miR-203 Regulates Nociceptive Sensitization after Incision by Controlling Phospholipase A2 Activating Protein Expression

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ABSTRACT

Background: After incision keratinocytes in the epidermis become activated to produce a range of pain-related mediators. microRNA 203 (miR-203) is known to be involved in keratinocyte growth, differentiation, and skin inflammation. We hypothesized that one or more of these mediators might be under the control of miR-203.

Methods: The expression of miR-203 and its target gene, phospholipase A2 activating protein (PLAA), were examined after hind paw incision in mice. We investigated the local effect of intraplantar PLAA peptide injection in normal mice and the effects of a selective secretory phospholipase A2 inhibitor (HK064) on PLAA or incision-induced mechanical allodynia. Last, we investigated the role of substance P signaling in regulating miR-203 and PLAA expression in vitro and in vivo.

Results: Levels of miR-203 were strongly down-regulated in keratinocytes after incision. Informatics-based approaches identified PLAA as a likely candidate for regulation by miR-203. PLAA caused mechanical allodynia and conditioned place aversion but not thermal sensitization. HK064 reduced mechanical allodynia after incision and after intraplantar injection of PLAA. Using preprotachykinin gene knockout mice or with neurokinin-1 selective antagonist LY303870 treatment, we observed that substance P-mediated signaling was also required for miR-203 and PLAA regulation after incision. Finally, using the rat epidermal keratinocyte cell line, we observed that a miR-203 mimic molecule could block the substance P-induced increase in PLAA expression observed under control conditions.

Conclusions: miR-203 may regulate expression of the novel nociceptive mediator PLAA after incision. Furthermore, the regulation of miR-203 and PLAA levels is reliant upon intact substance P signaling.

What We Already Know about This Topic

MicroRNAs inhibit gene expression and comprise a target for therapeutic intervention

MicroRNAs influencing pain in the incisional environment have not been characterized

What This Article Tells Us That Is New

In this rodent study, miR-203 is a microRNA that is down-regulated in incisions and controls phospholipase A2 activating protein, which in turn influences inflammation and pain after incision.

POSTOPERATIVE incisional pain is a unique but common form of acute pain. About 30–40% of patients suffer moderate to severe pain during the postoperative period despite advances in surgical techniques and perioperative analgesic strategies.1,2 Recent evidence indicates that the neurobiology of postoperative incisional pain may be different from inflammatory and neuropathic pain.3 Our group has demonstrated that sensitization of peripheral nerve fibers to mediators produced and released by keratinocytes in the epidermis supports one component of incisional pain.4,5 Furthermore, we found that neuropeptides, such as substance P (SP), when acting through the neurokinin-1 (NK1) receptor support nociceptive sensitization after incision and the production of cytokines by keratinocytes in the vicinity of the wounds.6 Other pain-related mediators, such as prostaglandin metabolites, are also produced and released from cells in the skin after incision,5 and are produced in keratinocytes in response to the injection of SP into normal skin.7 Poorly understood, however, are the processes linking incision and the resulting release of neuropeptides to changes in production of pain-related mediators.
MicroRNAs (miRNAs) represent a group of small non-coding RNAs possessing 19–25 nucleotide bases. They are transcribed from specific genes and generally undergo two cleavage steps that result in mature miRNAs. The mature miRNAs suppress gene expression by binding to the 3’-untranslated region of target message RNAs (mRNAs), thus reducing translation and promoting degradation. Because many genes may be targeted for miRNA regulation by virtue of containing specific consensus sequences, informatics-based approaches are often taken to identify target gene candidates. Recently, a number of studies have demonstrated that miRNAs are key regulators of several important physiologic and pathologic processes, such as embryonic development, organogenesis, and tumorigenesis. The roles of miRNA in the control of pain mechanisms are of growing interest.

One miRNA, miR-203, is highly and selectively expressed in mouse and human keratinocytes. This specific miRNA forms a gradient expression pattern in the epidermis, with low expression in the basal layer and high expression in the more differentiated suprabasal layers, consistent with its known roles in regulating keratinocyte proliferation and differentiation. It has been observed that miR-203 promotes epidermal differentiation and represses “stemness” in epidermal progenitors via the targeting of the transcription factor p63. Interestingly, miR-203 was found to be up-regulated in psoriatic plaques, which are characterized by the dysregulation of epidermal growth and differentiation. However, there is little information available concerning how miR-203 might regulate nociceptive sensitization and pain after incision. Furthermore, signaling systems controlling changes in the levels of miR-203 have not been described.

The current study, therefore, was designed to investigate the expression of miR-203 in hind paw skin before and after incision, and elucidate the possible molecular mechanism underlying miR-203-mediated regulation of keratinocyte function in incisional nociceptive sensitization. The demonstration of such regulation would be entirely novel in the field of pain research.

