samples used as the reference group.

We calculated ΔΔCq as mean ΔCq (miRNA/mRNA of interest in the channelopathy cohort) – mean of ΔCq (miRNA of interest in the reference group). Then, the fold change in expression was calculated as 2–(ΔΔCq). For a reduction of expression in the group of interest with respect to controls we transformed as the negative inverse of 2–(ΔΔCq) to provide with the fold change reduction in expression.[53]

Gene target and pathway enrichment analysis

In order to identify genes that represent putative targets of differentially expressed miRNAs, an *in silico* prediction analysis was performed using TargetScan, miRTarBase and tarbase repositories. Moreover, ClueGO app (v2.5.8) from Cytoscape 3.9.1[54] was applied to identify enriched KEGG/REACTOME pathways and biological processes starting from the lists of miRNA targets and pain-related genes. We performed an overrepresentation analysis based on an enrichment right-sided hypergeometric test that uses Benjamini–Hochberg as multiple testing correction. Enriched terms with a *P*-value <0.05 were considered statistically significant. Pathways involved in peripheral sensation, nociception and neuropathic pain were selected.

Correlation analysis and construction of miRNA–mRNA pathway network

Based on the expression of different miRNAs and mRNAs, Pearson's correlation coefficient was calculated for miRNA-target genes pairs. The normality of expression data was checked by a Shapiro–Wilk test. Non-normal distributed variables were log-transformed. Correlation coefficient ≥ 0.5 or ≤ -0.5, with *P*-value <0.05 was considered statistically significant. Cytoscape (v3.9.1)[54] was used to create and visualize the miRNA–mRNA pathway networks.

Statistical analyses

Comparisons of miRNA/mRNA expression values and immunofluorescence intensities in NAvNP and HC groups were performed according to Wilcoxon rank sum test.

The cut-off for the IFA test was determined by calculating the mean IFA value ± 3 SD in the HC group. The specificity and sensitivity of the test performed on skin biopsy specimens, IFA, was calculated by using the specificity formula TN/(FP + TN) and sensitivity formula TP/(TP + FN), where TN represents true negative, TP true positive, FN stands for false negative and FP false positive.[55,56] Statistical analysis was completed using the Statistical Programming Language R, version 3.6 and STATA11 software.

Data availability

All data are available in the main text or the Supplementary material. The raw data from miRNA and mRNA profiling are deposited in the institutional database and will be publicly available.

Results

Epidermal tissue miRNA profiling in NavNP patients

To reduce the potential variability of miRNAs due to inter-individual differences, we recruited the most phenotypically and genotypically homogeneous cohort of painful neuropathy patients enrolled during the PAIN-Net project (grant agreement number: 721841) in two consortium centres: Fondazione IRCCS Istituto Neurologico Carlo Besta, Milan, Italy (FINCB) and Maastricht University Medical Center+ (Maastricht UMC+), Maastricht, The Netherlands. The study population included 11 patients harbouring previously published GoF *SCN9A* mutations[38-41]: the W1538R, D1908G, V991L/M932L variants were present in different combinations in six patients, one subject additionally carried the I739V and the L1267V was found in five patients (Supplementary Table 1).

Patients had highly similar clinical features, with a length-dependent presentation of neuropathic pain symptoms and signs. All patients reported that first- and second-line analgesic drugs for neuropathic pain[57] did not provide satisfactory pain relief defined as a reduction of pain intensity >50% at the PI-NRS. Detailed clinical information is provided in Table 1.

The skin biopsy tissue used in the present study was collected from all NavNP patients and seven sex- and age-matched healthy individuals during the neurological visit with a 3-mm punch at the distal site of the leg following the standard procedure for the diagnostic assessment of IENFD[43,48] (Fig. 1). The unbiased miRNA profiling on epidermal specimens dissected from fixed skin biopsy sections (Supplementary Table 1) was performed with the microfluidic array containing 754 unique miRNAs hybridized on two card sets (pools A and B). The profiling allowed identification of 510 miRNAs expressed in epidermal tissue with 108 miRNAs present in at least 95% of samples. Because the reference miRNAs in human epidermis have not been previously identified, we used the NormFinder software[58] to determine the miRNAs with the highest rate of stability across all samples. MiR-200c-3p and miR-625-3p emerged as the most suitable normalization controls for pools A and B, respectively. Relative expression analysis (Supplementary Table 2) revealed four miRNAs that were significantly downregulated in NavNP patients compared to HC, surviving the Bonferroni multiple test correction (Fig. 2). Two of them, miR-30a-5p [*P*-value 4.40×10^{-4}, fold change (FC) −4.95] and miR-30d-5p (*P*-value 3.23×10^{-4}, FC −5.83), belong to the same miRNA family (miR-30 family), whereas miR-203a-3p (*P*-value 4.40×10^{-4}, FC −3.64) and miR-181a-2-3p (*P*-value 4.40×10^{-4}, FC −2.21) had no shared origin.

