Published in final edited form as:
Anesthesiology. 2012 September ; 117(3): 626–638. doi:10.1097/ALN.0b013e31826571aa.

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 hindpaw 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.

1. Introduction

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

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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) 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 noncoding 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 [8]. Since many genes may be targeted for miRNA regulation by virtue of their containing specific consensus sequences, informatics-based approaches are often taken to identify target gene candidates [9]. Recently, a number of studies have demonstrated that miRNAs are key regulators of several important physiological and pathological processes such as embryonic development, organogenesis, and tumorigenesis [10–12]. The roles of miRNA in the control of pain mechanisms are of growing interest [13].

One miRNA, miR-203, is highly and selectively expressed in mouse and human keratinocytes [14, 15]. 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 [15–17]. It has been observed that miR-203 promotes epidermal differentiation and represses ‘stemness’ in epidermal progenitors via the targeting of the transcription factor p63 [15, 18]. Interestingly, miR-203 was found to be up-regulated in psoriatic plaques which are characterized by the dysregulation of epidermal growth and differentiation [14]. 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 hindpaw 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.

2. Materials and Methods

2.1. Animal use

All experimental protocols were reviewed and approved by Veterans Affairs Palo Alto Healthcare System Institutional Animal Care and Use Committee prior to 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 8–9 weeks old of the C57BL/6J strain were obtained from Jackson Laboratory (Bar Harbor, ME). Breeding pairs of pre-protachykinin-A gene knockout (*ppt*-A$^{-/-}$) mice congenic in the C57BL/6J background were acquired from Jackson laboratories and a breeding colony was established as previously described [6]. 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*.

2.2. Hindpaw incision

The hindpaw incision model in mice was performed in our laboratory as described in previous studies [5, 6, 19]. Briefly, mice were anesthetized using isoflurane 2–3% delivered through a nose cone. After sterile preparation with alcohol, a 5 mm longitudinal incision was made with a number 11 scalpel on the plantar surface of the right hindpaw. 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.

2.3. Drug administration

SP administration—SP (Sigma, St. Louis, MO) prepared in 0.9% saline (Sigma) or vehicle were injected intraplantar of the right hindpaws 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 [6].

Phospholipase A2 activating protein (PLAA) administration—The full length endogenous murine PLAA contains 646 amino acid level [20] and the synthetic PLAA peptide contains 21 amino acid level, which was able to increase phospholipase A2 activity in a dose-dependent manner *in vitro* [21]. 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 intraplantar in the right hindpaws of normal mice at two different doses (100 μg/15 μl or 10 μg/15 μl) [22].

HK064 administration—5-(4-Benzyloxyphenyl)-4S-(7-phenylheptanoylamino) pentanoic acid (HK064), a selective secretory phospholipase A2 inhibitor (Sigma), was freshly dissolved in dimethyl sulfoxide (ATCC, Manassas, VA) according to manufacturer's instruction then further diluted in sterile water for injection with 5% Tween 80 (Sigma). The concentration was adjusted to 50 μg/100 μl so that 5 mg/kg dose could be administrated intraperitoneally in a volume of 100 μl/100g body weight. Mice received either HK064 solution or vehicle (90% water for injection, 5% dimethyl sulfoxide and 5% Tween 80) 1h before incision and each day 6 h before behavior testing or 1h 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 [23].

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/100g body weight. Mice received either LY303870 solution or vehicle 1h before incision and each day 4 h before tissue collection. The dose was chosen on the basis of our previous studies [6].

PLAA and λ-Carrageenan administration forconditioned place aversion—λ-Carrageenan was obtained from Sigma-Aldrich and 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 intraplantar in a volume of 15μl.

2.4. 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 diameter and 30 cm in height. After 20 min of acclimation, fibers of sequentially increasing stiffness with initial bending force of 0.2 gram were applied to the plantar surface of the hindpaw 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 hindpaw 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 4 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] as 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 towards the same area of the hindpaw 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 counter balanced 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 a middle neutral compartment. One association compartment is constructed of white opaque plastic walls with a floor made of metal rods while 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 to 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 two days (conditioning days 2 and 3) each mouse randomly received treatment or vehicle hind-paw injections and assigned to either the white or black association compartments in a counter-balanced 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. 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 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).

