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Ex vivo non-viral gene delivery of μ -opioid receptor (*OPRM1*) to attenuate cancer-induced pain

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Abstract

Virus-mediated gene delivery shows promise for treatment of chronic pain. However, viral vectors have cytotoxicity. To avoid toxicities and limitations of virus-mediated gene delivery, we developed a novel non-viral hybrid vector: HIV-1 Tat peptide sequence modified with histidine and cysteine residues combined with a cationic lipid. The vector has high transfection efficiency with little cytotoxicity in cancer cell lines including HSC-3 (human tongue squamous cell carcinoma) and exhibits differential expression in HSC-3 (~45-fold) relative to HGF-1 (human gingival fibroblasts) cells. We used the non-viral vector to transfect cancer with *OPRM1*, the μ -opioid receptor gene, as a novel method for treating cancer-induced pain. After HSC-3 cells were transfected with *OPRM1*, a cancer mouse model was created by inoculating the transfected HSC-3 cells into the hind paw or tongue of athymic mice to determine the analgesic potential of *OPRM1* transfection. Mice with HSC-3 tumors expressing *OPRM1* demonstrated significant antinociception compared to control mice. The effect was reversible with local naloxone administration. We quantified β -endorphin secretion from HSC-3 cells, and showed that HSC-3 cells transfected with *OPRM1* secreted significantly more β -endorphin than control HSC-3 cells. These findings indicate that non-viral delivery of the *OPRM1* gene targeted to the cancer microenvironment has an analgesic effect in a preclinical cancer model, and non-viral gene delivery is a potential treatment for cancer pain.

Keywords

Cancer pain; gene delivery; μ -opioid receptor; *OPRM1*; oral squamous cell carcinoma

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Conflict of interest statement

The authors declare that they have no conflict of interest in regard to this work.

1. Introduction

Oral cancer patients often suffer from severe function-induced pain when they eat, drink or speak. Quality of life is severely degraded in these patients [1; 14; 21]. The intensity and prevalence of pain is generally higher in patients with oral cancer than in patients with other types of cancer [32; 48]. Oral cancer pain escalates with disease progression. Although opioids are initially effective for oral cancer pain, patients develop tolerance and require ever larger narcotic doses. Unfortunately, high dose opioids have limited efficacy when administered over an extended duration and off-target effects are severe.

Gene therapy holds potential for treating cancer pain along with other types of chronic pain; a single injection could produce a long-term effect [56] and target molecules involved in the pathophysiology of pain [52]. Several potential gene targets for cancer pain therapy are already known. *EDNRB*, the gene for the endothelin B (ET_B) receptor, is silenced in human oral squamous cell carcinoma (SCC) tissues, and adenovirus-mediated re-expression of *EDNRB* produces antinociception in a mouse cancer model [50]. Similarly, we show that *OPRM1*, the μ -opioid receptor gene, is hypermethylated and transcriptionally silenced in human oral SCC. We also demonstrated that adenovirus-mediated re-expression of *OPRM1* produces antinociception in a mouse cancer model [49].

Although our adenovirus-mediated gene transduction approach showed promising analgesic effects in a preclinical model, its translational potential is limited [50]. Viral transduction induces a strong immune response and the duration of gene transduction is limited [22]. Toxicity with viral vectors remains a concern; study subjects have died during clinical trials of adenovirus vectors [10] and adeno-associated virus vectors [7]. To preclude complications related to viral vectors, several studies have employed intrathecal injection of naked plasmid [23; 34], liposomal-complexed DNA [46; 47], or polymer-complexed DNA [1; 33]. A clinical trial of intrathecally injected naked plasmid encoding interleukin 10 (IL-10) in patients with chronic neuropathic pain has also been proposed [30]. Non-viral vectors generate a smaller immune response compared with viral vectors [20; 44]. Moreover, non-viral transfection facilitates delivery of genes that are too large to be carried by viruses. The *in vivo* efficiency of currently available non-viral vectors is too low for therapeutic purposes [38]. To avoid limitations associated with available non-viral vectors we created a novel non-viral hybrid vector composed of a modified HIV-1 Tat peptide (mTat) and a cell-permeable peptide combined with a cationic lipid, FuGENE HD (FH) transfection reagent [55]. Our non-viral vector has excellent transfection efficiency with low cytotoxicity across a range of cell lines, including different types of cancer cells; transfection with our non-viral vector leads to high and long-term transgene expression (~7 months) after intramuscular injection of the vector *in vivo* [54].

We hypothesize that non-viral gene transfection can stably re-express silenced genes and treat cancer-induced pain as effectively as viral transduction. Here we demonstrate the effects of *OPRM1* transfection of cancer cells using a non-viral vector in two (paw and tongue) mouse cancer pain models. We show analgesia in response to *OPRM1* transfection in both models.

2. Methods

2.1. Cell culture

Human oral SCC, HSC-3, and human gingival fibroblasts, HGF-1, were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Grand Island, NY), and 1% penicillin G (Life Technologies) with streptomycin sulfate. All cells were cultured at 37 °C in a humidified atmosphere with 5% CO₂. This same medium was used for transfection.

2.2. Plasmid DNAs

Plasmid DNA encoding for *luciferase* (gWIZ luciferase) and the *green fluorescent protein* (*GFP*) (gWIZ GFP) under the control of the cytomegalovirus promoter/enhancer obtained from Genlantis (San Diego, CA), and plasmid DNA encoding *OPRM1-GFP* (Origene, Rockville, MD) were used. Vectors were propagated in competent *E. coli* DH5α cells (Life Technologies). Ultrapure endotoxin-free plasmid DNA was prepared using the QIAfilter kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Each plasmid DNA was diluted in sterile water to a final concentration of 1 µg/mL DNA.