Materials and Methods

Animal Use

All experimental protocols were reviewed and approved by Veterans Affairs Palo Alto Healthcare System Institutional Animal Care and Use Committee (Palo Alto, California) before beginning the work. All protocols conform to the guidelines for the study of pain in awake animals as established by the International Association for the Study of Pain. Male mice 8 to 9 weeks old of the C57BL/6J strain were obtained from Jackson Laboratory (Bar Harbor, ME). Breeding pairs of preprotachykinin-A gene knockout (PPT-

A\(^{-/-}\)) mice congenic in the C57BL/6J background were acquired from Jackson Laboratory, and a breeding colony was established as previously described. Mice were housed four per cage and maintained on a 12-h light/dark cycle and an ambient temperature of 22 ± 1°C, with food and tap water available ad libitum.

Hind Paw Incision

The hind paw incision model in mice was performed in our laboratory as described in previous studies. Briefly, mice were anesthetized using isoflurane 2 or 3% delivered through a nose cone. After sterile preparation with alcohol, a 5-mm longitudinal incision was made with a No. 11 scalpel on the plantar surface of the right hind paw. The incision was sufficiently deep to divide deep tissue, including the plantaris muscle, longitudinally. After controlling bleeding, a single 6-0 nylon suture was placed through the midpoint of the wound and antibiotic ointment was applied. Mice used in these experiments did not show evidence of infection in the paws at the time of behavioral or biochemical assays.

Drug Administration

SP Administration. SP (Sigma-Aldrich, St. Louis, MO) prepared in 0.9% saline (Sigma-Aldrich) or vehicle were injected intraplantarly of the right hind paws in normal mice. The dose for SP injection was 3 μg/15 μl, which was based on our previous dose–response studies demonstrating hyperalgesic effects.

Phospholipase A2 Activating Protein (PLAA) Administration. The full-length endogenous murine PLAA contains 646 amino acid levels and the synthetic PLAA peptide contains 21 amino acid levels, which were able to increase phospholipase A2 activity in a dose-dependent manner in vitro. PLAA peptide (Enzo Life Science, Plymouth Meeting, PA) was freshly prepared in 0.9% saline, which was the vehicle used for control injections. PLAA or vehicle was injected intraplantarly in the right hind paws of normal mice at two different doses (100 μg/15 μl or 10 μg/15 μl).

HK064 Administration. 5-(4-Benzoyloxyphenyl)-4S-(7-phenylheptanoylamino) pentanoic acid (HK064), a selective secretory phospholipase A2 inhibitor (Sigma-Aldrich), was freshly dissolved in dimethyl sulfoxide (ATCC, Manassas, VA) according to manufacturer’s instructions, then further diluted in sterile water for injection with 5% Tween 80 (Sigma-Aldrich). The concentration was adjusted to 50 μg/100 μl so that 5 mg/kg dose could be administered intraperitoneally in a volume of 100 μl/100 g body weight. Mice received either HK064 solution or vehicle (90% water for injection, 5% dimethyl sulfoxide and 5% Tween 80) 1 h before incision and each day 6 h before behavior testing or 1 h before PLAA administration. The selection of 5 mg/kg of HK064 is based on the finding that at this dose, it effectively protected rats from ischemia and reperfusion injury of the small intestine.

LY303870 Administration. The selective neurokinin receptor-1 (NK-1) antagonist LY303870 (Eli Lilly Co., Indianapolis, IN) was freshly prepared in 0.9% saline, which was the vehicle used for control injections. The concentration was
adjusted to 300 μg/100 μl so that 30 mg/kg dose could be administrated intraperitoneally in a volume of 100 μl/100 g body weight. Mice received either LY303870 solution or vehicle 1 h before incision and each day 4 h before tissue collection. The dose was chosen on the basis of our previous studies.4

PLAA and α-Carrageenan Administration for Conditioned Place Aversion. α-Carrageenan (Sigma-Aldrich) was used as a 1% solution in water. PLAA peptide was used in the dose of 10 μg/15 μl in 0.9% saline. Either drugs or their respective vehicles were injected intraplantarly in a volume of 15 μl.

Nociceptive Testing
Mechanical Allodynia. Mechanical nociceptive thresholds were assayed using von Frey filaments according to a modification of the “up-down” algorithm described by Chaplan et al.24 as described previously.5,6,19 Mice were placed on wire mesh platforms in clear cylindrical plastic enclosures of 10 cm in diameter and 30 cm in height. After 20 min of acclimation, fibers of sequentially increasing stiffness with initial bending force of 0.2 g were applied to the plantar surface of the hind paw adjacent to the incision, just distal to the first set of foot pads, and left in place 5 s with enough force to slightly bend the fiber. Withdrawal of the hind paw from the fiber was scored as a response. When no response was obtained, the next stiffer fiber in the series was applied in the same manner. If a response was observed, the next less stiff fiber was applied. Testing proceeded in this manner until four fibers had been applied after the first one, causing a withdrawal response allowing the estimation of the mechanical withdrawal threshold using a curve-fitting algorithm.25