2.5. Protein isolation and Western blot analysis

To obtain skin samples for Western blot analysis, mice were first euthanized by carbon dioxide asphyxiation, and an ovular patch of full-thickness skin providing 1.5- to 2-mm margins surrounding the hindpaw incisions was collected by dissection, frozen in liquid nitrogen and stored at −80°C until required for analysis. Mice hindpaws were dissolved at 4°C in T-PER Tissue Protein Extraction buffer (Thermo Scientific, Rockford, IL) in the presence of protease inhibitor cocktail (Roche, Mannheim, Germany). Equal amounts of protein (30 μg) were loaded for SDS-PAGE (10% Tris-HCl acrylamide gel) and electrotransferred onto a polyvinylidene difluorided membranes as described previously [29, 30]. Blots were probed with the primary antibody of PLAA polycolonal antibody (1:1,000, ProteinTech group, Chicago, IL) at 4°C overnight. β-actin (Sigma) was used as an internal control. The band intensity was quantified using National Institutes of Health image J analysis software (version 1.44, Bethesda, MD).

2.6. RNA isolation, Quantitative real-time Polymerase Chain Reaction (PCR) of mRNA and microRNA

The whole skin samples were dissected as described in the 2.5 section. 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 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 [19]. 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 each 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 0 points, the miR-203/U6 ratio s and PLAA/18s ratios are normalized to one.

2.7 Laser Capture Microdissection

For laser capture microdissection, all tissue processing was performed under RNase-free conditions. Mouse hindpaw skin was mounted in Tissue-Tek OCT embedding compound (Sakura Finetek USA, Inc., Torrance, CA), frozen on dry ice and sectioned in 10 μm slices. Each slide contained about six pieces of skin samples. After quick dehydration through ethanol and xylene, epidermis (except stratum corneum) was identified by their location and dissected with a Leica Laser Capture Microdissection System (Leica, Buffalo Grove, IL). As we described previously [31], laser capture microdissection caps were snapped onto 0.5-ml microcentrifuge tubes containing lysis buffer (RNAqueous®-Micro Kit, Applied Biosystems) for RNA isolation. Isolated RNA was subjected to reverse transcription and real-time PCR for detection of miR-203 expression as described in the 2.6 section.

2.8. Tissue processing and immunohistochemistry

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 fluorescein-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.

2.9. Tissue processing and miR-203 in situ hybridization

For *in situ* hybridization, all tissue processing was performed under RNase-free conditions. Briefly, mouse hindpaw skin was washed with RNaseZAP (Sigma) followed by diethylpyrocarbamate-treated water, and dissected in ice-cold phosphate buffered saline and fixed overnight in 4% paraformaldehyde (Sigma) 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.

microRNA Hybridizations 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 proteinase 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 x 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 anti-digoxin 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.

2.10. Cell culture and miRNA transfections

The epidermal keratinocyte (REK) cell line was generously 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 cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin, 0.4 μg/mL hydrocortisone, and 0.75 mM aminoguanidine. On reaching approximately 90% confluence, the cells were starved with the above culture medium supplemented with only 1% fetal bovine serum overnight. Cells were then washed twice with fresh Dulbecco's modified Eagle's medium before addition of SP (Sigma) or saline vehicle. At least five culture dishes were used for each time point and treatment condition.

Cells were transfected with miR-203 Pre-miR™ miRNA Precursor Molecules (Ambion, Austin, TX), or Pre-miR™ 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.

2.11. Statistical analysis

All data are expressed as mean ± SEM. Analysis of repeated parametric measures was accomplished using a one-way analysis of variance followed *post doc* Dunnett testing or a two-way analysis of variance followed by Bonferroni testing. For simple comparisons of two groups, a two-tailed Student *t* test was employed. *P* values less than 0.05 were considered significant (Prism 5; GraphPad Software, La Jolla, CA).

3. Results

3.1 Expression of miR-203 in hindpaw 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 hindpaw 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 hindpaw 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).

3.2 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).

3.3 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 as this signaling molecule has been implicated in epithelial regulation 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.

*http://www.targetscan.org. Last accessed June 24, 2011.
†http://www.microrna.org/microrna/home.do. Last accessed June 24, 2011.
‡http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/. Last accessed June 24, 2011.

To determine the functional relevance of the interaction between miR-203 and PLAA, we tested whether pre-miR-203 (a miR-203 mimic) could inhibit PLAA expression in REK cells stimulated by SP exposure. When REK cells were transfected with 25nM pre-miR-203, the level of PLAA mRNA measured after exposure to SP was significantly reduced (fig. 2C). When the concentration of pre-miR-203 was increased to 200nM, 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. Since pre-miR-203 reduced PLAA expression during SP exposure, these data confirm the interaction between miR-203 and PLAA mRNA.