2.3. Non-viral vector

The HIV-1 Tat (RKKRRQRRRR) covalently fused with ten histidine and two cysteine residues (C-5H-Tat-5H-C), modified Tat (mTat) was obtained from Biomart Corporation (Cambridge, Canada). To prepare mTat/DNA complexes, the peptide solution (1 mM) and plasmid DNA encoding the gene for either *GFP* or *OPRM1-GFP* (2 µg/well each) were mixed in 5% glucose solution to a final volume of 60 µL/well. The solution was quickly mixed for 5 seconds. After the mixture was incubated at room temperature for 30 minutes, the cap of the sample tube was opened to expose the solution to air. The sample was shaken vigorously for 90 minutes. The cap was opened intermittently to allow for air replenishment. Plasmid DNA (2 µg/well) encoding the gene for either *GFP* alone as a control or *OPRM1-GFP* was added to FuGENE HD (FH, Roche, Indianapolis, IN), and then incubated at room temperature for 15 minutes. Sixty µL complexes (containing 2 µg DNA and FH) were added into a 6-well plate. Two vector/DNA groups were used: group 1, mTat/FH/*GFP*, and group 2, mTat/FH/*OPRM1-GFP*.

2.4. In vitro plasmid DNA transfection for luciferase expression

For luciferase expression, cells were plated in a 96-well plate at a density of 2×10^5 cells/mL (100 µL/well), cultivated in the appropriate growth medium with 10% FBS. One hundred µL of media with 10% FBS was added to each well. After 24 hours in culture, the old culture medium was aspirated and 100 µL fresh media with 10% FBS was added to each well. The vector/DNA (luciferase plasmid) complexes were then added to each well and incubated. The cells were cultured for 48 hours at 37 °C in 5% (v/v) CO₂ after transfection. All transfection assays were carried out in quadruplicate simultaneously. Also, we tested the cells treated with plasmid only as the controls.

For GFP or OPRM1-GFP expression, cells were plated in a 6-well plate at a density of 2×10^5 cells/mL (1 mL/well), cultivated in the appropriate growth medium with 10% FBS. One

ml of media with 10% FBS was added to each well. After 24 hours in culture, 1 mL fresh media with 10% FBS was added to the cells. Sixty μ L of the vector/DNA (*GFP* or *OPRM1-GFP* plasmid) complexes were then added to each well and incubated with the cells for 48 hours at 37 °C in 5% (v/v) CO₂ after transfection. Transfection assays were carried out in quadruplicate simultaneously. To employ stable transfection for *ex vivo* gene delivery in mice, the medium was changed to selection medium containing 0.8 mg/mL of G418 sulfate (Gemini Bio-Products, West Sacramento, CA). After the cells were cultured for 1–2 weeks, media was changed to a lower concentration of selection media containing 0.4 mg/mL of G418 sulfate. The cells were cultured for 40 days in the selection medium.

2.5. Detection of transgene expression

Transgene expression was detected at a standardized representative time point of 48 hours after transfection. Luciferase expression level was measured with Bright-Glo™ Luciferase Assay System (Promega, Madison, WI) using a multi-detection microplate reader, SpectraMax® M5 (Molecular Devices, LLC, Sunnyvale, CA). Relative light units (RLU) were recorded in duplicates with 10-s integration. This detection system is designed to measure directly the expression level using 96-well plates. The use of RLU/well as the unit of measure allowed for accurate detection of expression levels and is considered a well-accepted method in the field.

2.6. Cell viability evaluation

Cell viability of HSC-3 cells after transfection with the transfection reagents was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, MTT assay. HSC-3 cells (1×10^6 /mL) in 100 μ L of DMEM supplemented with 10% FBS were seeded in 96-well plates and incubated overnight. Then HSC-3 cells were transfected with mTat/FH/*GFP* or mTat/FH/*OPRM1-GFP* complex and incubated for 24 or 48 hours. The 5 mg/mL MTT reagent in 1×PBS (10 μ L/well) was added to the plates and incubated for 4 hours. After incubation, the medium was aspirated and dimethyl sulfoxide (100 μ L/well) was added to stop the reaction. The optical density was quantified in a multi-detection microplate reader, SpectraMax® M5, at 570 nm wavelength. The percentage of cell viability was calculated by comparing the appropriate optical density to the control cells that were not transfected.

2.7. Zeta potential

Zeta potentials of HGF-1 and HSC-3 cells were measured at 25 °C by a Zetasizer Nano ZS90 (Malvern Instruments Ltd, UK). This instrument is equipped with a red laser of wavelength 630 nm and measures the electrophoretic mobility of the particles using phase analysis of scattered light in an experimental set up similar to Laser Doppler Velocimetry (M3PALS technique, Malvern Instruments Ltd). Zeta potential was derived from the electrophoretic mobility using the Smoluchowski model since the measurements were performed in aqueous solutions of moderate ionic strength (*i.e.*, electrical double layer thickness $\ll$ the particle size). Each sample was observed with 20 repeated measurements across 3 trials.