Thermal Hyperalgesia. Paw withdrawal response latencies to noxious thermal stimulation were measured using the method of Hargreaves et al.,26 which we have modified for use with mice.27 In this assay, mice were placed on a temperature-controlled glass platform (29°C) in a clear plastic enclosure similar to those described in the method of Hargreaves et al.26 After 30 min of acclimation, a beam of focused light was directed toward the same area of the hind paw as described for the von Frey assay. A 20-s cutoff was used to prevent tissue damage. In these experiments, the light beam intensity was adjusted to provide an approximate 10-s baseline latency in control mice. Three measurements were made per animal per test session, separated by at least 1 min.

Conditioned Place Aversion. To assess the affective component of PLAA-induced nociception, a counterbalanced conditioned place aversion (CPA) paradigm was employed, as described elsewhere.28 The CPA experiments were performed using standard conditioning chambers (MED Associates Inc., St. Albans, VT), which consists of three compartments: two outer compartments for active association and one middle neutral compartment. One association compartment is constructed of white opaque plastic walls with a floor made of metal rods, whereas the other compartment is made of black opaque plastic walls with a metal mesh floor. The smaller middle neutral compartment is made of gray opaque plastic walls and floor. The conditioning apparatus is equipped with motion photo-sensors with automatic data collection via a computer. Male C57Bl/6J mice (8–12 weeks old) were used in all experiments. Each experiment was carried out for 4 days starting with preconditioning day (day 1) with the mice freely exploring the three chambers for 15 min. Any mouse that spent more than 80% or less than 20% of the total experiment time in either of the association compartments was excluded. On the following 2 days (conditioning days 2 and 3), each mouse randomly received treatment or vehicle hind paw injections and was assigned to either the white or black association compartments in a counterbalanced fashion for 40 min. The day (day 2 or 3) and the paw (left or right) for the treatment or vehicle injection were randomized accordingly between groups. α-Carrageenan was injected 3 h before chamber placement, as the onset of hyperalgesia is delayed.26,28 As preliminary experiments showed the onset of hyperalgesic effect of local PLAA to be rapid, the mice were immediately placed in the chambers subsequent to injection. Experiments with α-carrageenan were carried out to be able to evaluate the extent, if any, of the PLAA-induced effects on place conditioning (positive controls). On day 4, mice were placed in the middle neutral compartment of the apparatus for 15 min with full access to the other two compartments and were assessed for the length of time spent in each compartment (postconditioning test).

Protein Isolation and Western Blot Analysis
To obtain skin samples for Western blot analysis, mice were first euthanized by carbon dioxide asphyxiation, and an oval patch of full-thickness skin providing 1.5- to 2-mm margins surrounding the hind paw incisions was collected by laser. The whole skin samples were dissected as described in Protein Isolation and Western Blot Analysis. According to the manufacturer’s instructions, total RNA from whole skin samples and cell culture were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). For samples collected by laser
capture microdissection, RNA was isolated with RNAqueous-Micro Kit (Applied Biosystems, Foster City, CA). Following on DNase treatment, RNA quality was determined by Nanodrop (NanoDrop Technologies, Wilmington, DE). For detection of the mRNA levels of PLAA and 18S, RNA (0.5 μg) was reverse-transcribed into complementary DNA using a First Strand Complementary DNA Synthesis Kit (Invitrogen). Real-time PCR was performed in an ABI prism 7900HT system (Applied Biosystems). All PCR experiments were performed using the SYBR Green I master kit (Applied Biosystems). The primer set for PLAA was purchased from SABiosciences (Valencia, CA). The primer set for 18S mRNA and the amplification parameters were described previously.5,32 Data analysis was performed using the Applied Biosystems SDS Software package (version 2.3) and were normalized by 18S mRNA expression. For detection of the microRNA levels, 25 ng RNA was reverse transcriptized using the Universal Complementary DNA Synthesis Kit (Exiqon, Woburn, MA). Quantitative real-time PCR primer sets (Exiqon) specific for miRNA 203 or for the internal control U6 small nuclear RNA were used to determine the expression of miRNA 203 by real-time PCR (7900HT System, Applied Biosystems) with SYBR Green master mix (Exiqon). Melting curves were performed to document single-product formation and miR-203 was normalized by U6 small nuclear RNA expression. For all time-points, the miR-203/U6 ratio s and PLAA/18s ratios are normalized to one.

Laser Capture Microdissection

For laser capture microdissection, all tissue processing was performed under RNase-free conditions. Mouse hind paw skin was washed with RNaseZAP (Sigma-Aldrich) followed by diethylpyrocarbamate-treated water, and dissected in ice-cold phosphate-buffered saline and fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline. Then tissue was infused with 0.5 m sucrose in phosphate-buffered saline overnight, then mounted in Tissue-Tek OCT embedding compound (Sakura Finetek), frozen on dry ice, and sectioned in 8 μm slices and mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA). All slides were stored at −80°C until use for in situ hybridization.