3.4 Expression of PLAA in hindpaw tissue after incision

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

3.5 Effects of peripheral administrations of PLAA on mechanical and thermal nociceptive sensitivity

Since PLAA exhibits 42% protein sequence identity with melittin which comprises approximately 50% of the dry weight of bee venom [21, 42], we examined whether PLAA itself would induce the nociceptive sensitization in the normal mouse hindpaw after local injection. Intraplantar PLAA peptide injection in the hindpaw induced prolonged mechanical allodynia lasting between 1/2 and 4 h, without any alteration of thermal sensitivities after administration at doses of 10 μg and 100 μg (figs. 4A and B).

3.6 Effects of peripheral administrations of PLAA onconditioned 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 carrageenan produced significant CPA when compared with their respective vehicle paired compartments (fig. 5A). No such effect seen in controls and the PLAA induced CPA was to some extent less than that of carrageenan, though not statistically different (fig. 5B). Either of the latter treatments produced significant CPA when compared to controls.

3.7 Effect of systematic administration of HK064 on PLAA- or incision-induced mechanical allodynia

PLAA is also a novel activator of phospholipase A2 that subsequently regulates the production of PGE2, TNF-α, and IL-1β, 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 (5mg/kg, intraperitoneally) 1 h before local application of PLAA (10 μg) injection in the normal mouse hindpaw reduced the PLAA induced- mechanical allodynia, lasting between 1/2 h to 6 h after PLAA injection (fig. 6A).

PLAA was upregulated 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 hindpaw 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.

3.8 Effects of peripheral SP administration on miR-203 and PLAA levels in hindpaw 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 downregulation. SP (3 μg/paw) was intraplantar injected in normal mouse hindpaw and tissue was harvested at 4 h after administration. Figure 7 demonstrated 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).

3.9 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 a significant attenuations of incision-decreased miR-203 in ppt-$A^{-/-}$ mice compared to 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 a significant attenuations of incision-decreased miR-203 and reduced elevations of PLAA mRNA compared to 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 hindpaw incision [6].

4. Discussion

A significant amount of work has been done over the past decade examining the mechanisms involved in supporting pain after surgical incision. Recently, attention has to a degree shifted towards 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 PGE2, IL-1β, nerve growth factor, and other pronociceptive mediators [5, 45–47]. Rather than pursuing well established biochemical signaling pathways, we hypothesized that changes in the level of a key skin miRNA species might be involved. 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 relieving mechanisms [13]. Recently Favereaux *et al* [48] 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 downregulated in the dorsal root ganglion adjacent to the one serving the injured nerve [49]. 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 upregulation of the pronociceptive machinery. Kusuda *et al* [50] studied the expression levels of three miRNAs, miR-1, -12, and -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 Freunds 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 downregulated by the global reduction in miRNA function [51]. 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 muscle inflammation. Thus miRNA is also a tool useful form probing biological functions of genes [52].

The dysregulation of miRNA-203 signaling has been proposed to support psoriasis, 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.

Since 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 since 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 PLA2 in cultured keratinocytes [58]. In addition to stimulating PLA2 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 upregulated 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 carrageenan in the conditioned place aversion 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 which 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 PLA2 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 showing 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 plays 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 [6, 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/NK-1 regulates miR-203 through its promotor's methylation. It does not appear that neuropeptide mediated epigenetic modification of this gene's promoter has been investigated, though 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 due at least in part to the activation of PLA2. 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 responsible for incisional pain. We also acknowledge that significant work is still required to establish the precise molecular mechanisms involved. The use of sequence-specific mimic and inhibitor molecules of miRNAs as well as transgenic animal models manipulating miRNA expression may help in these efforts. On a cautionary note, since miR-203 has many effects on cell growth, such as on differentiation, proliferation and migration [14, 17, 74], care needs to be used in applying miR-203 targeted strategies. Unwanted effects on wound healing may occur. Future studies might also include both

investigations as to how miRNA levels are themselves regulated, and to identify additional miRNAs and their targets in neural and somatic tissues involved in supporting and suppressing pain and inflammation.

Acknowledgments

The work was also supported by National Institutes of Health (Bethesda, Maryland) award GM079126.