2.8. Quantitative real-time polymerase chain reaction with reverse transcription (QRT-PCR)

For *caveolin* mRNA expression, the total RNA in the harvested HSC-3 and HGF-1 cells was isolated with RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The cells were homogenized in a lysis buffer. The lysis buffer containing the homogenate was centrifuged for 1 minute at 13,000 *g* at 4 °C. The supernatant was applied to RNeasy column, rinsed, and eluted. RNAs were measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and cDNA was synthesized using a total of 1 µg RNA using QuantiTect® Quantiscript reverse-transcriptase and RT Primer Mix, (Qiagen), according to the manufacturer's protocol. Real-time PCR was performed using Opticon Monitor 3® (Bio-Rad, Hercules, CA, USA) with Rotor-Gene™ SYBR® Green (Qiagen), according to standard protocols. The sequences of caveolin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene specific primers are as follows: caveolin forward: 5'-AAGACCTGCCTAATGGTTCTGC-3'; reverse: 5'-CTCGTACACAATGGAGCAATGAT-3'; GAPDH forward: 5'-ACACCCACTCCTCCACCTTT-3'; GAPDH reverse: 5'-TAGCCAAATTCGTTGTCATACC-3'. Standard curves were generated for each gene, and the amplification was found to be 90%–100% efficient. The relative quantification of gene expression was determined by the comparison of threshold values. All the results were normalized to GAPDH gene. The results presented are the average of four replicate experiments. All the graphic data for mRNA expression are presented as the fold expression relative to the reference control cells.

For *OPRM1* mRNA expression, the total RNA in the harvested HSC-3 cells was isolated with RNeasy Mini Kit, according to the manufacturer's instructions. The transfected HSC-3 cells were first homogenized for 30 seconds in a lysis buffer. The lysis buffer containing the homogenate was centrifuged for 1 minute at 13,000 *g* at 4 °C. The supernatant was applied to RNeasy column, rinsed and eluted. RNA concentration was measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and treated with DNase I. mRNA was reverse transcribed with Random Hexamers. An 8-µL cDNA aliquot was amplified in 25 µL of 2× TaqMan universal PCR master mix and 2.5 µL of 20× TaqMan primer and probe mix (Applied Biosystems, Carlsbad, CA) under the following PCR conditions: 2 minutes at 50 °C, 10 minutes at 95 °C, 50 cycles of 95 °C for 15 seconds, and 60 °C for 1 minute. The TaqMan gene expression assay for *OPRM1* (Hs01053957_ml, Applied Biosystems) was used as it does not detect residual genomic DNA. Human β-glucuronidase (*GUSB*, 4326320E, Applied Biosystems) was used as the endogenous control. Threshold analysis was carried out using the software supplied with the Opticon Monitor™ 3 (Bio-Rad, Hercules, CA). Each sample was measured in quadruplicate. Relative *OPRM1* transcript levels were determined using the 2^{-C_t} method with *GUSB* as the internal control.

2.9. Fluorescence microscope

To observe transgene GFP protein expression, 48 hours after transfection, the medium was removed and the cells were rinsed twice with PBS. Images of GFP expression were captured using a fluorescence microscope, Axiovert 200 and AxioCan digital camera both from Carl

Zeiss (Thornwood, NY), and processed with a SPOTTM software version 4.6 (Diagnostic Instruments, Inc, Sterling Heights, MI). Transfection efficiency was measured by the following steps: (1) randomly counting five fluorescence (F) field views and five black (B) field views for each well; and (2) calculating the transfection efficiency rate (%), the mean cell numbers of F were divided by the mean cell numbers of B and multiplied by 100.

2.10. Enzyme-linked immunosorbent assay (ELISA)

The μ -opioid receptor (MOR) was measured in HGF-1 and HSC-3 cells transfected with *GFP* plasmid or *OPRM1* plasmid using an ELISA (MyBioSource, Inc., San Diego, CA). Cells were seeded at a density of 3×10^5 cells/mL in 10 cm culture plate. After 24 hours incubation, medium was removed and cells were rinsed with ice-cold PBS, then cells were scraped from the plate and collected. ELISA was performed according to manufacturer's instructions. Samples were run in quadruplicate and measured using a spectrophotometer at the recommended wavelength.

The opioid peptides β -endorphin, met-enkephalin, and leu-enkephalin were quantified in the supernatant of *OPRM1*-expressing HGF-1 and HSC-3 cells with or without *OPRM1* antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂ (CTOP, Sigma-Aldrich, St. Louis, MO) using an ELISA (MD Bioproducts, St. Paul, MN). Four sample groups were prepared as follows: (1) non-transfected cells; (2) *GFP*-expressing cells; (3) *OPRM1*-expressing cells; and (4) *OPRM1*-expressing cells treated with CTOP (final concentration 10^{-10} , 10^{-11} , and 10^{-12} M) for 1 hour at 37 °C. Cells were seeded at a density of 2×10^5 cells/mL (500 μ L/well) and were grown to 70–80% confluence in 24-well plates, then incubated for 24 hours in 1 mL fresh DMEM with 10% FBS and supplements. Cell supernatant was collected and 10 μ L Halt Protease Inhibitor (Thermo Scientific, Rockford, IL) was added. Samples were transferred to the β -endorphin ELISA plate, and ELISA was performed according to manufacturer's instructions. Samples were run in quadruplicate and measured using a spectrophotometer at the recommended wavelength. The minimum detectable dose of β -endorphin, met-enkephalin, or leu-enkephalin ELISA kit was 10 pg/mL, 1 pg/mL, and 100 pg/mL, respectively.