Hybridizations of microRNA were performed as described previously.33 Briefly, the sections were dried at 37°C for 45 min and fixed with 4% paraformaldehyde for 20 min at room temperature. After the treatment with protease K (2 μg/ml) at 37°C, sections were incubated in prehybridization solution (Biochain, Hayward, CA) at 54°C, followed by incubation overnight at 54°C in hybridization solution (Biochain) containing 25 nmol of digoxin-labeled locked nucleic acid miR-203 detection probes (Exiqon). Hybridization temperature was 15°C below the predicted Tm value of the miR-203 detection probe, and 54°C calculated from 69°C of miR-203 Tm. After hybridization, sections were washed in 5 · saline-sodium citrate for 10 min and 1 · saline-sodium citrate for 10 min twice at 54°C, followed by washing in 0.2 · saline-sodium citrate twice at 37°C. Blocking was performed for 1 h at room temperature in the blocking solution (Biochain). Then, the slides were incubated with alkaline phosphatase-conjugated antidigoxin antibody (1:1,000, Biochain) overnight at 4°C. Staining was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Roche). For control experiment, sections were incubated with locked nucleic acid scrambled microRNA probe (Exiqon), which led to low-intensity nonspecific staining patterns in preliminary experiment.

Tissue Processing and Immunochemistry

The technique of immunohistochemical analysis was described previously.5,32 Briefly, the hindpaws were fixed in 10% buffered formalin for 24 h. Blocking of the sections took place overnight at 4°C in Tris-buffered saline containing 5% dry milk, followed by exposure to the primary antibody against PLAA (1:200, ProteinTech group) overnight at 4°C. Sections were then rinsed and incubated with fluorescin-conjugated secondary antibodies against the primary antibodies (1:500, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h. Confocal laser-scanning microscopy was carried out using a Zeiss LSM/510 META microscope (Thornwood, NY). Control experiments included incubation of slices in primary and secondary antibody-free solutions. For specificity of PLAA antibody, the preabsorption of the antibody with blocking peptide was conducted before adding to the section. Signals in sections from normal and experimental mice were negative or negligible.

Cell Culture and miRNA Transfections

The epidermal keratinocyte (REK) cell line was provided by Dr. Howard Baden, M.D. (Department of Dermatology, Massachusetts General Hospital, Boston, Massachusetts) and grown as described previously.34–36 In brief, cells were...
Cells were transfected with miR-203 PremiR miRNA Precursor Molecules (Ambion, Austin, TX), or PremiR miRNA Precursor Molecules-Negative Control #1 (Ambion) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Transfected cells were harvested after 48 h for PLAA mRNA assay.

Statistical Analysis
All data are expressed as mean ± SEM. Analysis of repeated parametric measures was accomplished using a one-way ANOVA followed post hoc Dunnett test or a two-way ANOVA followed by Bonferroni correction. For simple comparisons of two groups, a two-tailed Student t test was employed. P < 0.05 was considered significant (Prism 5; GraphPad Software, La Jolla, CA).

Results
Expression of miR-203 in Hind Paw Tissue after Incision
As miR-203 is a skin- and keratinocyte-specific microRNA and is a known regulatory molecule in epithelial cell biology,15,17 we measured changes in miR-203 level. As shown in figure 1A, levels of miR-203 in hind paw tissue were greatly decreased after incision and reached their nadir at 24-h time point. In situ hybridization revealed that miR-203 was expressed in the epidermis in the normal hind paw skin, especially in the suprabasal layer versus basal layer (fig. 1B), which was consistent with others’ findings.15,17 Keratinocytes appeared to be the main cell type expressing miR-203. In order to quantitatively examine the changes in miR-203 expression specifically in the epidermis, the epidermis was harvested using laser capture microdissection before and at 24 h after incision; miR-203 in the epidermis was dramatically down-regulated (5.5-fold) at 24 h (fig. 1C).

Predicted Targets of miR-203
To elucidate the molecular mechanism underlying miR-203-mediated regulation of keratinocyte function after incision, we used in silico analysis based on the computer-aided algorithm, TargetScan5.2** for predicting target genes.37–39 This algorithm suggested that the gene for PLAA was the gene most strongly expressing miR-203 regulatory elements in mice (table 1) with five complementary targeting sites in its 3’-untranslated region (table 2). We then employed two additional computational miRNA prediction algorithms, miRanda†† and Microcosm‡‡, and verified that PLAA is a target for miR-203 regulation (data not shown).