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Final Boxed Summary Statement

What we already know about this top

- microRNAs inhibit gene expression and comprise a target for therapeutic intervention
- microRNAsinfluencing pain in the incisional environment have not been characterized

What this article tells us that is new

- In this rodent study, miR-203 is an microRNAs that is downregulated in incisions and controls phospholipase A2 which in turn influences inflammation and pain after incision

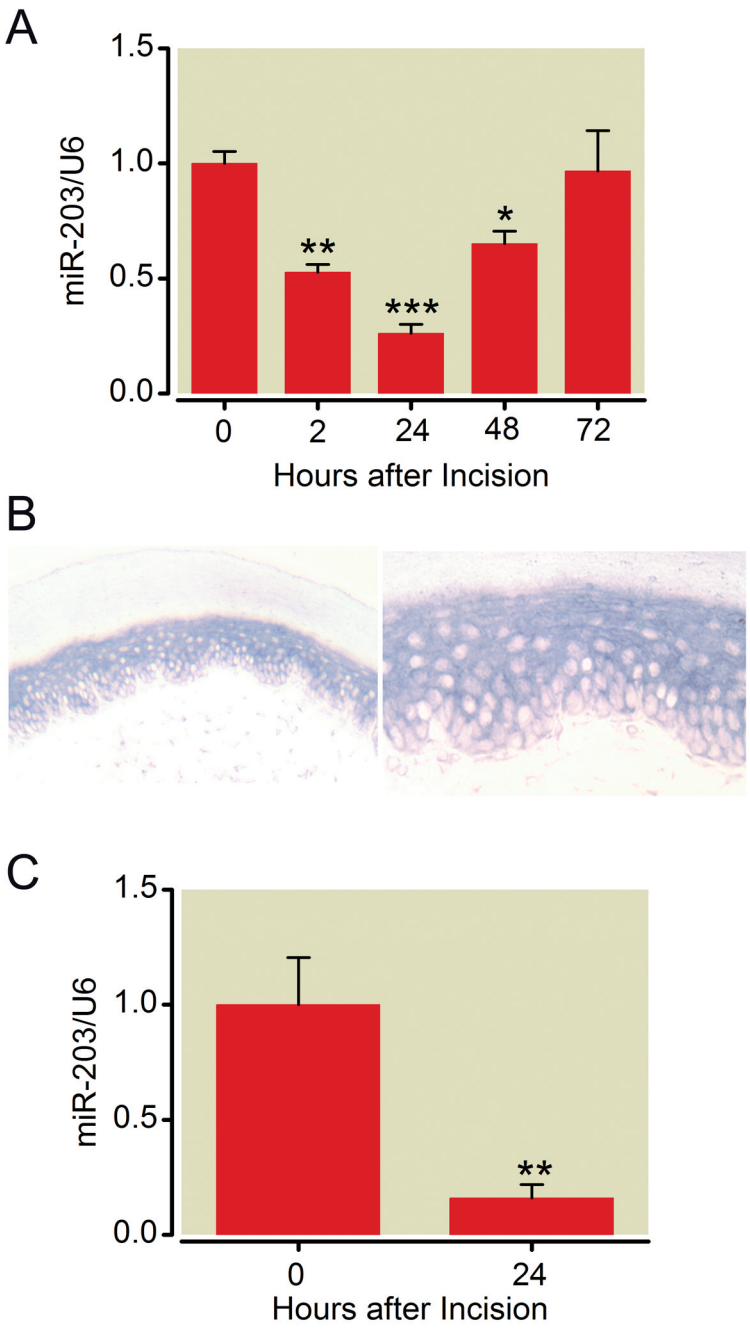

Figure 1.

miR-203 expression in the hindpaw skin before and after incision. (A) miR-203 level was significantly down-regulated in the whole hindpaw skin samples collected after incision. (B) In situ hybridization with oligonucleotide probes specific to miR-203 in normal mouse hindpaw skin. These images demonstrate that miR-203 (purple) was localized in the epidermis, especially in the suprabasal layer. Magnification x100 (left) and x400 (right). (C) miR-203 level in the epidermis collected by laser capture microdissection was significantly reduced at 24h after incision. Values are displayed as the mean ± SEM, n = 6, *p<0.05, **p<0.01 or *** p<0.001 *vs.* 0h just before incision.

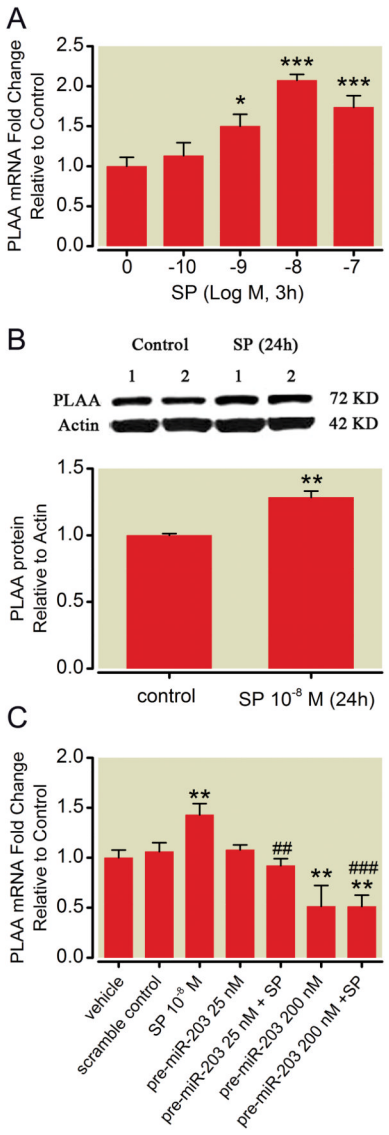

Figure 2.

