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Epigenetic Silencing is a Novel Mechanism Controlling Cancer-Induced Pain

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Epigenetic Silencing is a Novel Mechanism Controlling Cancer-Induced Pain

by

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DISSERTATION

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DOCTOR OF PHILOSOPHY

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in the

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of the

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by
Chi Tonglien Viet
To my parents, Chanh Viet and Dieulien Tong, and brother, Dan Viet, for their love.
Abstract: Cancer pain creates a poor quality of life for cancer patients. Chronic cancer pain is a major public health problem in which little progress has been made in treatment. In this dissertation we propose that pain is the result of imbalance between algesic and analgesic mediators within the cancer microenvironment. Furthermore, this imbalance arises from downregulation by hypermethylation of genes coding for analgesic mediators, and re-expression of these mediators by demethylating drugs produces analgesia. We determine whether EDNRB and OPRM1, genes of receptors mediating analgesia, are hypermethylated in the tumors of oral cancer patients, compared to normal and oral dysplasia controls. To determine whether the expression of these genes mediates analgesia, we create an orthotopic oral cancer mouse model, where we re-express the EDNRB or OPRM1 gene using adenoviruses. Re-expression of EDNRB results in mechanical analgesia, and re-expression of OPRM1 results in mechanical and thermal analgesia in the mouse cancer pain model. Finally, we determine the analgesic potential of demethylating drugs decitabine and zebularine in the mouse cancer model. We show that combination therapy with these two drugs results in an antiproliferative and analgesic effect in the mouse model.
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Chapter 1

Introduction

Cancer Pain

For most cancer patients, uncontrollable pain creates a poor quality of life (Hodder et al. 1997; Chaplin et al. 1999; Hammerlid et al. 2001; Connelly et al. 2004). Cancer patients suffer pain for months and even years, preventing them from functioning normally. Because improved chemotherapy and radiotherapy prolong survival, chronic cancer pain is becoming a major clinical problem without a proper solution.

There is no effective treatment for cancer pain. Although opiates are initially effective, escalating doses are required, and cancer patients develop opiate tolerance. The high doses of opiates required for cancer pain cause nausea, vomiting, constipation, and respiratory depression, which further reduce quality of life and can increase mortality. Effective pain relief has been elusive because the cause of cancer pain is unknown.

Cancer pain limits function independently of other factors. In studies of symptom clusters in patients with metastatic bone pain, patients' “worst pain” item clustered together with the functional interference items of walking ability, general activity, normal work, and enjoyment of life. Subsequent radiation therapy to alleviate metastatic bone cancer pain resulted in disintegration of this entire symptom cluster (Hadi et al. 2008a; Hadi et al. 2008b). Multivariate analysis of cancer-related breakthrough pain in a cross-sectional survey showed an
independent contribution to impaired functioning for activity, walking, work, social relationships, and sleep (Portenoy et al. 1999). Specifically for oral squamous cell carcinoma (SCC) pain, patients experience significantly high function-related pain even before metastasis, and increased functional restriction upon nodal metastasis (Connelly et al. 2004; Kolokythas et al. 2007). Intensity of oral SCC pain as measured by the Opioid Escalation Index is higher than pain of other cancers (Mercadante et al. 1997). As a result, oral cancer patients have poor quality of life because their intense pain keeps them from functioning, well before any regional metastasis.