Expression of PLAA after SP Exposure in REK Cell Line
Identifying PLAA as a Direct Target of miR-203
We first pursued the functional relationship between miR-203 and PLAA using an in vitro culture system. The neuropeptide SP was used to stimulate the cultures, because this signaling molecule has been implicated in epithelial regul-
tion after incision, in inflammatory skin diseases, and in the setting of complex regional pain syndrome.6,40,41 Cultures of REK cells were exposed to 10^{-10} to 10^{-7} M concentrations of SP, and enhanced levels of PLAA mRNA were observed at 3 h (fig. 2A). Levels of PLAA protein were enhanced at 24 h (fig. 2B). Maximal mRNA responses to SP occurred using the 10^{-8} M concentration, and we used these parameters for subsequent experiments.

To determine the functional relevance of the interaction between miR-203 and PLAA, we tested whether premiR-203 (a miR-203 mimic) could inhibit PLAA expression in REK cells stimulated by SP exposure. When REK cells were transfected with 25 nM premiR-203, the level of PLAA mRNA measured after exposure to SP was significantly reduced (fig. 2C). When the concentration of premiR-203 was increased to 200 nM, basal PLAA levels were decreased consistent with miR-203 controlling PLAA mRNA stability and expression (fig. 2C). Transfection of the scrambled sequence control did not alter PLAA expression. Because premiR-203 reduced PLAA expression during SP exposure, these data confirm the interaction between miR-203 and PLAA mRNA.

Expression of PLAA in Hind Paw Tissue after Incision

Next, we examined the changes in PLAA mRNA and protein levels in the hind paw tissue after incision. Figure 3A showed that the level of PLAA mRNA was significantly increased after hind paw incision. The level of PLAA protein was remarkably up-regulated at 24 h after incision, as well (fig. 3B). PLAA protein was not detectable by immunostaining in the epidermis in normal hind paw, but was strongly up-regulated at 24 h after incision (fig. 3C). Therefore, incision induced gene and protein expression of PLAA in the epidermis.

Effects of Peripheral Administrations of PLAA on Mechanical and Thermal Nociceptive Sensitivity

Because PLAA exhibits 42% protein sequence identity with melittin, which comprises approximately 50% of the dry

Table 1. Mouse PLAA Was the Target Gene of miR-203

<table>
<thead>
<tr>
<th>Mouse Ortholog of Target Gene</th>
<th>Gene Name</th>
<th>Conserved Sites</th>
<th>Poorly Conserved Sites</th>
<th>Representative miRNA</th>
<th>Total Context Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAA</td>
<td>Phospholipase A2-activating protein</td>
<td>1</td>
<td>4</td>
<td>mmu-miR-203</td>
<td>−1.50</td>
</tr>
<tr>
<td>B3GNT5</td>
<td>UDP-GlcNAc: betaGal β-1, 3-N-acetylglucosaminyltransferase 5</td>
<td>1</td>
<td>4</td>
<td>mmu-miR-203</td>
<td>−1.31</td>
</tr>
<tr>
<td>AFF4</td>
<td>AF4/FMR2 family, member 4</td>
<td>2</td>
<td>5</td>
<td>mmu-miR-203</td>
<td>−1.25</td>
</tr>
<tr>
<td>C22orf9</td>
<td>Chromosome 22 open reading frame 9</td>
<td>1</td>
<td>2</td>
<td>mmu-miR-203</td>
<td>−1.00</td>
</tr>
<tr>
<td>LRRTM2</td>
<td>Leucine rich repeat transmembrane neuronal 2</td>
<td>1</td>
<td>3</td>
<td>mmu-miR-203</td>
<td>−0.99</td>
</tr>
</tbody>
</table>

Shown are the top five molecular targets sorted by total context score, which was predicted using TargetScan 5.2 (Whitehead Institute for Biomedical Research, Cambridge, MA). [37–39]. PLAA = phospholipase A2 activating protein.

Table 2. Predicted miR-203 Target Sites on PLAA 3’-untranslated Region

<table>
<thead>
<tr>
<th>Target Sites</th>
<th>Predicted Consequential Pairing of Target Region (Top) and miRNA (Bottom)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position 415–421 of PLAA 3’ UTR</td>
<td>5’...UCAAAUGCAUGAUCAUUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...AUUACGUAAUCAAACCUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...AGAUGUACUAUUUCUUUAAGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...</td>
</tr>
<tr>
<td>Position 452–458 of PLAA 3’ UTR</td>
<td>5’...UCAAAUGCAUGAUCAUUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...AUUACGUAAUCAAACCUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...</td>
</tr>
<tr>
<td>Position 591–597 of PLAA 3’ UTR</td>
<td>5’...UCAAAUGCAUGAUCAUUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...AUUACGUAAUCAAACCUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...</td>
</tr>
<tr>
<td>Position 737–743 of PLAA 3’ UTR</td>
<td>5’...UCAAAUGCAUGAUCAUUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...AUUACGUAAUCAAACCUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...</td>
</tr>
<tr>
<td>Position 771–777 of PLAA 3’ UTR</td>
<td>5’...UCAAAUGCAUGAUCAUUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...AUUACGUAAUCAAACCUUCAA...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...3’...GAUCAGGGUUUUUAGUG...5’...UGUUUAUACGUAUUCAAUGAG...</td>
</tr>
</tbody>
</table>