Changes in phospholipase A2 activating protein (PLAA) expression after substance P (SP) exposure and pre-miR-203 transfection in epidermal keratinocyte (REK) cells. Both PLAA mRNA (A) and protein (B) expression were up-regulated at 3h or 24h after SP exposure in REK, respectively. Values are displayed as the mean ± SEM. *p<0.05, **p<0.01 or ***p<0.001 *vs.* control group. (C) Repression of PLAA mRNA by pre-miR-203 transfection in REK cells after SP exposure. REK cells were transfected with the pre-miR-203 (25nM and 200nM) or scramble control after the 3h exposure of SP (10^{-8}M). Values are displayed as the mean ± SEM. **p<0.01 *vs* scramble control group; $^{\#\#}$ p<0.01 or $^{\#\#\#}$ p<0.001 *vs.* SP (10^{-8}M) treated group.

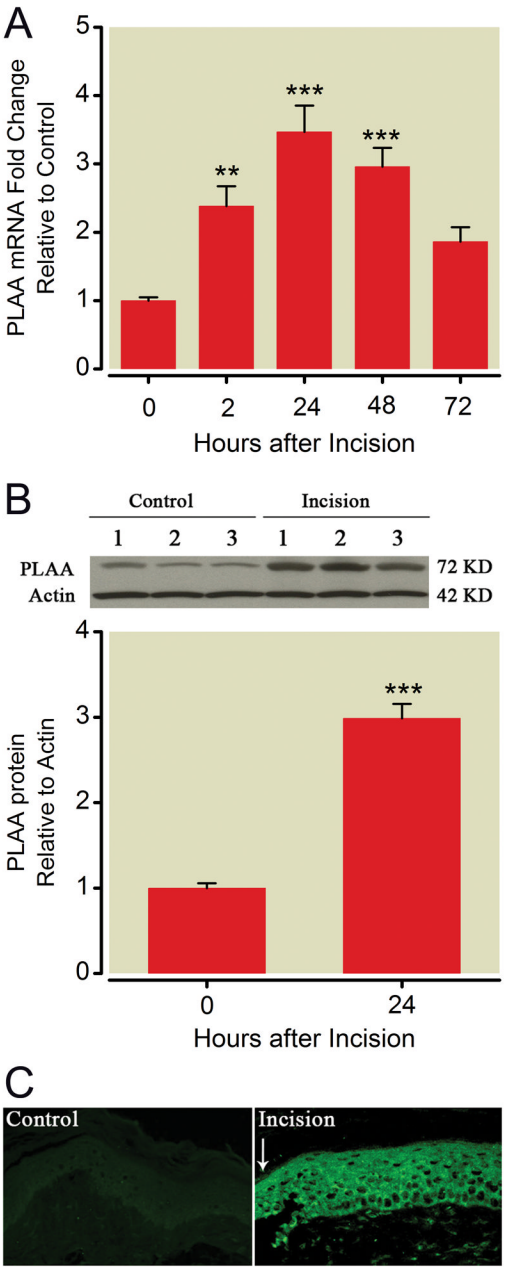

Figure 3.

Phospholipase A2 activating protein (PLAA) expression in the hindpaw skin before and after incision. Both PLAA mRNA (A) and protein (B) levels were up-regulated after incision. Values are displayed as the mean ± SEM, n = 6, **p<0.01, *** p<0.001 *vs.* 0h just before incision. (C) Immunostaining of PLAA was increased in the epidermis at 24h after paw incision. Arrow: the edge of incision. Magnification x200.