Epigenetics as a Novel Mechanism of Cancer Pain

Chronic cancer pain is a major public health problem in which little progress has been made in treatment in the past century. In this dissertation we hypothesize a novel concept: that epigenetic changes within the cancer itself holds the key to cancer pain. The key to inducing endogenous analgesia in the cancer is therefore reversal of epigenetic gene silencing within the cancer. Currently there are no studies on epigenetic modulation of cancer pain. However, genes moderating endogenous analgesia are structurally capable of epigenetic silencing through DNA methylation. This dissertation seeks to demonstrate that this epigenetic key could unlock endogenous mediators of analgesia in the cancer microenvironment. Our research combines current knowledge of cancer pain mediators with novel demethylating drug treatments.
Cancer pain is caused by localized molecular mediators. Cancer pain has been hypothesized to result from either a tumor-mass effect or the activation of primary afferent nociceptors by mediators liberated by the cancer (Bjordal et al. 1995; Morton 1995; Diener 1996; Fujita et al. 1998; Mantyh et al. 2002). The tumor-mass effect is unlikely because even small carcinomas are painful (Connelly et al. 2004). Moreover, we have demonstrated that mediators produced by the cancer and secreted into the cancer microenvironment have a more significant role in cancer pain than does tumor size (Pickering et al. 2007). Therefore, therapy targeted to the cancer microenvironment could antagonize pain-producing mechanisms at the source while freeing the patient of systemic drug toxicity.

Endogenous analgesic mechanisms are present in cancer: the endothelin axis and opioid system. We hypothesize that cancer pain results from an imbalance in pain-producing and pain-relieving effects within the cancer microenvironment. The endothelin axis provides a compelling example of this imbalance. Endothelin-1 (ET-1) is a vasoactive peptide produced in high levels by many cancers and shown by us and others to have a clear role in cancer pain (Wacnik et al. 2001; Peters et al. 2004; Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010a; Quang et al. 2010b). ET-1 activates two receptor subtypes, the endothelin A receptor (ET\textsubscript{A}) and the endothelin B receptor (ET\textsubscript{B}) (Schmidt et al. 2007). Activation of ET\textsubscript{A} receptors on the primary afferent nerves within the cancer microenvironment leads to significant pain (Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010a). In opposition to this pain effect, we also showed that activation of ET\textsubscript{B} receptors by a selective agonist in a mouse squamous cell
carcinoma (SCC) model leads to the production and release of beta-endorphin, an opioid peptide that produces analgesia. The level of analgesia was approximately a 40% increase in paw withdrawal threshold, a similar level of analgesia to that observed with high dose morphine.

The likely mechanism for this opioid effect is that oral SCC consists of malignant keratinocytes, which bear ET\textsubscript{B} receptors and are known to secrete opioids (Wintzen et al. 1996; Zanello et al. 1999; Wintzen et al. 2000) that modulate the activity of the surrounding primary afferent nociceptors in skin (Lewin et al. 1993; Haberberger et al. 2000). Other carcinomas similarly secrete opioids (Melzig et al. 1998; Nagahama et al. 1998; Bagnato et al. 2008). Opioids bind and activate opioid receptors and activate a series of downstream events to produce an analgesic effect. We reversed this analgesia with co-administration of the opioid antagonist naloxone (Quang et al. 2010b). Naloxone is a competitive antagonist with highest affinity for the mu-opioid receptor, which is encoded by the \textit{OPRM1} gene.

**Analgesic receptors could be downregulated by promoter methylation.**

We have shown in a human oral SCC cell line that mRNA for ET-1 is nearly doubled, while mRNA for ET\textsubscript{B} receptors is severely down-regulated compared to normal oral keratinocytes (Quang et al. 2010b). Two lines of investigation reveal the epigenetic mechanism. First, in lung cancer, ET\textsubscript{B} receptors are down-regulated due to gene methylation (Knight et al. 2009). Second, our \textit{in vitro} preliminary data confirm that down-regulation of ET\textsubscript{B} receptors in oral cancer is also through promoter methylation. \textit{OPRM1}, the gene encoding the mu-opioid receptor, is also capable of being silenced by promoter methylation (Hwang et al. 2008).
Demethylation could produce an analgesic effect. Although at low ET-1 concentrations ET_B receptors mediate local inflammatory pain, at high concentrations these ET_B receptors mediate analgesia, attenuating the acute pain generated by ET-1 (Piovezan et al. 2000; Khodorova et al. 2002). This counterbalancing, pain-relieving effect is lost in cancers because ET_B receptors are not expressed. We therefore propose, based on our preliminary results, that if the promoters for ET_B receptors could be demethylated then the receptor could be expressed and activated by the high levels of ET-1 produced by the cancer, leading to the release of opioids within the cancer microenvironment. This endogenous mechanism would induce beta-endorphin release. Demethylation of OPRM1 could re-express mu-opioid receptors, which would moderate beta-endorphin production and produce analgesia.