2.11. SCC mouse model

The cancer pain mouse model was produced as previously described [39]. Experiments were performed on adult female BALB/c, athymic, immunocompromised mice weighing 16–20 g at the time of SCC inoculation. The mice were housed in a temperature-controlled room on a 12:12 hour light cycle (0700–1900 hour light), with unrestricted access to food and water. All the procedures were approved by the New York University Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. Twenty mice were divided into two inoculation groups and inoculated with the respective cell types: (1) HSC-3 expressing *GFP* and (2) HSC-3 expressing *OPRM1*. Cells (10^7) from each group, after 40-day stable transfection, were suspended in Matrigel (Becton Dickinson & Co., Franklin Lakes, NJ) to a total volume of 50 μ L and inoculated into the plantar surface of the right hind paw. Inhalational isoflurane at 2–5% was used for anesthesia to inoculate.

2.12. Paw volume measurement and cellular proliferation for SCC paw model

Paw volume measurements were performed with a plethysmometer (IITC Life Sciences, Woodland Hills, CA). The paw was inserted into a water cell of which pressure is changed due to the immersion. The volume change was calibrated in milliliters and shown on an electronic monitor. The measurements were accurate by 1 μ L. Triplicate measurements were taken for each mouse. Paw volume measurements were made at 0, 13, and 17 days relative to inoculation of HSC-3 cells. Each mouse was used as its own control, and relative changes in paw volume were calculated based on day 0 baseline. Cellular proliferation was also measured in the cancers using Ki67, a nuclear protein used to evaluate proliferation. Cancer sections from animals in the *OPRM1* and *GFP* groups were stained for Ki67 (rabbit anti-Ki67, 1:400; Abcam, Cambridge, MA). After incubation in primary antibody, sections were rinsed in PBS three times for 10 min each and then incubated in the FITC-AffiniPure goat anti-rabbit secondary antibody (1:400; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. The number of positive cells were counted in 3 randomly selected sections from 3 mice per treatment group.

2.13. Mechanical allodynia measurement in the paw cancer mouse model

Paw withdrawal testing was performed as described previously [39]. Testing was performed by an observer blinded to the experimental groups. Mice were placed in a plastic cage with wire mesh floor, which allowed access to the paws. One hour was allowed for acclimation prior to testing. The probe was applied to the mid-plantar right hind paw. Paw withdrawal threshold was determined in response to pressure from an electronic von Frey anesthesiometer (2390 series, IITC Life Sciences, Woodland Hills, CA). The amount of pressure (g) needed to produce a paw withdrawal response was measured six times on each paw separated by 3 minute intervals to allow resolution of the previous stimulus. The results of the six values were averaged for each paw for that day. Behavioral measurements were performed on post-inoculation days (PID) 13 and 17, when tumor growth was present in all animals.

2.14. Thermal hyperalgesia measurement in the paw cancer mouse model

Thermal hyperalgesia of the hind paw was quantified according to the Hargreaves' method [19]. Mice were acclimated to the test room and chamber for 30 minutes twice a week for two weeks. Mice were then acclimated in the Plantar Test Apparatus for 30 minutes prior to actual testing. The device consisted of a glass surface upon which the mice were placed individually in Plexiglass cubicles. The glass surface temperature was maintained at 30 °C. The thermal nociceptive stimulus originating from a focused projection bulb was manually manipulated to permit the stimulus to be delivered separately to the hind paw of each test animal. This stimulus was positioned under the right footpad of each mouse. The elapsed time required for a paw withdrawal response was measured automatically and considered an index of the heat nociceptive threshold. Paw withdrawal to heat was calculated as a mean of six measurements, carried out at 3 minute intervals. An automatic 20 sec cut-off was used to minimize tissue damage.

2.15. Orofacial nociceptive measurement in the tongue cancer mouse model

The Dolognawmeter is a validated device and assay invented to measure oral function and nociception in mice with oral cancer [6]. In brief, each mouse was placed into a confinement tube with two obstructing dowels in series. The mouse voluntarily gnaws through the two dowels to escape from confinement within the tube. Each obstructing dowel is connected to a digital timer. When the dowel is severed by the gnawing of the mouse, the timer is automatically stopped and records the duration of time to sever each of the two dowels. To acclimatize the mice and improve consistency in gnawing duration, all mice were trained for 5 sessions in the Dolognawmeter. Training involves placing the animals in the device and allowing them to gnaw through the obstructing dowels in exactly the same manner that they do so during the subsequent experimental gnawing trials. A baseline gnaw-time value to sever the second dowel was established for each mouse as the mean of the final three training sessions. After baseline gnaw-times were established for each mouse, the mice were inoculated with HSC-3 expressing *GFP* ($n = 15$) or *OPRM1* ($n = 10$). Gnaw time was measured on days 4, 8, 11 and 15 following tongue cancer inoculation.

2.16. Local naloxone administration prior to behavioral testing

Mice inoculated with HSC-3 expressing *GFP* ($n = 5$) or *OPRM1* ($n = 5$) were used for mechanical allodynia or thermal hyperalgesia quantification. Separate groups of mice were used for each behavioral test. Testing was performed on PID 19. Naloxone (500 $\mu\text{g/kg}$) dissolved in 20 μL PBS or 20 μL PBS (control) was injected into the right hind paw at the site of the tumor. Behavioral testing was performed at intervals up to 180 minutes post-injection, and then 24 hours post-injection. Four measurements were taken for each time point.

2.17. Statistical Analysis

Statistical analysis was performed using Sigma Plot for Windows (version 11.0). Data was analyzed using Student's *t*-test, One-Way ANOVA, ANOVA on the Ranks, Holm-Sidak, or Dunn's test as appropriate. Statistical significance was set at $p < 0.05$. Results were presented as mean $\pm$ standard error of the mean (SEM).