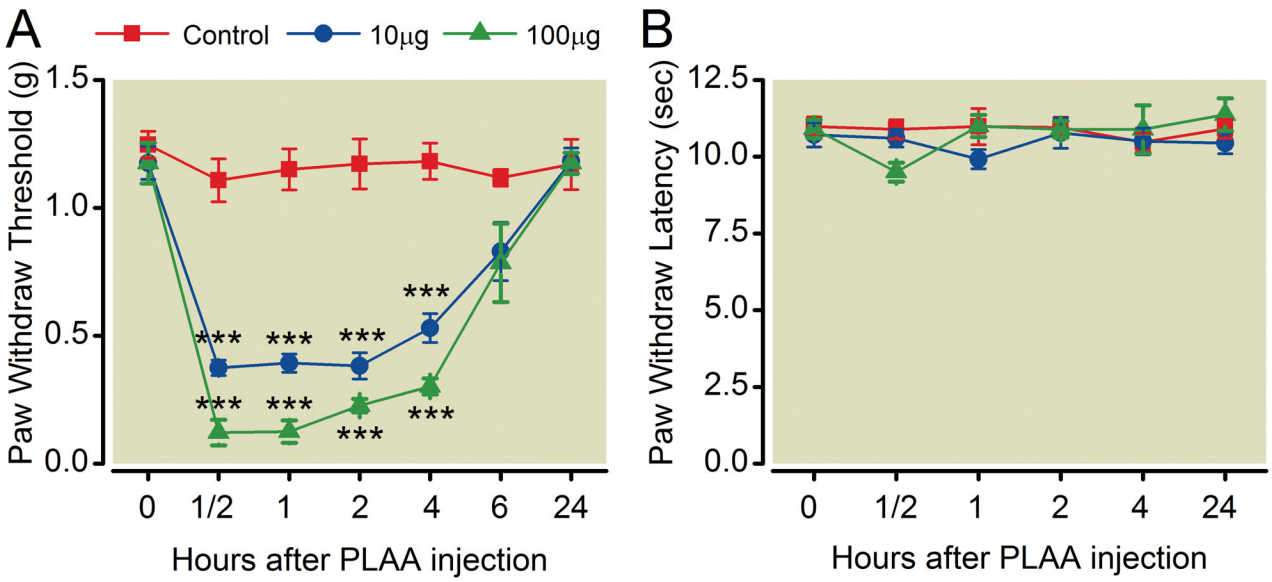

Figure 4.

Changes in hindpaw nociceptive sensitivities induced by intraplantar injection of phospholipase A2 activating protein (PLAA) peptide in normal control mice. Local application of PLAA peptide increased mechanical allodynia (A) without any alteration of thermal sensitivity (B). Values are displayed as the mean ± SEM, n = 6, *** $p<0.001$ *vs.* control group.

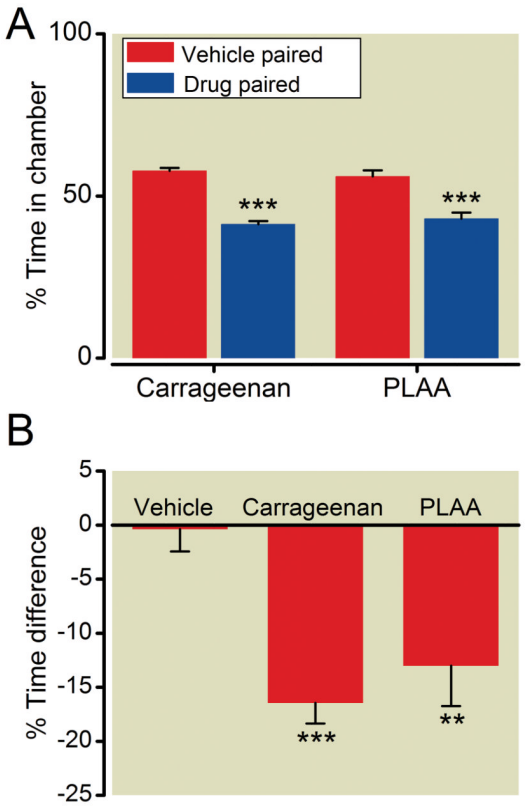

Figure 5.

Comparison of phospholipase A2 activating protein (PLAA) and carrageenan induced conditioned place aversion (CPA). (A) Comparison of the time-difference between the treatment paired and vehicle paired compartment shows both PLAA and carrageenan to induce CPA. (B) Treatment with either drug causes the mice to develop CPA compared to vehicle treatment alone. Data presented as Mean ± S.E.M, n = 10, **p<0.01, ***p<0.001 *vs.* vehicle treatment.