Demethylating drugs are potential analgesics. Decitabine and zebularine are nucleoside analogs that inhibit methylation by trapping the catalytic DNA methyltransferases (DNMTs) and causing their degradation. They have clear antineoplastic effects, and are currently demethylating drugs of choice due to their efficacy and manageable side effects as described below. In our preliminary studies these drugs show promise as effective therapeutics against cancer pain.

Multiple phase II trials of decitabine for hematological malignancies have shown no systemic accumulation of the drug, and the toxicity profile of transient myelosuppression is predictable and manageable (Blum et al.; Cashen et al. 2010; Daskalakis et al. 2010). A recent North American phase III trial for myelodysplastic syndromes demonstrated superior quality of life during decitabine treatment even
though grade 3/4 neutropenia and thrombocytopenia were somewhat higher. Gastrointestinal toxicities were mild and infrequent (Atallah et al. 2007; Jabbour et al. 2008; Daskalakis et al. 2010). Solid tumors have also been effectively treated with decitabine in a recent phase I trial, where the only myelosuppression effect that was dose limiting was febrile neutropenia. In these very heavily pretreated solid tumor and lymphoma patients, the only non-haematological adverse effect was rare fatigue (Stewart et al. 2009).

Although decitabine cannot be given orally (Cheng et al. 2003), the new demethylating drug zebularine is both orally available and less toxic (Cheng et al. 2003; Yoo et al. 2004; Yoo et al. 2008). It has a half life of approximately 44 hours at 37°C in PBS at pH 1.0 and 508 hours at pH 7.0 (Yoo et al. 2004). Zebularine has a preference for tumor cells relative to normal fibroblasts, and is minimally cytotoxic in vitro and in vivo (Yoo et al. 2004). By reactivating hypermethylated genes, it has an anti-proliferative effect in multiple cancer models, including breast, bladder, and colon (Cheng et al. 2004; Yoo et al. 2008; Billam et al. 2009).

Zebularine also inhibits the degradation of decitabine by cytidine deaminase (Marquez et al. 2005; Daskalakis et al. 2010) and potentiates the effects of decitabine in breast cancer cell lines (Billam et al. 2009). Recently, it has been shown that when human bladder cancer cells are treated transiently with decitabine and then treated continuously with zebularine, remethylation was hindered and gene expression of reactivated genes was maintained (Cheng et al. 2004). These two drugs therefore act together to initiate and maintain demethylation of genes. Although clinical trials have not yet been done for zebularine, this drug shows
minimal side effects with long-term administration in mice with intestinal tumors. No weight change, toxicity, or histopathology was detected (Yoo et al. 2008). Because zebularine affected the expression of only a small percent of genes in the most actively dividing tissue, chronic demethylation treatment might not dramatically disturb global gene expression patterns, and could be less detrimental to normal tissues than expected (Yoo et al. 2008).

**Oral Cancer is an Optimal Cancer Pain Model**

Due to the intense, well-localized, and function-related pain that oral SCC patients exhibit (Connelly et al. 2004), oral SCC serves as a promising model to study cancer pain’s restriction on daily function. Oral SCC pain is more circumscribed, unlike cancers of other primary sites, such as the gastrointestinal tract or pelvis, which are more visceral (Hirshberg et al. 1996; Kim et al. 2000; Rigor 2000). Function-related pain in oral SCC predominates over spontaneous pain. Functional intensity, functional sharpness, and functional aching are associated with restricted speech, swallowing, eating, and drinking (Connelly et al. 2004; Kolokythas et al. 2007). The above characteristics of oral SCC pain are possibly due to the dense trigeminal nerve innervation of the oral cavity and to the high concentration of nociceptive mediators produced by the cancer.