3. Results

3.1. The transfection efficiency of luciferase plasmid with mTat/FH in gingival fibroblasts and oral SCC cells

To clarify differences in transfection of human gingival fibroblasts (HGF-1) and oral SCC cells (HSC-3), transfection efficiency was measured by luciferase activity. Plasmid DNA without the vector/DNA was used for control. The luciferase expression in HSC-3 cells was significantly higher (~45-fold) than in HGF-1 cells ($p < 0.001$) (Figure 1).

3.2. The effect of transfection with mTat/FH on viability of HSC-3 cells

To investigate the effect of transfection reagents following transfection, cell viability with the mTat/FH/*GFP* or mTat/FH/*OPRM1-GFP* complex for transfection in HSC-3 cells

evaluated by MTT assay. There was no significant difference among these groups ($p = 0.072$) (Figure 1B).

3.3. Characterization of transfection parameters in gingival fibroblasts and oral SCC cells

To characterize transfection parameters in HGF-1 and HSC-3 cells, we first measured gene expressions of caveolin in both cells using QRT-PCR. The gene expression of *caveolin* in HSC-3 cells was significantly higher (~5-fold) than HGF-1 cells ($p < 0.001$) (Figure 2A). In addition, the zeta potentials of both cell types were determined to assess the charge of cell membrane. The zeta potential of HSC-3 cells (-9.1 ± 2.2 mV) was significantly less negative compared to HGF-1 cells (-14.8 ± 1.9 mV) ($p < 0.05$) (Figure 2B).

3.4. Fluorescence protein expression in oral SCC cells transfected with a non-viral vector/OPRM1-GFP

To explore the biological role of *OPRM1* re-expression on cancer cells, the non-viral vector mTat/FH with plasmid DNA encoding *OPRM1* gene tagged with *GFP* was transfected in HSC-3 *in vitro*. Green fluorescence protein expression was observed and used as a surrogate for *OPRM1* expression subsequent to transfection. Figure 3A shows that *GFP* expression was observed in oral SCC cells transfected with *OPRM1-GFP* plasmid. Similarly, *GFP* expression was observed in HSC-3 cells transfected with *GFP* control plasmid. No *GFP* was detected in control non-transfected HSC-3 cells. The transfection of *OPRM1-GFP* plasmid showed similar expression efficiency to that of the control *GFP* plasmid ($65.5 \pm 4.9\%$ and $66.6 \pm 3.5\%$, respectively; $p = 0.97$) (Figure 3B).

3.5. OPRM1 mRNA and MOR expression in gingival fibroblasts and oral SCC cells stably transfected with OPRM1 plasmid

QRT-PCR quantification of *OPRM1* mRNA expression showed that HSC-3 cells transfected with *OPRM1-GFP* plasmid had a significantly higher expression (~40-fold) than HSC-3 cells transfected with the control *GFP* plasmid ($p < 0.001$) (Figure 4A). The expression level of *GFP* plasmid transfection group was similar to the control non-transfected cells. Furthermore, the results of ELISA showed that MOR expression in *OPRM1-GFP* plasmid-transfected HSC-3 cells (31.8 ± 4.7 pg/ 10^6 cells) was significantly higher than in control *GFP*-transfected cells (3.8 ± 0.8 pg/ 10^6 cells) ($p < 0.01$). Also, MOR expression in *OPRM1-GFP* plasmid-transfected HGF-1 cells (4.7 ± 0.6 pg/ 10^6 cells) was significantly higher than in control *GFP*-transfected cells ($p < 0.05$) though it was significantly lower than in *OPRM1-GFP* plasmid-transfected HSC-3 cells ($p < 0.001$, Figure 4B). The expression level in *GFP*-transfected groups were also similar to the control non-transfected cells groups.

3.6. Oral SCC tumors re-expressing μ -opioid receptor produced lower levels of mechanical allodynia than control tumors in the paw cancer models

HSC-3 cells that were transfected with either *GFP* or *OPRM1* were inoculated into the right hind paw to create the cancer mouse model. The paw was chosen as a site of oral cancer inoculation because the current gold standard assay to quantify mechanical or thermal hypersensitivity is the paw withdrawal assay. The two groups, (1) HSC-3 tumors expressing *GFP* and (2) HSC-3 tumors expressing *OPRM1*, had paw volumes that were not

significantly different from each other. Average paw volumes at baseline for both groups was 0.17 mL. Average paw volumes at PID 17 were 0.29 mL and 0.26 mL, respectively. There was no difference in cellular proliferation, as measured by Ki67, in the HSC-3 tumors expressing *GFP* or *OPRM1* (Figure 5). Therefore, re-expressing *OPRM1* did not significantly change tumor proliferation. Re-expression of *OPRM1* did, however, affect mechanical allodynia. Tumors re-expressing *OPRM1* showed a significantly higher mechanical threshold than control tumors expressing *GFP*. Figure 6A shows that mice with tumors re-expressing *OPRM1* had significantly higher mechanical thresholds than the control mice on both PID 13 and 17. Mice with tumors expressing *OPRM1* had thresholds 32% and 44% below day 0 baseline (group average = 4.6 g) on PID 13 and 17, respectively (Figure 6B). Control mice, on the other hand, had thresholds 77% and 70% below day 0 baseline (group average = 4.9 g).

