Nociceptin/orphanin FQ opioid peptide-receptor expression in pachyonychia congenita

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Nociceptin/orphanin FQ opioid peptide (NOP)-receptor (NOP-R) is a member of the opioid receptor family. NOP-R activation has demonstrated analgesic effects in preclinical pain models without the addiction risks associated with other opiate targets. Pachyonychia congenita (PC) is a palmoplantar keratoderma characterized by neuropathic pain in affected skin. A cohort of KRT6A gene mutation PC patients with no other explanation for their neuropathic pain offered a unique opportunity to assess potential of NOP-R as a therapeutic target. Plantar biopsies from 10 PC patients and 10 age/gender matched controls were performed at the ball (PC-affected) and the arch (PC-unaffected) of the foot. NOP-R expression was assessed by immunohistochemistry. Localization of NOP-R in subsets of epidermal nerve fibers was investigated using the pan-neuronal marker PGP9.5, markers for unmyelinated peptidergic fibers (calcitonin gene-related peptide [CGRP] and substance P [SP]), as well as for myelinated Aδ and Aβ fibers (neurofilament H [NFH]). Robust NOP-R expression was detected in epidermal keratinocytes and in a subset of PGP9.5+ fibers in both epidermis and dermis, confirmed by western blot and absorption experiments with NOP-R peptide. NOP-R expression in keratinocytes was significantly reduced in PC-affected plantar skin compared with PC-unaffected skin. In addition, NOP-R expression occurred in dermal NFH+ myelinated fibers in all groups, although few CGRP+ fibers co-expressed NOP-R. Furthermore, most SP+ fibers also co-expressed NOP-R. These findings indicate that NOP-R is expressed on epidermal keratinocytes, as well as on epidermal and dermal nerve fibers and has potential as a promising target to treat neuropathic pain in PC.

KEYWORDS
dermis, epidermis, neuropathic pain, nociceptin/orphanin FQ opioid peptide (NOP) receptor, pachyonychia congenita

1 | INTRODUCTION

Nociceptin/orphanin FQ opioid peptide (NOP)-receptor (NOP-R) is a member of the opioid receptor family. Several NOP-R agonists have demonstrated anti-nociceptive and anti-hypersensitive effects in experimental pain models. Activation of the NOP-R by its endogenous peptide ligand nociceptin/orphanin FQ (N/OFQ) or non-peptide agonists has been shown to induce potent anti-hypersensitive effects in rodent models of neuropathic pain and anti-nociceptive effects in non-human primate models of acute and inflammatory pain. Moreover, NOP-R does not bind classic opioid ligands, and its endogenous peptide ligand N/OFQ has low affinity for the three classic opioid receptors: μ, δ, and κ. NOP-R activation induced analgesia is not naloxone reversible. Therefore, NOP-R and its agonists hold potential as neuropathic pain treatments without the addiction risk of conventional opioid drugs. The localization and functional plasticity of the N/OFQ-NOP-R system at multiple sites of the pain pathway including the dorsal root ganglion (DRG), superficial dorsal horn of the spinal cord, sensory trigeminal complex, and periaqueductal gray is in line with the system’s well-described role in modulating nociceptive signaling in a site, pain state, and species-dependent manner (reviewed in Schröder et al, 2014). However, the distribution and...
function of the N/OFQ-NOP-R system in the periphery have been less well characterized, and study in human tissue is limited.

Pachyonychia congenita (PC) is an autosomal dominant palmo-plantar keratoderm affecting the palms of the hands and soles of the feet. It is caused by a mutation in any one of the keratin genes, KRT6A, KRT6B, KRT6C, KRT16, or KRT17. Focal plantar hyperkeratosis, thickened nails, oral plaques, and intense pain (in the feet) are the distinct characteristics of PC. Pressure on affected areas of the soles is painful, often causing patients to limit their activity. Mechanisms of pain in PC have recently been elucidated with pathologic, functional, and questionnaire studies supporting that pain in PC is neuropathic in nature. With the phenotype of chronic pain and focal hypersensitivity in affected skin, PC is an attractive model system to study expression of NOP-R distribution and assess whether targeting NOP-R has potential for the treatment of neuropathic pain. In the present study, we rigorously characterize NOP-R expression in human plantar skin and then assess whether it is altered in PC-affected plantar skin. All subjects studied possessed the KRT6A mutation and offered a homogenous population that is well suited for such purposes.