Target sites predicted using TargetScan 5.2 (Whitehead Institute for Biomedical Research, Cambridge, MA). [37–39]. PLAA = phospholipase A2 activating protein; UTR = untranslated region.
weight of bee venom, \textsuperscript{21,42} we examined whether PLAA itself would induce the nociceptive sensitization in the normal mouse hind paw after local injection. Intraplantar PLAA peptide injection in the hind paw induced prolonged mechanical allodynia lasting between 0.5 and 4 h, without any alteration of thermal sensitivities after administration at doses of $10^{-8}$ g and $10^{-5}$ g (figs. 4A and B).

Effects of Peripheral Administrations of PLAA on Conditioned Place Aversion

The preconditioning report showed that there was no overall side preference to the outer compartments in any of the
groups. Both PLAA and \( \lambda \)-carrageenan produced significant CPA when compared with their respective vehicle paired compartments (fig. 5A). No such effect was seen in controls, and the PLAA-induced CPA was less than that of \( \lambda \)-carrageenan, though not statistically different (fig. 5B). Either of the latter treatments produced significant CPA when compared with controls.

**Effect of Systematic Administration of HK064 on PLAA- or Incision-induced Mechanical Allodynia**

PLAA is also a novel activator of phospholipase A2 (PLA2), which subsequently regulates the production of prostaglandin E2, tumor necrosis factor-\( \alpha \), and interleukin-1\( \beta \), all pain-related signaling molecules in the skin up-regulated after incision.\(^{43,44} \)

Therefore we hypothesized that inhibition of PLA2 activity would attenuate the mechanical allodynia induced by PLAA administration. Systemic administration of secretory PLA2 (sPLA2) inhibitor, HK064 (5 mg/kg, intraperitoneally), 1 h before local application of PLAA (10 \( \mu \)g) injection in the normal mouse hind paw reduced the PLAA-induced mechanical allodynia, lasting between 0.5 to 6 h after PLAA injection (fig. 6A).

PLAA was up-regulated after incision and inhibition of sPLA2 activity attenuates the PLAA-induced mechanical allodynia; therefore, we hypothesized that sPLA2 inhibitor would also reduce mechanical allodynia after hind paw incision in mice. The HK064-treated animals displayed reduced mechanical allodynia at 24 h and 48 h after incision (fig. 6B), without any alteration of thermal sensitization (fig. 6C). The PLA2 inhibitor did not have any effect on the nociceptive thresholds of control animals.

**Effects of Peripheral SP Administration on mir-203 and PLAA Levels in Hind Paw Tissue**

We established earlier in the course of our experiments that SP can enhance expression of PLAA in mir-203-dependent fashion in our REK cell culture experiments. We therefore hypothesized that SP could promote PLAA expression in the skin as well as mir-203 down-regulation. SP (3 \( \mu \)g/paw) was intraplantarly injected in normal mouse hind paw and tissue was harvested at 4 h after administration. Figure 7 demonstrates that SP induced a significant decrease of mir-203 (fig. 7A), which was concomitant with the elevations of PLAA mRNA and protein (figs. 7B and C).
Effects of Preprotachykinin Gene Deletion and Neurokinin-1 (NK-1) Receptor Blockade on the Expressions of miR-203 and PLAA after Incision.

Because both incision and SP injection reduced miR-203 expression and enhanced PLAA mRNA and protein expression in epidermis, we hypothesized that SP signaling was involved in the miR-203 regulation after incision. Figure 8A showed that deleting the SP precursor gene PPT-A produced significant attenuations of incision-decreased miR-203 in PPT-A⁻/⁻ mice compared with the wide-type mice at 24 h after incision, and concomitantly reduced elevations of PLAA mRNA at 24 h and 48 h (fig. 8B). Likewise, blocking SP-mediated signaling by administration of the NK-1 selective antagonist LY303870 (30 mg/kg, intraperitoneally) also produced significant attenuations of incision-decreased miR-203 and reduced elevations of PLAA mRNA compared with the vehicle-treated mice at 24 h after incision (figs. 8C and D). Previous reports demonstrate that PPT-A⁻/⁻ mice and LY303870-treated mice display less nociceptive sensitization after hind paw incision.⁶

Discussion

A significant amount of work has been done during the past decade examining the mechanisms involved in supporting pain after surgical incision. Recently, attention has to a degree shifted toward understanding how tissue adjacent to the incisions is stimulated to produce the wide variety of mediators ultimately found in the peri-incisional “inflammatory soup.” One approach involves understanding how neuropeptide-releasing primary afferent fibers might be involved. Recent reports indicate that neuropeptides like SP and calcitonin gene-related peptide may stimulate keratinocytes in the skin to produce prostaglandin E₂, interleukin-1β, nerve growth factor, and other pronociceptive mediators.⁵⁻⁷