3.7. Oral SCC tumors re-expressing OPRM1 demonstrated lower levels of thermal hyperalgesia than control tumors in the paw cancer model

Using the Hargreaves' test [19] as a measure for thermal hyperalgesia, we determined that mice with tumors re-expressing *OPRM1* experienced a significantly longer latency period than the control groups. The day 0 baseline latency for the *GFP* and *OPRM1* groups were 4.0 s and 4.1 s, respectively. Figure 7A shows that mice with tumors re-expressing *OPRM1* had significantly longer thermal thresholds than the control mice on both PID 13 and 17. The latencies for the *GFP* group on PID 13 and 17 were 40% and 45% below baseline, whereas the latencies for the *OPRM1* group were only 21% and 31% below baseline on the same days. *OPRM1* re-expression resulted in significantly lower thermal hyperalgesia (Figure 7B).

3.9. Oral SCC tumors re-expressing OPRM1 demonstrated less orofacial nociception in the tongue cancer model

HSC-3 cells that were transfected with either *GFP* or *OPRM1* were inoculated into the tongue to create the tongue cancer mouse model. The *OPRM1* exhibited significantly less orofacial nociception, as demonstrated by reduced change in gnaw time relative to baseline as compared to the *GFP* group at 15 days post-inoculation (Figure 8).

3.10. Mechanical and thermal antinociception are reversed by local naloxone administration

To determine whether the antinociceptive effect that resulted from *OPRM1* expression was naloxone-dependent, we tested the effect of local naloxone administration on mechanical and thermal hypersensitivity on PID 19. Five mice in the *GFP* and *OPRM1* groups were used for each type of hypersensitivity test. Figure 9 shows the results after naloxone administration for mechanical and thermal behavioral testing. The absolute values were compared to baseline values on day 0 prior to cancer inoculation. The mechanical antinociceptive effect present in the *OPRM1* group was reversed with naloxone administration and lasted 60 minutes after injection. While the pre-injection mechanical threshold in the *OPRM1* group was 43% below baseline, the threshold decreased to 75% below baseline ten minutes after naloxone injection. At 24 hours post-injection the mechanical threshold was again at 43% below baseline. Naloxone did not cause the same

significant decrease in mechanical threshold in the *GFP* group. The thermal antinociceptive effect was also reversed in the *OPRM1* group after local naloxone administration to the right hind paw and the effect lasted 60 minutes. The thermal threshold of the *OPRM1* group dropped from 33% pre-injection to 62% ten minutes after injection. By 24 hours post-injection the thermal threshold had increased back to 25% below baseline. Naloxone did not cause a significant decrease in thermal latency in the *GFP* group. Of note, however, the *GFP* group had an increase in both mechanical and thermal thresholds immediately after injection, attributable to sedation from isoflurane anesthesia used during drug injection.

3.11. β -endorphin, met-enkephalin and leu-enkephalin secretion in gingival fibroblasts and oral SCC cells expressing *OPRM1*

The mechanical and thermal antinociceptive effects in the *OPRM1* groups were reversible by naloxone, suggesting an opioid-dependent antinociceptive mechanism. We wanted to determine whether the effect was due to endogenous opioid secretion into the cancer microenvironment. We measured β -endorphin in the supernatant of HSC-3 cells that were stably transfected with *OPRM1* plasmid. We also determined the effect of CTOP, a selective μ -opioid receptor antagonist, on β -endorphin secretion. Figure 10 shows that *OPRM1*-expressing HSC-3 cells (185 ± 13 pg/mL) had significantly increased β -endorphin secretion compared to non-transfected cells (69 ± 13 pg/ml) and *GFP*-expressing cells (74 ± 13 pg/mL) (One-way ANOVA, Holm-Sidak test, $p < 0.001$). *OPRM1*-expressing HSC-3 cells treated with CTOP showed a significant dose-dependent (10^{-10} to 10^{-12} M) decrease in β -endorphin secretion (Figure 10). In contrast, β -endorphin was not detected from *OPRM1*-expressing HGF-1 cells. *OPRM1*-expressing HGF-1 and HSC-3 cells did not have significance met-enkephalin and leu-enkephalin secretion compared to non-transfected cells and *GFP*-expressing cells (data not shown).

4. Discussion

Using an *ex vivo*, non-viral, gene transfer method in this study, we demonstrated that re-expression of *OPRM1* led to significant attenuation of cancer pain behavior in a cancer mouse model. The magnitude of pain attenuation following transfection with our novel method was similar to that resulting from adenoviral transduction of the *OPRM1* gene [49]. There are limitations of the model we used. We used an orthotopic xenograft model which is created by inoculating human oral squamous cell carcinoma (HSC-3) into immunocompromised mice. The BALB/c *Foxn1^{nu}* lack cell mediated immunity which might impact the pain response. Using naloxone we demonstrated that the analgesic mechanism of our non-viral method entailed secretion of endogenous opioids into the cancer microenvironment. The stroma is a potential source of β -endorphin in the cancer microenvironment; however, we favor the cancer as the primary source of β -endorphin. In a previous publication we showed that cancer cells secrete β -endorphin following transduction with *OPRM1*. We confirmed these findings in the current study using non-viral transfection. We also confirmed that non-viral transfection is an effective approach for gene therapy in the treatment of cancer pain. It should be noted that inhalational isoflurane was administered prior to the injection of naloxone which might explain the antinociceptive effect observed shortly after administration of naloxone in the control group.