Validation of miRNA-target genes and functional pathway enrichment

Prior to expression analysis, we performed *in silico* analysis using publicly available databases (TargetScan, miRTarBase and Tarbase) to identify miR-30 family, miR-181a-2-3p and miR-203a-3p putative gene targets.

Because miRNAs typically act as context-dependent regulatory elements, we sought to validate the effect of our four miRNAs on

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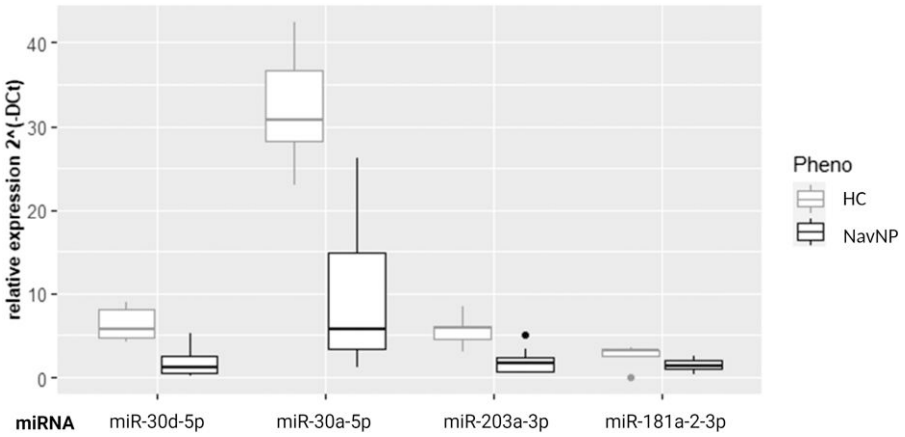

Figure 2 Downregulated miRNA in epidermis of painful SCN9A-related neuropathy patients (NavNP). Microfluidic analysis of miRNA profiling in total RNA extracted from the epidermis of 11 NavNP patients and 7 healthy controls (HC) demonstrated a significant reduction of miR-30d-5p (*P*-value 3.23×10^{-4}, FC −5.83), miR-30a-5p [*P*-value 4.40×10^{-4}, fold change (FC) −4.95], miR-203a-3p (*P*-value 4.40×10^{-4}, FC −3.64), miR-181a-2-3p (*P*-value 4.40×10^{-4}, FC −2.21) expression in NavNP patients compared to HC. Bar graph indicates the $2^{-(\Delta\Delta Cq)}$. The comparisons are made applying Wilcoxon rank sum test and corrected for Bonferroni multiple test. The fold change in expression was calculated as $2^{-(\Delta\Delta Cq)}$. We provide the fold change reduction in expression in the NavNP group compared to HC applying the negative inverse of $2^{-(\Delta\Delta Cq)}$.

in silico predicted targets in epidermal microenvironments, utilizing a custom-designed microfluidic array containing 93 pain genes. Intraepidermal nociceptors interact with surrounding cells, mainly keratinocytes, to detect and transduce thermal, mechanical or chemical stimuli.[59] This crosstalk has been suggested to be essential for evoking pain perception and activation of appropriate protective behaviours.[7,9,12,13,60-62] Therefore, we designed the microfluidic array hybridized with mRNA probes of genes that are implicated in cutaneous nociception and keratinocyte–intraepidermal sensory nerve crosstalk (Supplementary Table 3). Epidermal mRNA profiling was performed in all the subjects (i.e. 11 NavNP and 7 HC) and allowed identification of six differentially expressed genes (DEGs) in NavNP patients considering nominal *P*-values < 0.05 among which five were downregulated (*ACAP2*, *EDN1*, *GSK3B*, *NEDD4*, *SOX5*) and one was upregulated (*MEF2C*). All these DEGs were *in silico* predicted miRNA-targets (Table 2, Supplementary Table 4 and Fig. 3A).

Over-represented KEGG and REACTOME pathways and biological processes were investigated, allowing us to find significantly enriched terms (Table 2).

Pairwise Pearson correlation was used to identify the functional relationships between miRNA and target mRNA expression (Supplementary Table 5). The correlation analysis was applied to each miRNA and its putative target that was significantly altered in NavNP patients (Table 2 and Fig. 3B). Our analysis showed correlations between expression values of *NEDD4*, miR-30a-5p ($r = 0.52$) and miR-30d-5p ($r = 0.5$), whereas miR-181-2-3p expression correlated with *EDN1* ($r = 0.6$) and *GSK3B* ($r = 0.71$) and miR-203-3p with *GSK3B* ($r = 0.52$), *MEF2C* ($r = 0.51$) and *SOX5* ($r = 0.69$).