In addition to these pain characteristics, oral SCC is a promising model for studying epigenetics because environmental factors, lifestyle, and individual variation all contribute to carcinogenesis. Finally, in addition to being a promising model for discovering pain mechanisms, oral SCC kills more people each year in the
United States than melanoma, cervical cancer, or ovarian cancer (Silverman 1998; Parkin et al. 1999). These patients experience excruciating pain during their final months of life that is refractory to traditional analgesics. Innovative treatment modalities with improved efficacy are necessary to improve quality of life for these patients.

**Hypothesis**

In this dissertation we hypothesize that endogenous analgesic mechanisms exist within cancer, and that cancer pain is caused by the imbalance between nociceptive and analgesic mechanisms. This imbalance could be caused by epigenetic silencing of genes that mediate analgesic mechanisms. The key to effective cancer pain treatment lies in re-expressing these genes through demethylation with chemotherapeutics.

To test our hypothesis, we focus on two genes that have known roles in mediating endogenous analgesia: **EDNRB** and **OPRM1**. We use oral SCC as our cancer pain model due to the advantages discussed. We determine whether these two genes are hypermethylated in cancer tissue of oral SCC patients, when compared to control contralateral normal tissue and tissue of oral dysplasia patients. To further define the role of these genes in endogenous analgesia, we establish a mouse oral SCC model with targeted re-expression of the genes by gene delivery with adenoviruses, and determine the extent of analgesic effect. Adenovirus-mediated re-expression serves as a controlled analog to the effect of demethylating drugs on these genes. Lastly, we determine the clinical feasibility of effective cancer
pain management through demethylating agents, by testing the analgesic potential of combined decitabine and zebularine treatment.

Our findings for each of the genes, EDNRB and OPRM1, will be presented in separate chapters, and will culminate with the findings of demethylation drug therapy as a potential treatment for cancer pain.

References


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Chapter 2

Re-expression of the Methylated \textit{EDNRB} Gene in Oral Squamous Cell Carcinoma Attenuates Cancer-Induced Pain

Abstract

Endothelin-1 is a vasoactive peptide that activates both the endothelin A (\(\text{ET}_A\)) and endothelin B (\(\text{ET}_B\)) receptors, and is secreted in high concentrations in many different cancer environments. While \(\text{ET}_A\) receptor activation has an established nociceptive effect in cancer models, the role of \(\text{ET}_B\) receptors on cancer pain is controversial. \textit{EDNRB}, the gene encoding the \(\text{ET}_B\) receptor, has been shown to be hypermethylated and transcriptionally silenced in many different cancers. In this study we demonstrate that \textit{EDNRB} is heavily methylated in human oral SCC lesions, which are painful, but not methylated in human oral dysplasia lesions, which are typically not painful. \(\text{ET}_B\) mRNA expression is reduced in the human oral SCC lesions as a consequence of \textit{EDNRB} hypermethylation. Using a mouse cancer pain model that is created using a human oral squamous cell carcinoma we show that \(\text{ET}_B\) receptor re-expression attenuates cancer-induced pain. These findings identify \textit{EDNRB} methylation as a novel regulatory mechanism in cancer-induced pain and suggest that demethylation therapy targeted at the cancer microenvironment has the potential to thwart pain-producing mechanisms at the source, thus freeing patients of systemic analgesic toxicity.
INTRODUCTION