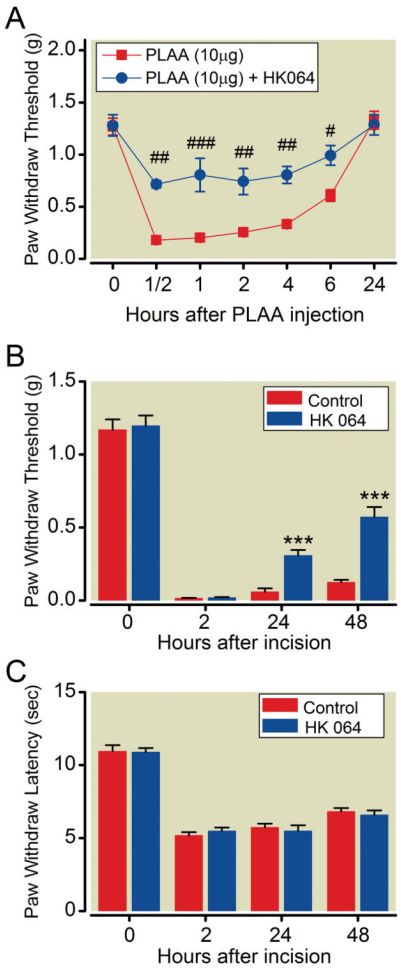

Figure 6.

Assessment of secretory phospholipase A2 inhibitor (HK064) effects on phospholipase A2 activating protein (PLAA)- or incision-induced mechanical and thermal allodynia. Blocking secretory phospholipase A2 activity reversed PLAA- (A) or incision-(B) induced mechanical allodynia, but did not change incision-induced thermal hyperalgesia (C). Values are displayed as the mean ± SEM, n = 6, **p<0.01, *** p<0.001 *vs.* control group; $^{\#}$ p<0.05, $^{\#\#}$ p<0.01 or $^{\#\#\#}$ p<0.001 *vs.* only PLAA (10μg) treated group.

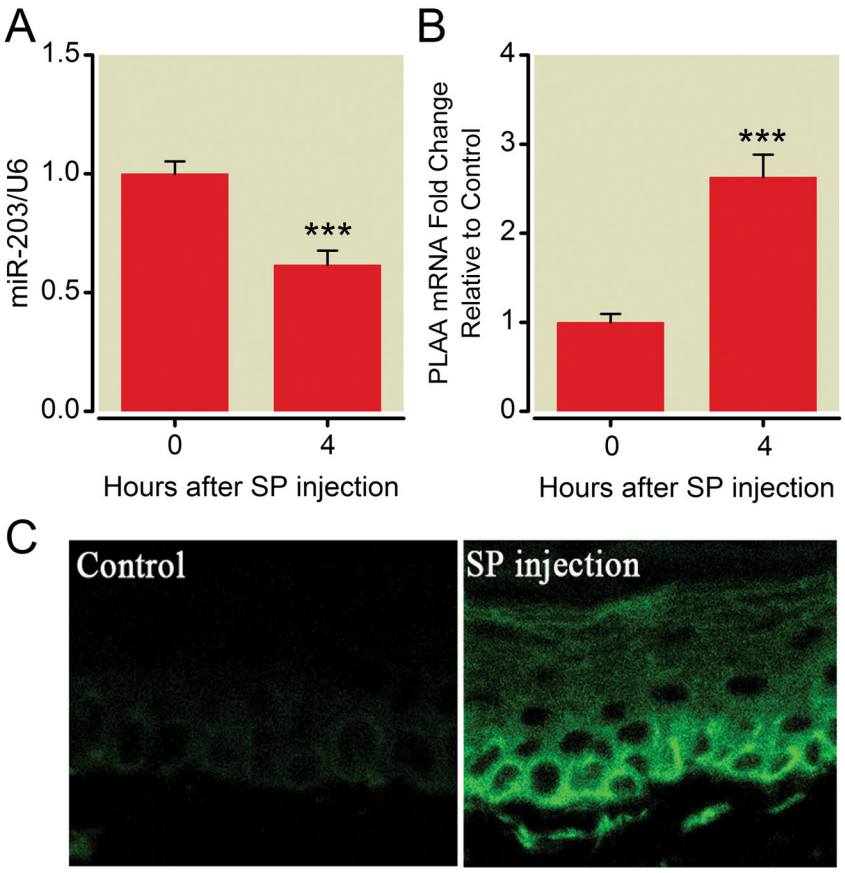

Figure 7.

Changes in miR-203 and phospholipase A2 activating protein (PLAA) mRNA expression induced by intraplantar injection of substance P (SP) in normal control mice. Local SP injection induced the downregulation of miR-203 (A), and upregulation of PLAA mRNA (B) and protein (C) at 4h after SP injection. Values are displayed as the mean ± SEM, n = 6, *** $p<0.001$ *vs.* 0h just before incision. (C) Magnification x200.