Integration of pain-related mRNA profiling

Pain gene expression profiling, independently of miRNA target validation, allowed the comprehensive analysis of broad spectrum of pain-related transcripts (Supplementary Table 3) and the identification of six additional DEGs (*IKBKAP*, *UQCR11*, *TRPV1*, *CNTRL*, *IL1R2* and *NTRK2*) that have not emerged as *in silico* predicted miRNA targets (Supplementary Table 4), but are found to be key genes in pain-related processes.

miRNA–mRNA pathway functional network analysis

To construct the functional network, we integrated the four dysregulated miRNAs and all the DEGs found with the *in silico* predicted pain-related pathways in NavNP patients (Fig. 4). The predicted pathways and biological processes were clustered into four groups according to their involvement in peripheral pain signalling as pain transduction, transmission, modulation and post-transcriptional modification.

Network analysis showed the interactions between the identified miRNAs and related mRNAs in the epidermis of NavNP patients. They included a relationship between the miR-30 family and downregulated *NEDD4* expression, whose downregulation in neuronal tissues was previously associated with neuropathic pain.[63] *ACAP2*, a transcription factor involved in neurite outgrowth[64] and vesicle transport,[65] was significantly downregulated in NavNP patients with no significant correlation with the miR-30 family even though it is an *in silico* predicted gene target. Its regulation might be related to a yet unknown additional context-related factor. We found that miR-181a-2-3p expression was correlated with downregulated *EDN1* that has been reported to be involved in hyperalgesia[66] and *GSK3B* that has been associated with neuropathic pain conditions and nerve regeneration.[67-69] The expression of miR-203-3p correlated with upregulated *MEF2C* that was reported to be involved in neuronal cell maintenance and pain perception,[70] and with downregulated *GSK3B* and *SOX5* that are involved in peripheral nerve integrity and chronic pain.[71,72] Besides these miRNA-target genes, pathways and biological processes were further connected with five pain-related DEGs, including *TRPV1*, *NTRK2*, *IL1R2*, *IKBKAP* and *CNTRL*, each differently contributing to several pain signalling functions.[73-79]

This miRNA–mRNA pathway network illustrates the complex molecular signature of targeted epidermal tissue in NavNP patients, suggesting that miRNA fine tuning goes beyond the effect on the putative gene target, indirectly altering the expression of other key genes interacting in pain-related pathways. These findings are in keeping with previous *in vitro* studies highlighting the functional role of epidermis in conveying noxious stimuli *via* synaptic-like contacts with intraepidermal nerve fibres.[9,11]

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Table 2 Differentially expressed genes in epidermis of NavNP patients matched with peripheral sensitization pathways

Gene symbol	P-value	Benjamini–Hochberg FDR	FC	miRNA	GO Term
NEDD4	0.0012	0.020	−1.78	hsa-miR-30a-5p, hsa-miR-30d-5p	Cellular component organization, cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to stress, generation of neurons, intracellular signal transduction, intracellular transport, ion channel transport, negative regulation of nitrogen compound metabolic process, negative regulation of response to stimulus, negative regulation of signal transduction, regulation of cell communication, regulation of cellular component organization, regulation of cellular process, regulation of gene expression, regulation of nitrogen compound metabolic process, regulation of signal transduction, signal transduction, stimuli-sensing channels, transport of small molecules, vesicle-mediated transport, protein ubiquitination, ion channel transport
GSK3B	0.0012	0.020	−1.79	hsa-miR-203a-3p, hsa-miR-181-2-3p	Cell–cell signalling, cellular component organization, cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to stress, cytoskeleton organization, generation of neurons, intracellular signal transduction, intracellular transport, negative regulation of nitrogen compound metabolic process, negative regulation of response to stimulus, negative regulation of signal transduction, regulation of cell communication, regulation of cellular component organization, regulation of gene expression, regulation of nitrogen compound metabolic process, response to nitrogen compound, response to oxygen-containing compound, signal transduction, synaptic signalling, trans-synaptic signaling, vesicle-mediated transport, axon guidance, neurotrophin signalling pathway
EDN1	0.0041	0.046	−2.13	hsa-miR-181a-2-3p	Cell–cell signalling, cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to stress, cellular response to transforming growth factor beta stimulus, cytoskeleton organization, generation of neurons, intracellular signal transduction, negative regulation of nitrogen compound metabolic process, negative regulation of response to stimulus, regulation of cell communication, regulation of cellular component organization, regulation of gene expression, regulation of nitrogen compound metabolic process, regulation of signal transduction, response to nitrogen compound, response to oxygen-containing compound, signal transduction, synaptic signalling, TNF signalling pathway, transcription regulator activity, trans-synaptic signalling
SOX5	0.0041	0.046	−1.81	hsa-miR-203a-3p	Cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to growth factor stimulus, cellular response to transforming growth factor beta stimulus, generation of neurons, regulation of cellular process, regulation of gene expression, regulation of nitrogen compound metabolic process, response to transforming growth factor beta, transcription regulator activity
MEF2C	0.0204	0.150	1.76	hsa-miR-203a-3p	Cell–cell signalling, cellular component organization, cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to stress, cellular response to transforming growth factor beta stimulus, generation of neurons, intracellular signal transduction, negative regulation of nitrogen compound metabolic process, regulation of cell communication, regulation of cellular component organization, regulation of gene expression, regulation of nitrogen compound metabolic process, regulation of signal transduction, response to nitrogen compound, response to oxygen-containing compound, signal transduction, synaptic signalling, transcription regulator activity, trans-synaptic signalling, signalling by NTRK1 (TRKA)
ACAP2	0.0332	0.199	−1.29	hsa-miR-30a-5p, hsa-miR-30d-5p	Cellular response to chemical stimulus, cellular response to endogenous stimulus, cellular response to growth factor stimulus, cellular response to nerve growth factor stimulus, intracellular transport, response to nerve growth factor, vesicle-mediated transport