2 | MATERIALS AND METHODS

2.1 | Patients

Ten subjects (50% female) with genetically confirmed PC (KRT6A mutations) and 10 control subjects (50% female) underwent 3 mm plantar skin punch biopsies at the level of the tarsal/metatarsal joint and at the arch. Demographic information for the subjects is provided in Table 1. The tarsal/metatarsal region contained affected skin in PC subjects while the arch biopsy contained unaffected epidermis. Control subjects underwent biopsies at both locations in order to address any potential anatomic differences. All biopsies were obtained after local anesthesia with subcutaneous 0.5 cc 2% lidocaine with epinephrine. PC subjects were part of an institutional review board (IRB)–approved protocol through the International Pachyonychia Congenita Research Registry (western institutional review board (WIRB) #20040468). Control subjects underwent biopsies as part of a Johns Hopkins-approved protocol. Both PC and control subjects had no known risk factors or symptoms for peripheral neuropathy, and normal peripheral nerve examinations including vibration threshold, pin sensation, reflexes, light touch, and von Frey filament assessment (all detected using a 0.4-g filament).

2.2 | Immunohistochemistry

Skin samples were processed for immunohistochemical analysis using an established protocol. Briefly, skin biopsies were fixed in Zamboni’s fixative overnight, followed by a phosphate-buffered saline rinse. Samples were incubated in cryoprotectant overnight. Specimens were sectioned perpendicular to the skin surface at 50 μm intervals. Three to four sections were immunohistochemically stained for each marker using single or combined immunofluorescence to reveal patterns of coexpression/distribution: rabbit anti-KOR-3 (NOP receptor, Santa Cruz Biotechnology, Santa Cruz, CA, sc-15309, 1:100), goat anti-KOR-3 (NOP receptor, Santa Cruz Biotechnology, sc-9760, 1:100), mouse anti-β3 (Bio-Rad, Hercules, CA., 47850, 1:2500), mouse anti-calcitonin gene-related peptide (CGRP) (Millipore, Burlington, MA., MAB15360, 1:1000), guinea pig anti-substance P (SP, Abcam, Cambridge, MA, AB10353), chicken anti-NF200 (Avex Lab, Tigard, OR. Catalog #: NFH, 1:2000). Antibody details and purposes are shown in the Table S1, Supporting Information. For combined immunostaining, the primary antibodies used were raised in different species, and detected with species-specific secondary antibodies raised in donkey conjugated with fluorescent dyes, Cy2, Cy3, or Cy5 (all at 1:300 dilution; Jackson Immunoresearch, West Grove, Pennsylvania). The common nuclear dye DAPI was used for counterstaining (Thermo Scientific, Grand Island, NY., Cat # D-1306). PGP9.5 was used as a panaxonal marker. Large-myelinated fibers, including those forming touch complex and Meissner corpuscles, were identified by their immunoreactivity to neurofilament H (NFH). Peptide-containing unmyelinated sensory fibers were identified by immunoreactivity to CGRP or SP. Control experiments included omission of primary or secondary antibody and yielded no immunoreactivity for all the above markers. In order to demonstrate that keratinocyte NOP-R staining was specific, pre-absorption of NOP-R antibody (Santa Cruz Biotechnology, sc-9760) with its specific blocking peptide (Santa Cruz Biotechnology, sc-9760 p, 1:20) was performed, resulting in loss of staining (see Figure 1B). Immunohistochemical staining against NOP-R was also performed on human lumbar DRG to further characterize neuronal expression (Figure 1D).

2.3 | Western blot

Fresh human skin biopsies were obtained and homogenized using a glass-made microhomogenizer in extraction buffer (T-PER reagent; Thermo Scientific, Grand Island, NY.) containing protease inhibitor mixture. Protein concentrations were determined using Pierce BCA protein assay Kit. Samples (20 μg) were loaded into lanes of a SDS polyacrylamide gel 4% to 15% (Bio-rad) and run at 95 V for 2.5 hours. Proteins were transferred to PVDVF membrane (Bio-rad) and probed with rabbit anti-KOR-3 (catalog # sc-15309) and goat anti-KOR-3 (catalog # sc-9760) antibodies (both from Santa Cruz at 1:1000 dilution).
2.4 | Imaging and quantitative analysis

Images were obtained using a Zeiss Z1 confocal microscope equipped with selective optical filters. Sequential scanning was performed to prevent bleed-through of the different fluorophores. Confocal fluorescence microscopy was used to assess colocalization and patterns of expression of two or more markers. Because NOP-R labeling is relatively homogenous in the epidermis, we applied a technique of unbiased sampling. Briefly, immunostaining was performed under a uniform protocol on three sections per biopsy, and all images were captured at the same exposure condition and were processed with Zeiss Zen 2012 software for measuring signal intensity in a 50 × 40 μm box (~2000 μm²), at random about every third optical field through the whole length of each evaluated section. Comparisons of NOP-R staining intensity were made between control and PC groups, and within the PC group between affected and unaffected skin areas.