Gene therapy has shown promise in treating chronic pain, but most published preclinical and clinical trials employed viral vectors including adenovirus, adeno-associated virus, retrovirus, or herpes simplex virus (HSV) [14; 53]. In a recent series of studies, Fink and colleagues delivered an HSV-based vector expressing different pain or analgesic mediators to peripheral sensory neurons. These studies demonstrated reduction of pain-related responses in rodent models of inflammatory pain, neuropathic pain, and bone cancer-induced pain. The tested mediators included δ -opioid agonist peptide enkephalin [24; 31], μ -opioid agonist peptide endomorphin-2 [18; 52], glutamic acid decarboxylase [17; 27], IL-4 [15], IL-10 [59], vascular endothelial growth factor [3], and tumor necrosis factor α [16]. In addition, the investigators performed a phase I human clinical trial using the HSV vector encoding enkephalin for treatment of moderate to severe intractable pain caused by head and neck cancer [8]. Viral vectors such as those that employ adenoviruses have proven to be cytotoxic. Non-viral vectors are less toxic and overcome other problems encountered in viral vector-mediated therapy, including elevated immune response, limited DNA carrying capacity, recombination, and limited stability of gene expression. In the current study, we stably expressed *OPRM1* using our non-viral vector. The sustained gene expression that we achieved would not have been possible with a viral vector (due to vector cytotoxicity). Our vector, mTat/FH, has low cytotoxicity and high transfection efficiency *in vitro* [55]. Furthermore the transfection efficiency (expression ~65% at 2 days after transfection) is higher than the transduction efficiency with an adenoviral vector (~50%) [11]. In addition, our *in vivo* studies using an mTat/FH vector demonstrate long-term transgene expression (~7 months) with little immune response after intramuscular injection of the vectors [54]. Our vector mTat/FH/DNA complex exhibits high expression efficiency because the zeta (electric) potential of the complex is less negatively charged and the particle size of the complex is smaller than the mTat- or FH-complex. The positive charge and small size of our mTat/FH/DNA complex allows the vector to easily pass through the cell membrane. Subsequently, there is greater transfection efficiency [55]. The increased efficiency of non-viral gene transfection is beneficial for patient care because a smaller vector dose is sufficient.

Interestingly, our current study demonstrated that our non-viral vector, mTat/FH, preferentially transfects oral cancer cells without affecting cell viability. To investigate the mechanism by which cancer cells are preferentially transfected, we studied several parameters related to transfection. Because our previous study demonstrated that internalization of mTat/FH vector is mediated by caveolae-mediated endocytic pathway [55], we measured mRNA expression of *caveolin*, an integral component of caveolae, in oral cancer cells and in normal fibroblasts. We found that the *caveolin* gene expression was higher in oral cancer cells than normal fibroblasts. We also found that the zeta potential of oral cancer cells was less negative than that in normal cells. Our findings related to zeta potential in oral cancer cells are similar to findings in cancer cells from other tissues including liver, breast, stomach, and pancreas. Cancers in these other tissues have been found to have more positive electric charge compared to normal cells [29; 35; 58]. Surface electric charge of cancer cells seems to be more suitable for transfection compared to normal fibroblasts. In addition, *in vitro* studies demonstrate that several types of cancer cells are softer than their normal tissue counterparts [13; 25; 26; 43; 45]. Cancer cells have proven to

be approximately 70% softer than normal cells that line the body cavity [4]. A reduction in membrane tension stimulates endocytosis [40; 41].

Our non-viral approach targets cancer cells while an HSV-based gene transfer technique targets neurons. The cancer cells targeted in our approach induce production of endogenous opioids. Secreted opioids remain in the cancer microenvironment and produce peripheral analgesia. We demonstrated that peripheral analgesia proved to be reversible with local naloxone administration. Most other gene transfection approaches that have been developed for cancer pain target the central nervous system, especially dorsal root ganglion (DRG) and spinal cord [12; 14]. Our rationale for targeting cancer cells rather than neurons is that cancer cells secrete a host of mediators that facilitate cancer-neuron crosstalk; either algesia or analgesia, can be generated depending on the mediator. A large body of literature, including previous publications from our lab, support the plausibility of an endogenous opioid approach for pain relief [2; 5; 9; 28; 36; 39]. Because almost all forms of cancer pain are generated at the site of the cancer, therapy targeted to the cancer microenvironment could reverse pain-producing mechanisms at the source and free patients of systemic drug toxicity.

In our non-viral approach we only transfected cancer cells but the same transfection technique could be employed to target neurons for the treatment of pain. Previously published work has demonstrated non-viral transfection in neurons. Plasmid DNAs with liposomes have low immunogenicity, a high level of transgene expression and high transfection efficiency [42], including transfection in DRG neurons following intrathecal injection [51]. To increase specificity, nerve growth factor peptides have been used to promote binding of naked DNA complexes to TrkA-positive DRG neurons [57]. A fragment of the tetanus toxin non-toxic subunit has been used to target the tetanus toxin receptor on DRG neurons [37].

We propose that non-viral gene transfection is a potentially effective, long-term treatment modality for cancer pain. We have demonstrated that the cancer microenvironment can be manipulated by selective gene transfer to the cancer through hybrid non-viral gene transfection. Our non-viral approach might be a viable option to treat cancer pain at its source in patients. Our non-viral approach might also be refined to achieve gene transfection in neurons adjacent to the cancer.

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Figure 1.

(A) The transfection efficiency of plasmid DNA encoding the gene for luciferase with mTat/FH in gingival fibroblasts (HGF-1) and oral SCC cells (HSC-3). Plasmid DNA without the vector was used for control. *** $p < 0.001$. (B) Cell viability with the mTat/FH/*GFP* or mTat/FH/*OPRM1*-*GFP* complex for transfection in HSC-3 cells evaluated by MTT assay. As a control (100% viability), non-transfected cells were used. There was no significant difference among these groups ($p = 0.072$).