MicroRNAs targeting these genes are also reported. The table shows altered target genes (*NEDD4*, *GSK3B*, *EDN1*, *SOX5*, *MEF2C* and *ACAP2*) associated with the enriched terms. FC = fold change; GO = gene ontology.

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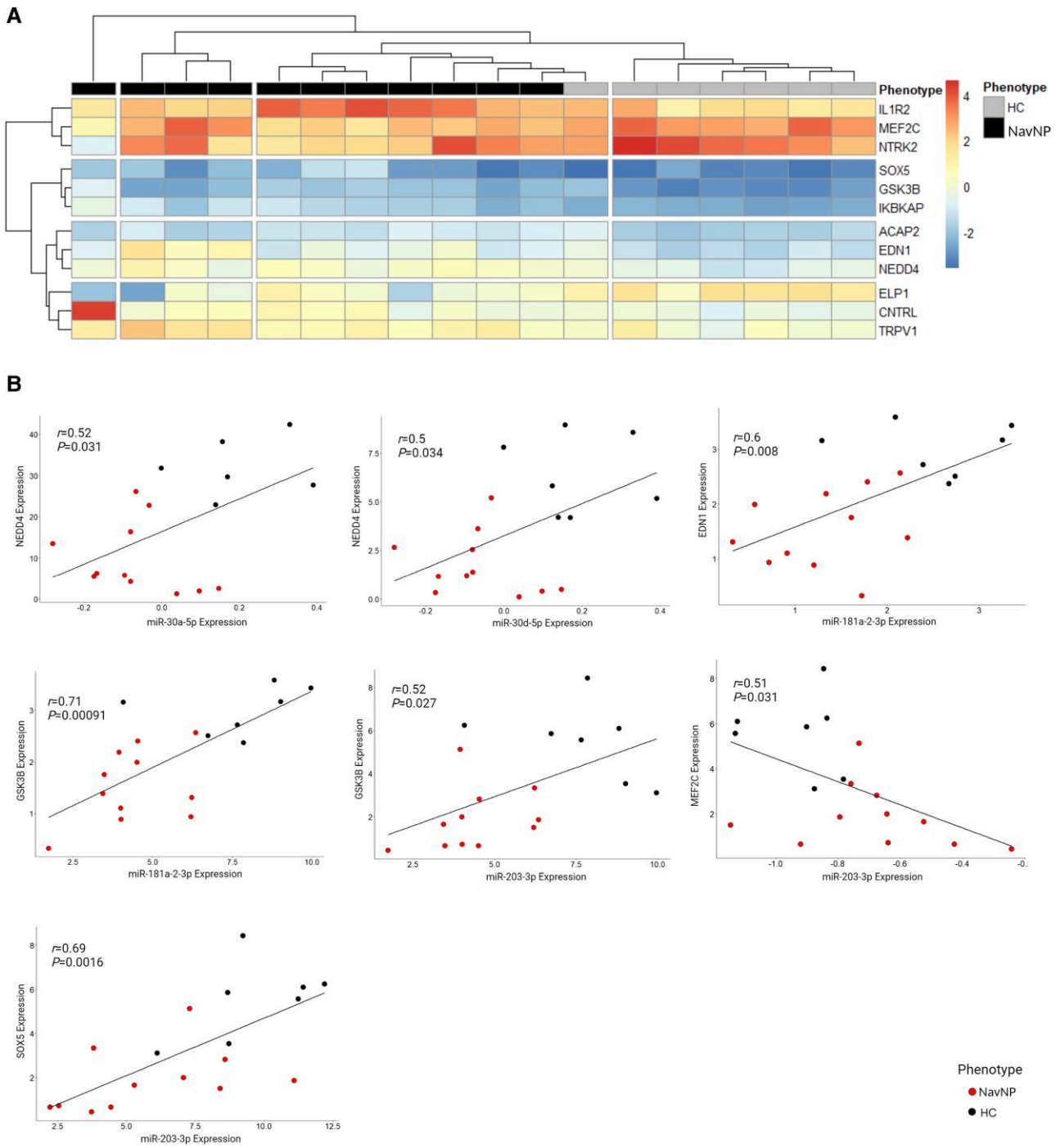