2.5 | Statistics

Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, California). Group averages for other measurements are presented as mean ± SEM. Comparisons between PC-affected and PC-unaffected samples was performed by paired Wilcoxon non-parametric tests and Mann-Whitney rank sum analysis was used for control and PC comparisons. PC-affected skin was compared to anatomically-matched biopsies (ball of the foot) from healthy controls while PC-unaffected skin from the arch was compared to arch samples from control subjects.

3 | RESULTS

3.1 | Study subjects

PC subject age range was 19 to 71 years (49.0 ± 15.4 years) while controls were 28 to 62 years (46.6 ± 10.7). Each group was
composed of five men and five women. All PC subjects possessed KRT6A mutations. Out of ten PC subjects, nine reported pain that affected their daily life and limited walking. Foot pain had prompted 7 of 10 to purchase special shoes or make modifications to their shoes. Standing, walking or heat made pain worse in 8 of 10 subjects.

### 3.2 | NOP-R expression in epidermis and DRG

Robust NOP-R expression was detected in epidermal keratinocytes throughout the whole epidermis in a relatively homogenous pattern (Figures 1A and 2A). Staining was nearly completely inhibited when NOP-R antibody was pre-incubated with blocking peptide (Figure 1B) or when primary antibody was omitted (not shown) indicating that the immunohistochemical staining pattern was specific. Western blot analysis of homogenized fresh frozen human plantar skin demonstrated that both NOP-R antibodies recognized a band at the expected molecular weight of the NOP-R (Figure 1C). Staining of human DRGs demonstrated NOP-R was expressed on small, mid, and large diameter neurons (Figure 1D).

### 3.3 | Correlation of NOP-R expression and epidermal innervation

We assessed the relationship between NOP-R expression and intraepidermal nerve fiber density, and found weak correlations (Pearson correlation coefficient is 0.009 and 0.245 for the PC-unaffected and PC-affected skin, respectively).

### 3.4 | NOP-R expression in keratinocytes and nerve fibers in PC-affected vs PC-unaffected skin

Double immunohistochemical staining for PGP9.5 and NOP-R demonstrated that a subset of PGP9.5+ fibers in both epidermis (Figure 2A) and dermis (Figure 2D) were also NOP-R positive in normal plantar skin. Quantitative analysis revealed that epidermal keratinocytes in PC-affected skin had significantly lower NOP-R expression than in PC-unaffected skin (Figure 3). Double immunofluorescence with NOP-R and NFH (a marker for Aβ fibers) antibodies showed that nearly all NFH+ nerve fibers in upper dermis also expressed NOP-R in both PC-unaffected and PC-affected skin (Figure 4). In contrast, colocalization of NOP-R with peptidergic nerve fiber markers (CGRP or SP) was only found in a small subset of CGRP+ fibers (Figure 5A-D). SP+ fibers are rare in plantar skin but nearly all expressed NOP-R (Figure 5E-H). This co-localization pattern was similar between PC-unaffected and PC-affected as well as control plantar skin.

### 4 | DISCUSSION

Patients with peripheral neuropathy often complain of neuropathic pain that preferentially affects the soles of the feet. The discomfort is aggravated by prolonged standing or walking and often interferes with sleep. PC is a palmoplantar keratoderma characterized by callus development and severe pain and sensitivity to pressure in affected areas. While PC is perceived as a dermatological condition, neuropathic pain in affected regions is patients’ largest complaint affecting quality of

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**FIGURE 2**  Nociceptin/orphanin FQ opioid peptide-receptor (NOP-R) expression in epidermis and dermis. Epidermal cells (keratinocytes) and subsets of PGP9.5 labeled nerve fibers in epidermis and dermis are NOP-R positive. Double immunostaining of NOP-R (rabbit anti-KOR-3, sc-15309, red) and PGP9.5 (green) shows subsets of PGP9.5 labeled fibers are NOP-R positive in the epidermis (arrow in A-C) and upper dermis (arrows in D-F). DAPI counterstain is blue. Scale bars = 30 μm
We recently demonstrated that affected PC patient plantar skin had neuropathological alterations including reduction in epidermal innervation and an increase in Merkel cell density compared to PC-unaffected and healthy control skin. In the current study, we demonstrate that NOP-R is abundantly expressed in human plantar epidermis, localized primarily to keratinocytes, but also to epidermal and dermal nerve fibers including large-myelinated fibers (NFH+) and small unmyelinated peptidergic fibers (CGRP+). There was a small, but statistically significant reduction in NOP-R immunoreactivity in PC-affected skin compared to PC-unaffected skin or anatomically-matched control skin. Taken together, these findings have implications for treatment of neuropathic pain in PC.