Rather than pursuing well established biochemical signaling pathways, we hypothesized that changes in the level of a key PLAA mRNA at 24 h and 48 h (fig. 8B). Likewise, blocking SP-mediated signaling by administration of the NK-1 selective antagonist LY303870 (30 mg/kg, intraperitoneally) also produced significant attenuations of incision-decreased miR-203 and reduced elevations of PLAA mRNA compared with the vehicle-treated mice at 24 h after incision (figs. 8C and D). Previous reports demonstrate that PPT-A⁻/⁻ mice and LY303870-treated mice display less nociceptive sensitization after hind paw incision.⁶

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Our principal observations were: (1) miR-203 is strongly down-regulated after incision; (2) a major predicted target of regulation of miR-203, PLAA, appears to be strongly up-regulated as a consequence of diminished miR-203 levels; (3) PLAA causes both mechanical nociceptive sensitization and spontaneous nociception in mice; (4) PLAA, a known PLA2 activator, requires activation of sPLA2 for full expression of its pro-nociceptive effects; (5) SP is one mediator capable of causing both the down-regulation of miR-203 and enhanced expression of PLAA in mouse skin and keratinocyte cultures; and (6) SP signaling is involved in both miR-203 and PLAA regulation after incision. To our knowledge, receptor-mediated regulation of miRNA levels has not before been described as participating in a pain-signaling pathway.

Though the field is relatively new, it is expected that the study of miRNA will provide new insights into pain-producing and pain-relieving mechanisms. Recently Favereaux et al. discovered that miR103 regulates Cav1.2 l-type calcium ion channel subunits in spinal cord dorsal horn tissue. In fact, three subunits of this channel are regulated by the same miRNA. Administration of miR-103 thus down-regulating expression of channel subunits reduced neuropathic sensitivity in a rodent model. A more general study of dorsal root ganglion tissue after spinal nerve ligation revealed that a group of 59 miRNA species were down-regulated in the dorsal root ganglion adjacent to the one serving the injured nerve. Subsequent informatics and in vitro experiments confirmed miRNA regulation of key genes involved in nociceptive signaling, such as the P2X4 receptor and the α-2/δ-1 subunit of the voltage-dependent calcium ion channel. Again, the down-regulation of miRNAs was observed to be the cause of the up-regulation of the pronociceptive machinery. Kusuda et al. studied the expression levels of three miRNAs: miR-1, miR-12, and miR-206. They found differences in the direction of expression of these miRNA species, which were dependent on the specific tissue used (dorsal root ganglion vs. spinal cord) and the pain model used (complete Freund’s adjuvant-induced inflammation vs. sciatic nerve partial ligation). Using a novel nociceptor-selective knockout of the gene coding for dicer, a critical protein involved in miRNA maturation, Zhao et al. were able to identify genes both up- and down-regulated by the global reduction in miRNA function. This work and that in other fields suggests that miRNAs may provide mechanisms whereby the expression of many target genes can be integrated into the function of a small group of regulatory molecules. In addition, miRNA directed against acid-sensing ion channels was used to demonstrate a role for these channels in the setting of nociception.

Fig. 8. Effect of incision on miR-203 and phospholipase A2 activating protein (PLAA) mRNA levels in PPT-A null mutant (PPT-A−/−) mice and neurokinin-1 antagonist LY303870-treated mice after incision. Incision induced less down-regulation of miR-203 level (A and C) and less up-regulation of PLAA mRNA level (B and D) in PPT-A−/− mice and LY303870-treated group compared with wild-type mice and vehicle-treated group, respectively. Values are displayed as the mean ± SEM. For parameters measured in PPT-A−/− mice, n = 5; for all the other groups, n = 6. *P < 0.05, **P < 0.01, or ***P < 0.001 versus wild-type group or vehicle-treated group. miR-203 = microRNA 203; mRNA = messenger RNA; PLAA = phospholipase A2 activating protein; PPT = preprotachykinin-A gene knockout; WT = wild-type.
miR203 versus Paw Incision

Sun et al.

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Although miRNA is also a tool useful for probing biologic functions of genes,52 the dysregulation of miRNA-203 signaling has been proposed to support psoriatic plaques, and its specific roles have been identified.14 In particular, miR-203 is over-expressed in psoriatic plaques. More broadly, miR-203 is felt to promote the differentiation of keratinocytes in the epithelium via a process that may involve the activation of protein kinase C.53 Other studies examining the role of miR-203 in the epithelium have concluded that this miRNA species may promote differentiation by repressing what has been termed the “stemness” or the proliferative potential of calls in the basal layer of the epidermis thus promoting stratification.15 This mechanism probably involves the ability of miR-203 to repress expression of p63, a transcription factor expressed in keratinocytes in the basal layer of the epidermis. Several studies have attempted to link miR-203 levels to the development of epithelial cancers, for example in the esophagus.54,55 In light of the demonstrated roles for miR-203 in epithelial differentiation, it seems reasonable to observe a decrease in miR-203 levels in skin actively involved in regeneration, such as occurs in tissue surrounding surgical wounds. Despite this work, miR-203 had not before been linked to pain or the production of PLAA.