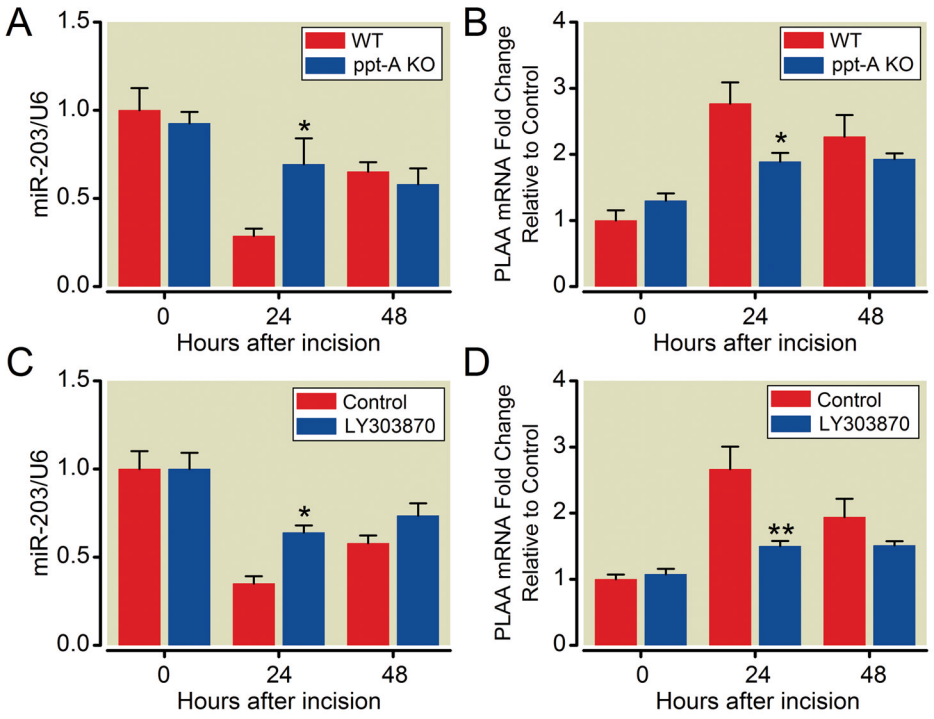

Figure 8.

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 downregulation of miR-203 level (A and C) and less upregulation of PLAA mRNA level (B and D) in ppt-A$^{-/-}$ mice and LY303870 treated group compared with wide type mice (WT) 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 *vs.* wild-type group or vehicle treated group.

Table 1

Mouse PLAA was the Target Gene of miR-203.

Mouse ortholog of target gene	Gene name	Conserved sites	Poorly conserved sites	Representative miRNA	Total context score
PLAA	Phospholipase A2-activating protein	1	4	mmu-miR-203	−1.50
B3GNT5	UDP-GlcNAc: betaGal beta-1, 3- N-acetylglucosaminyltransferase 5	1	4	mmu-miR-203	−1.31
AFF4	AF4/FMR2 family, member 4	2	5	mmu-miR-203	−1.25
C22orf9	Chromosome 22 open reading frame 9	1	2	mmu-miR-203	−1.00
LRRTM2	Leucine rich repeat transmembrane neuronal 2	1	3	mmu-miR-203	−0.99

Shown are the top 5 molecular targets sorted by total context score, which was predicted from TargetScan 5.2 (http://www.targetscan.org). TargetScan is from Whitehead Institute for Biomedical Research (Cambridge, MA) [37–39].

Table 2

Predicted miR-203 Target Sites on PLAA 3′-untranslated Region were Identified by TargetScan 5.2 Software.

Target sites	Predicted consequential pairing of target region (top) and miRNA (bottom)
Position 415–421 of PLAA 3′ UTR	5' . . . UCAAUAGCAUGAUGCAUUUCAA . . . ||||||| 3' GAUCACCAGGAUUUGUAAAGUG
Position 452–458 of PLAA 3′ UTR	5' . . . AUUUACUUGAAAAAUCAUUUCAA . . . ||||||| 3' GAUCACCAGGAUUUGUAAAGUG
Position 591–597 of PLAA 3′ UTR	5' . . . UGUUAUAUUACAUAUCAUUUCAG . . . ||||||| 3' GAUCACCAGGAUUUGUAAAGUG
Position 737–743 of PLAA 3′ UTR	5' . . . AGUAGUGUCAUCUUUCAUUUCAG . . . ||| || ||||| 3' GAUCACCAGGAUUU-GUAAAGUG
Position 771–777 of PLAA 3′ UTR	5' . . . UUGAUCAUAUUCAUUAUUUCAAA . . . |||||| 3' GAUCACCAGGAUUUGUAAAGUG

UTR = untranslated region.

TargetScan is from Whitehead Institute for Biomedical Research (Cambridge, MA) [37–39].