Figure 3 miRNA target prediction and gene expression validation. (**A**) Unsupervised heat map of the ΔCq values, the difference between the tested gene and geometric mean as normalization factor, of significantly dysregulated miRNA-targets in NavNP patients (black) compared to HC (grey), highlighting the two separated phenotypic classes. Heat map colours correspond to mRNA expression as indicated in the colour key: red (lower expression) and blue (higher expression). (**B**) Scatter correlation plots between miRNAs and their gene targets. Pearson coefficient and *P*-value are shown in the graphs. NavNP patients are coloured in red and HC in black showing a different expression distribution between phenotypic groups.

miR-30 family interaction in the epidermis of NavNP patients

Evidence from preclinical models suggested a role of the miR-30 family as promising regulators of neuropathic pain mechanisms,[80-83] also suggesting their direct involvement in the regulation of peripherally expressed VGSC subunits.[81,84,85] Our integrative miRNA–mRNA profiling identified *NEDD4* as a potentially important player in the epidermal microenvironment. *NEDD4* is known to participate in the regulation of axonal growth, neuronal hyperexcitability[63,86] and Nav1.7 modulation.[63,87-89] To investigate its effect on Nav1.7, which has been previously proposed as a potential biomarker for painful neuropathy,[90] we performed IFA in skin biopsy sections from 11 NavNP patients carrying W1538R, L1267V, D1908G and/or V991L/M932L haplotypes and in 18 HC (Supplementary Table 7). Remarkably, Nav1.7 signal intensity was significantly increased in patients' keratinocytes as compared to HC (*P*

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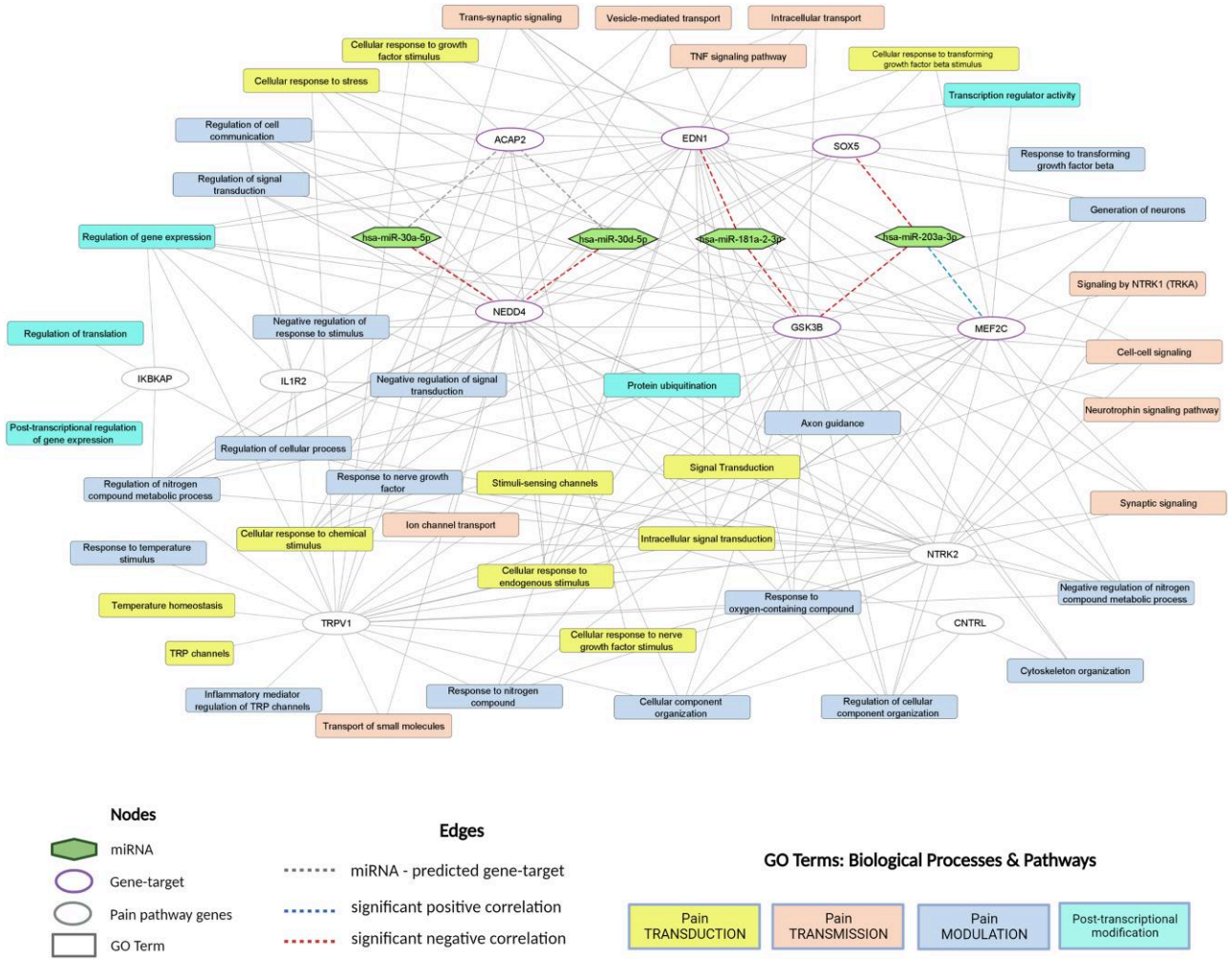