Figure 2. Characterization of transfection parameters in HGF-1 cells and HSC-3 cells. (A) *Caveolin* mRNA expression in HGF-1 and HSC-3 cells measured by QRT-PCR. (B) Zeta potentials of HGF-1 and HSC-3 cells. * $p < 0.05$ and *** $p < 0.001$.

Figure 3.

Expression of GFP after transfection of plasmid DNA encoding *GFP* or *OPRM1-GFP* with a non-viral vector into oral SCC cells. (A) Green fluorescence images of non-transfected (as control), *GFP* plasmid transfected (as *GFP*), and *OPRM1-GFP* plasmid transfected HSC-3 cells (as *OPRM1*). Images are shown at 40× magnification. (B) The transfection efficiency of plasmid DNA encoding *GFP* or *OPRM1-GFP* with a non-viral vector in HSC-3 cells. Calculation is described in Materials and Methods. There is no significant difference between *GFP* and *OPRM1* ($p = 0.97$).

Figure 4.

Expression of *OPRM1* mRNA and μ -opioid receptor (MOR) in HGF-1 and HSC-3 cells transfected with *OPRM1* or *GFP* plasmid. Abbreviations represent non-transfected as control, *GFP* plasmid transfected as GFP, and *OPRM1* plasmid transfected HSC-3 cells as OPRM1. (A) Relative expression of *OPRM1* mRNA in control by QRT-PCR. Data represent expression relative to the level of the HGF-1 and HSC-3 cells control, set at 1. *** $p < 0.001$. (B) Expression of MOR in HGF-1 and HSC-3 cells stably transfected with *OPRM1* or *GFP* plasmid was measured by ELISA. Statistical comparison was analyzed between *OPRM1* plasmid and other groups. ** $p < 0.01$ and *** $p < 0.001$.

Figure 5.

Cancers produced with HSC-3 cells transfected with *OPRM1* did not show increased proliferation, as measured by Ki67, relative to tumors produced with HSC-3 cells transfected with *GFP* plasmid. (A) Representative cancer sections from the *OPRM1* and *GFP* groups stained for the proliferative marker Ki67. Bottom row shows sections stained for the marker and the nuclear stain Dapi. (B) There was no difference in cells positive for Ki67 in the *OPRM1* and *GFP* groups.

Figure 6.

Mice with HSC-3 tumors re-expressing *OPRM1* demonstrate lower mechanical allodynia than control. (A) Bar graph shows absolute mechanical withdrawal threshold (g) in mice with HSC-3 tumors re-expressing *GFP* or *OPRM1* on PID 13 and 17. (B) Bar graph shows percent change in mechanical withdrawal threshold from day 0 baseline. Mechanical thresholds of mice with tumors expressing *OPRM1* are significantly higher than the control groups on PID 13 and 17, indicating lower mechanical allodynia. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 7.

Mice with HSC-3 tumors re-expressing *OPRM1* demonstrate increased thermal hyperalgesia relative to control. (A) Bar graph depicts absolute thermal withdrawal threshold (sec) in mice with HSC-3 tumors re-expressing *GFP* or *OPRM1* on PID 13 and 17. (B) Bar graph shows percent change in thermal withdrawal threshold relative to day 0 baseline. Mice with tumors expressing *OPRM1* have significantly longer thermal latency than control mice, indicating lower thermal hyperalgesia. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Figure 8.

Mice with HSC-3 tongue tumors re-expressing *OPRM1* demonstrate reduced orofacial nociception relative to mice with HSC-3 tongue tumors re-expressing *GFP*. Mice with tongue cancer re-expressing *OPRM1* (n = 10) exhibited decreased gnaw time compared to control (*GFP*) mice (n = 15) at day 15 following cancer inoculation (* $p < 0.01$).

Figure 9.

Mice with HSC-3 tumors re-expressing *OPRM1* display naloxone-reversible antinociception. (A) Mice are treated with an intra-tumor injection of naloxone (500 µg/kg) on PID 19. Graph shows percent change in mean paw withdrawal threshold from day 0 baseline. Mice in the *OPRM1* group have less mechanical allodynia than control mice, but this effect is reversed with naloxone administration. Naloxone reversal brings the threshold of the *OPRM1* group to a lower threshold than the *GFP* group. The effect lasts 60 minutes; the *OPRM1* group returns to its pre-injection baseline by 24 hours. (B) Mice in the *OPRM1* group have less thermal hyperalgesia than the *GFP* group, represented by a smaller change from baseline latency. After naloxone administration, however, mice in the *OPRM1* group have increased thermal hyperalgesia indicated by a decrease in threshold, whereas the *GFP* group does not see a similar change. This effect lasts 60 minutes. By 24 hour, mice in the *OPRM1* group have returned to their pre-injection threshold, which is higher than the *GFP* group. * $p < 0.05$ and ** $p < 0.01$.

Figure 10.

An opioid peptide, β-endorphin secretion in the supernatant of *OPRM1* expressing HSC-3 cells with or without the μ-opioid inhibitor CTOP. Data are expressed as mean value ng/mg protein ± standard deviation from quadruplicates (white bar, non-transfected cell control; gray bar, *GFP* expressing cells; black bar, *OPRM1* expressing cells; and dot bars, *OPRM1* expressing cells treat with CTOP (10^{-10} to 10^{-12} M)). Statistical comparison was analyzed between *OPRM1* plasmid and other groups. * $p < 0.05$ and *** $p < 0.001$.