We rigorously assessed NOP-R expression in human skin. There was abundant staining in epidermal keratinocytes as well as nerve fibers in both the dermis and epidermis. The specificity of immunohistochemistry staining was confirmed through western blots using two separate antibodies, as well as through absorption experiments and the control experiments omitting the primary antibody. The observation that NOP-R is abundantly expressed in keratinocytes is noteworthy and is consistent with the growing literature that keratinocytes play a dynamic role in sensation and not a simple supportive/structural role. The pattern of NOP-R expression was observed in both PC patients and controls. Previous reports have shown that NOP-R and N/OFQ is expressed in mouse epidermal keratinocytes while preliminary human studies suggest there is NOP-R expression in human epidermal keratinocytes of healthy subjects. We have extended this observation to plantar skin and formally quantified NOP-R expression demonstrating that keratinocyte NOP-R expression was significantly reduced in PC-affected keratinocytes compared to either PC-unaffected keratinocytes or control skin. The reduction in NOP-R in PC-affected skin was not correlated with the decrease in epidermal innervation suggesting that this finding is not an indirect consequence of reduced innervation. These findings provide insight into the neuropathic pain that these patients experience as the reduced NOP-R expression could make PC patients less responsive to endogenous N/OFQ.

We selected patients with PC possessing defined KRT6A mutations in order to assess potential alterations in NOP-R expression in a neuropathic pain population. On the surface this may appear to be a curious choice, as PC is an ultra-rare disease that has recently been appreciated to have neuropathic pain. Painful diabetic neuropathy is much more common although factors other than glucose control contribute to the development of peripheral neuropathy in diabetes including lipids, hypertension, and other vascular risk factors and while diabetic neuropathy is common, neuropathic pain affects only a subset of patients. In contrast, neuropathic pain is a uniform finding among PC patients. Therefore, a homogenous KRT6A population with established neuropathic pain and alterations in mechanical thresholds and epidermal innervation provided a unique “proof of concept” opportunity.
Previous studies have suggested peripheral NOP-R as a target for neuropathic pain treatment. NOP-R mRNA and protein expression has been reported in small-, medium-, and large-size DRG neurons in rats\(^{30-32}\) and NOP-R activation inhibited N- and T-type Ca\(^{2+}\) currents in small- and medium-size DRG neurons.\(^{33-35}\) Anand et al\(^{36}\) demonstrated NOP-R expression in most TRPV1 positive small DRG neurons in humans and NOP-R activation inhibited capsaicin-induced TRPV1-mediated intracellular Ca\(^{2+}\) increase in rat and human DRG neurons. Similarly, intraplantar administration of N/OFQ in mice\(^{37}\) and subcutaneous tail administration of N/OFQ in rhesus monkeys inhibited capsaicin-induced allodynia.\(^{7}\) Additionally, experimental painful diabetic neuropathy studies in rats demonstrated that mechanical hyperalgesia was inhibited by intraplantar administration of the NOP-R agonist Ro-65-6570, which in turn was blocked by intraplantar administration of the NOP-R selective antagonist J-113397.\(^{5}\) Our human immunohistochemical studies expand these observations. We demonstrate that NOP-R is expressed in human DRG across a broad range of neuron sizes. Consistent with this, we also observed that NOP-R is expressed in a subset of CGRP-positive, most SP-positive small caliber fibers as well as on medium and large caliber myelinated NFH-positive A\(\delta\) and A\(\beta\) fibers within the dermis and epidermis. Taken together, these findings demonstrate that NOP-R is abundantly expressed in the peripheral nervous system and support a role for NOP-R in neuropathic pain treatment.
Limitations to this study include the small sample size and a lack of more detailed functional assessments. This is offset by the unbiased sampling approach. The small, but statistically significant difference that we observed in NOP-R expression in PC-affected skin has uncertain functional implications although the abundant presence of NOP-R in human epidermis and dermis validates it as a therapeutic target.

This study demonstrates that NOP-R is expressed in the peripheral nervous system. We observed NOP-R in keratinocytes, small unmyelinated, and large-myelinated nerve fibers as well as in human lumbar DRG. In plantar skin affected by PC, we observed that NOP-R expression was reduced relative to nearby unaffected skin or anatomically-matched control skin. Taken together, these results support peripheral NOP-R as a promising therapeutic target for treatment of neuropathic pain in PC, an ultra-rare disease with limited treatment options.

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REFERENCES

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.