Figure 4 miRNA–mRNA pathway functional network. Experimental miRNA and mRNA profiling data together with *in silico* terms enrichment are used to reconstruct functional biological network in the epidermis of NavNP patients. Four downregulated miRNAs (green hexagon) are paired with their gene targets (white ellipse with purple edge). Significant correlations between miRNA–mRNA pairs are represented with red and light blue dotted lines. All genes were then mapped within pain-related terms, grouped into four main classes, defined according to the scientific literature, as follows: (i) transduction (yellow rectangle); (ii) transmission (light orange rectangle); (iii) modulation (light blue rectangle); and (iv) post-transcriptional modification (cyan rectangle). Dysregulated genes that are not miRNA targets are represented with white ellipse with grey edge and are paired with pain-related pathways. The network represents the complex interactions occurring in the epidermis, related to pain signalling.

$= 8.798 \times 10^{-5}$, interobserver agreement $P < 0.001$), with a sensitivity of 81.8% and a specificity of 100%. Moreover, it negatively correlated with *NEDD4* expression and both members of miR-30 family (Fig. 5C–E).

To test the possibility that Nav1.7 expression was related to patients' *SCN9A* genetic background due to the presence of quantitative trait locus (eQTL), we interrogated the GTEx Portal database (released on 7 July 2022) and found that none of the genetic variants carried by NavNP patients represents an eQTL, making unlikely the possibility that Nav1.7 immunoreactivity was related to patients' genetic background. Finally, using RNAscope we confirmed the localization of *SCN9A* mRNA in the epidermis of NavNP patients (Supplementary Fig. 1). Therefore, the increased Nav1.7 signalling intensity in keratinocytes found in NavNP patients might be induced by post-transcriptional and/or post-translational mechanisms.

Discussion

Our findings demonstrate substantial molecular differences between NavNP patients and healthy individuals and provide clues that strengthen the role of altered pain signalling in the skin of patients carrying pathogenic variants in *SCN9A*, which could contribute to future drug development and innovative clinical trial design. Moreover, we generated robust protocols for molecular profiling of skin biopsy utilized for diagnostic assessment of IENFD that may widen the possibility to prospectively investigate individual patients, while also allowing additional examination on already collected specimens. This latter approach may facilitate correlation of individual molecular differences and responsiveness to treatments among patients enrolled in clinical trials.

The identification of four downregulated miRNAs (i.e. miR-30a-5p, miR-30d-5p, miR-181a-2-3p, miR-203a-5p) in the epidermis of NavNP patients provides the first robust biomarkers describing the complex pain signalling in targeted human tissue, potentially bridging the gap with the preclinical findings. Indeed, previous studies suggesting the role of the miR-30 family in regulating peripherally expressed VGSC subunits and related pathways focused only on rodent models.[80,81,83–85] We demonstrated, through functional network analysis, that the miR-30 family is required for regulation of expression of well-known pain therapeutic targets in human epidermis. However,