Because few targets for miR-203 regulation had been described, and none with obvious links to our pain- and inflammation-related phenotypes of interest, we undertook an informatics-based approach to selecting probable targets. Using three separate algorithms, PLAA was identified as a regulatory target containing multiple consensus sequences. This appeared to be a plausible pain- and inflammation-related target because it is known to have homology to melittin, the principal protein component of bee venom.56 Melittin injection into skin leads to mechanical hyperalgesia in humans,57 and is known to be able to stimulate PLAA in cultured keratinocytes.58 In addition to stimulating PLAA resulting in increased prostaglandin levels, PLAA is able to enhance the expression of COX-2 and tumor necrosis factor-α, molecules capable of supporting pain and inflammation.56 Both of these molecules are up-regulated in the vicinity of surgical wounds.19,59 In our own experiments we were able to demonstrate dose-dependent mechanical allodynia, and were further able to demonstrate that intraplantar PLAA injection has a spontaneous noxious quality similar to that of α-carrageenan in the conditioned place avoidance paradigm. Similar to results reported for melittin and sPLA2, mechanical rather than thermal sensitization was more prominent after PLAA administration.60,61 Thus PLAA production may not comprehensively explain the many dimensions of nociceptive sensitization that occur after incision. We went on to show that the selective sPLA2 inhibitor HK064 could partially reverse the mechanical allodynia measured after incision or PLAA injection, but had no effect on thermal sensitization in incised mice. Others have shown that PLAA acting either at the site of tissue injury or within the central nervous system can support pain through the generation of arachidonic acid and the subsequent conversion of this molecule to pronociceptive metabolites, such as prostaglandin E2.62,63 Besides the products of arachidonic acid metabolism, other PLA2 metabolites such as lysophospholipids may also act as pronociceptive substances. Lysophospholipids show distinct activities on sensory transient receptor potential ion channels and modulate pain processing.64 Another lysophospholipid, lysophosphatidylcholine, possesses proinflammatory properties and is involved in central nervous system and peripheral pain hypersensitivity.65–67 Furthermore, lysophosphatidylcholine that undergoes autotaxin-mediated conversion to lysophosphatidic acid induces neuropathic pain through the activation of lysophosphatidic acid-1 receptor.68–70 Finally, studies have shown that local or spinal injection of platelet-activating factor, the acetylation of lysophospholipids, induced pain hypersensitivity, and the platelet-activating factor/platelet-activating factor receptor system play a role in tissue injury-induced pain.71

Aside from identifying PLAA as a target of incision-regulated miR-203 expression, we attempted to identify mechanisms underlying this regulation. It had been demonstrated recently that neuropeptides such as SP released from primary afferent fibers near wounds can regulate nociception, the generation of inflammatory mediators, and healing.5,72 Many of these actions require signaling through the NK1 receptor expressed on keratinocytes. Our results show that SP can in fact reduce miR-203 levels and stimulate PLAA production. Mice with a SP preprotachykinin gene deletion showed little change in miR-203 and PLAA levels after incision. Our studies did not, however, probe the intracellular mechanisms linking SP to reductions in miR-203 levels. However, the regulation of this and other miRNAs has been studied particularly by those interested in the control of epithelial cell growth and oncogenesis in epithelial tissues. These studies have shown that hypermethylation of the miR-203 promotor decreases expression, resulting in enhanced rates of growth.73 It could be hypothesized that SP/NK1 regulates miR-203 through the methylation of its promotor. It does not appear that neuropeptide-mediated epigenetic modification of this gene’s promotor has been investigated, but such studies could be carried out in our system.

In summary, our evidence suggests that a skin-specific miRNA species, miR-203, is capable of interacting with the PLAA gene to enhance expression after incisions. PLAA, like its homolog, bee venom melittin, is capable of supporting spontaneous and evoked nociception. This appears to be because of, at least in part, to the activation of PLAA. This pathway relies on intact SP/NK1 signaling, which is a mechanism controlling other aspects of wound area inflammation and tissue repair. Aside from issues related to miR-203, this work suggests that we look more comprehensively at the roles miRNA and other forms of epigenetic signaling might have in controlling pain after surgery. Incisional pain is a complex pain phenomenon and involves many pathways. We contribute evidence for a previously unexplored mechanism as contributing to incisional pain, but do not claim that it could be entirely
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