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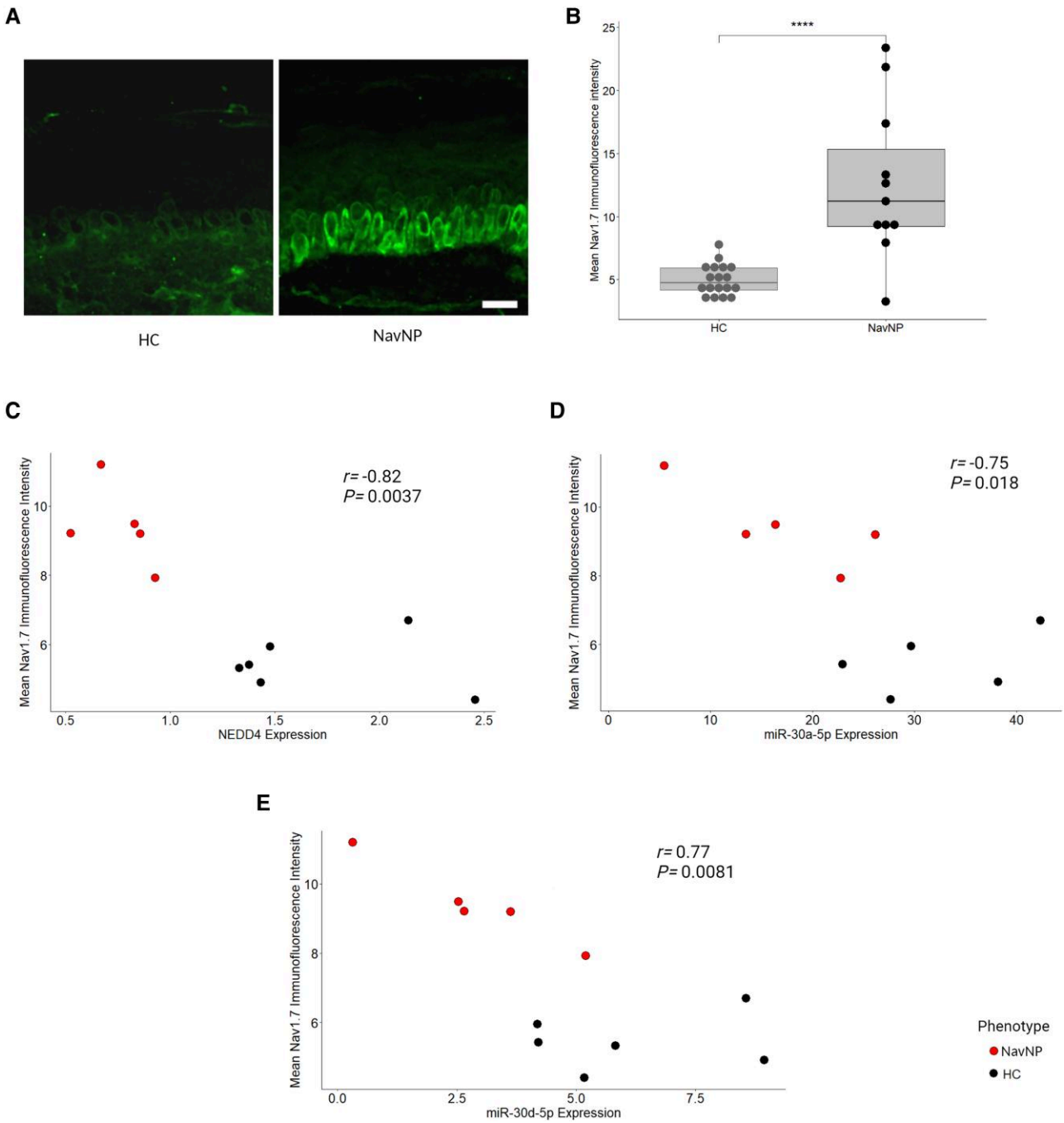

Figure 5 miR-30 family regulates Nav1.7 signalling in keratinocytes. (**A**) Representative confocal microscope image of epidermis. Nav1.7 (green) in keratinocytes of NavNP patients and HC. Scale bar = 20 µm. (**B**) Boxplot of mean Nav1.7 immunofluorescence intensity for two studied groups, NavNP patients and HC, respectively. NavNP patients show significantly higher Nav1.7 signal intensity than HC ($P = 8.798 \times 10^{-5}$). ***$P < 0.001$ according to Wilcoxon rank sum test. (**C**) Scatter correlation plot between mean Nav1.7 immunofluorescence intensity and *NEDD4* expression values in NavNP patients and HC samples. (**D** and **E**) Scatter correlation plot between mean Nav1.7 immunofluorescence intensity and (**D**) miR-30a-5p and miR-30d-5p (**E**) expression values in NavNP patients and HC samples. Spearman coefficient and *P*-value are shown in the graph.

synergy with the other two newly identified miRNAs (i.e. miR-181a-3p and miR-203a-5p) may be required for the modulation of pain-related pathways, rather than single gene targeting, which could be relevant for future development towards precision neuropathic pain medicine.

Our analysis revealed a correlation between miR-30 family and *NEDD4* that is especially interesting in the context of known interactions of *NEDD4* with Nav1.7 and other sodium channels.[87,88] Indeed, the *NEDD4* family has been reported to participate, along with other enzymes, in clathrin-mediated Nav1.7 endocytosis[89] and to be essential for Nav1.7 dowregulation.[63] Preclinical studies in models of neuropathic pain have suggested that targeting Nav1.7 regulation through the modulation of this pathway may attenuate neuronal hyperexcitability and nociceptor hypersensitivity.[63,91,92]

mRNA profiling allowed the validation of the identified targets, and experimental data were used to reconstruct the miRNA–mRNA network and to highlight dysregulated mRNAs in NavNP

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patients. This discovery demonstrates the complexity of signalling interactions in epidermal tissue and its potential role in neuropathic pain generation and maintenance *via* crosstalk with IENF,[10] which express Nav1.7[93] and other sodium channel subtypes.[94]

Analysis of Nav1.7 subunit protein level in NavNP patients yielded results in keeping with previously published studies showing high immunofluorescence signal intensity in keratinocytes of subjects with neuropathic pain.[90,95] The negative correlation between Nav1.7 immunofluorescence and *NEDD4* and miR-30 family expression suggests a possible interaction in the epigenetic fine tuning at post-translational level.

Basic research on miRNA biogenesis and action led to discoveries of non-canonical miRNA processing, highly context-dependent, suggesting their active role in regulating transcription and translation.[96,97] For instance, during alternative miRNA-activated binding, translation can yield an increase of protein expression. This event occurs during cell quiescence and is dependent on base-pairing in the presence of Argonaute 2 (*Ago2*) and fragile X mental retardation–related protein 1 (*FXR1*).[98] Epidermal cells such as keratinocytes have the capability to enter the quiescence state of proliferative arrest, and to maintain regulated re-entry into cell cycle as part of their regenerative functioning in tissue repair and immunological responses.[99,100] Indeed, Nav1.7 increased immunoreactivity is restricted to basal layer keratinocytes, which are known for their ability to enter the quiescent state.[101] Non-canonical miRNA binding might drive this phenomenon. Further functional studies are necessary to elucidate the mechanisms underlying Nav1.7 upregulation in epidermal cells and the relationship with miRNA candidates, as well as comprehensive proteomic profiling that would provide other miRNA–protein functional pairs characterizing the cutaneous pain signalling in sodium channel–related peripheral neuropathy.

Taken together, these results support the hypothesis of epidermis-targeted fine-tuning of neuropathic pain signalling involving druggable targets. Further proteomic profiling of human epidermis and miRNA–mRNA protein interaction may unravel mechanisms of protein translation essential for future drug development studies. This study was performed in a small cohort of patients harbouring *SCN9A* pathogenic variants, thus limiting the generalization of the findings. However, the highly homogeneous characteristics and pain-related genetic background of our study population allowed us to overcome the limitations of the heterogeneity of neuropathic pain patients and of inter-individual differences of miRNA profile. Further miRNA mimicking combined with electrophysiological assay would strengthen the functional role of candidate miRNAs in NavNP patients, which would require patient-derived 3D skin model. However, our data support the role of *NEDD4* and other genes as major players for modulating neuropathic pain pathways in human skin.

While selective sodium channel blockers are new promising treatments,[102] our results suggest additional targeted molecules for neuropathic pain drug development. We speculate that topical administration of miRNA designed cocktail mimicking the four miRNA candidates (i.e. miR-30a-5p, miR-30d-5p, miR-181a-2-3p and miR-203a-5p) might be considered as a potential candidate. miRNA therapeutics represent a cutting-edge drug design technology with highly specific target binding, potentially improving drug efficacy and reducing systemic side effects, which are a major cause of withdrawal especially in neuropathic pain.

In summary, we used human pain-related integrative miRNA and mRNA molecular profiling of the epidermis, the host tissue of the distal ends of small nerve fibres, to study a deeply phenotyped cohort of *SCN9A*-related painful neuropathy patients. We identified four miRNAs strongly discriminating patients from healthy individuals and confirmed their effects on differentially expressed gene targets involved in nociception pathways. These findings pinpoint the complex epidermal miRNA–mRNA network based on tissue-specific experimental data, supporting the idea of a cross-talk between epidermal cells and axons, modulated by pain-related molecular signatures. This targeted molecular profiling approach advances understanding of specific neuropathic pain fine signatures and may accelerate the process towards personalized medicine for neuropathic pain. Moreover, the molecular fingerprint of specific subgroups of patients could guide the design of clinical trials to improve recognition of individual endotypes and potentially increase the rate of responders.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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