Endothelin-1 (ET-1) is a vasoactive peptide that is produced and secreted into the cancer microenvironment in high concentration by many cancers including prostate, ovarian, breast, renal, bladder, cervical, bone and oral (Bagnato et al. 1997; Honore et al. 2000; Wacnik et al. 2001; Rosano et al. 2002; Peters et al. 2004; Carducci et al. 2006; Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010; Quang et al. 2010). Oral squamous cell carcinoma (SCC), in particular, expresses extremely high levels of ET-1, compared to other cancers (Pickering et al. 2008). ET-1 binds two receptors, the endothelin A (ET_A) and the endothelin B (ET_B) receptors. ET_A receptors have an established role in neuropathic and inflammatory pain (Davar et al. 1998; Jarvis et al. 2000; Baamonde et al. 2004). Activation of ET_A receptors also contributes to cancer pain. We have confirmed that it is the ET_A receptors on the primary afferent nerves within the cancer microenvironment that leads to significant pain (Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010).

Unlike ET_A receptor activation, evidence suggests that ET_B receptor activation has an anti-nociceptive effect in both non-cancer and cancer models (Khodorova et al. 2003; Peters et al. 2004). Khodorova and colleagues demonstrated that ET_B receptor activation on keratinocytes leads to an analgesic effect. Oral SCC consists of malignant keratinocytes. However, EDNRB, the gene encoding ET_B receptors, is frequently methylated in cancer. EDNRB methylation results in transcriptional silencing and has been demonstrated in cancers of many types, including lung,

In this study, we hypothesize that EDNRB is silenced through promoter methylation in oral SCC and that hypermethylation of this gene contributes to cancer induced pain. To investigate the role of EDNRB methylation in oral SCC pain we use a two-pronged approach. Firstly, to establish the clinical significance of EDNRB silencing on oral SCC pain, we quantify EDNRB promoter methylation in the biopsied tissues of oral dysplasia patients, and tumor and contralateral normal tissue from oral SCC patients. Secondly, we use a mouse oral cancer pain model to determine the behavioral effect of EDNRB re-expression in vivo.

MATERIALS AND METHODS

Tissue Collection

All procedures were approved by the University of California, San Francisco Committee on Human Research. We enrolled oral SCC patients with the following inclusion criteria: 1) biopsy-proven oral SCC and 2) no history of prior surgical, chemotherapeutic, or radiation treatment for head and neck SCC. We collected tissue at time of surgery from the primary cancer site and contralateral normal epithelium as control. Samples were flash frozen in liquid nitrogen and stored in -80°C. Demographic and health information were recorded for each patient. Cancer patients were staged according to the American Joint Commission on Cancer tumor-node-metastasis (TNM) staging system (Greene et al. 2002). Oral dysplasia tissue
was obtained from paraffin-embedded tissues archived from excisional biopsies. Patient demographics and tumor characteristics are shown in Table 1.

**RNA and DNA Extraction**

Thirty mg of each tissue collection sample was homogenized with a Mini Beadbeater-1 (BioSpec Products, Bartesville, OK) and subject to RNA/DNA extraction with AllPrep DNA/RNA Kit (Qiagen, Valencia, CA). RNA was eluted in a total volume of 50 ul and DNA was eluted in a total volume of 100 ul. RNA and DNA yield and quality were assessed with spectrometry (Nanodrop Technologies, Wilmington, DE).

**Quantitative Reverse Transcription PCR (RT-PCR) Analysis**

mRNA was reverse transcribed with Random Hexamers. An 8 ul cDNA aliquot was amplified in 25 ul of 2x TaqMan universal master mix and 2.5 ul of 20x Taqman primer and probe mix (Applied Biosystems, Carlsbad, CA). The primers and probes were designed and synthesized by Applied Biosystems. The Taqman gene expression assay used for EDNRB was Hs00240747_m1 and does not detect residual genomic DNA. Human GUSB (product 4326320E) was used as the endogenous control.

**Quantitative Methylation Analysis**

Quantitative methylation analysis of the *EDNRB* promoter was performed through the Genome Analysis Core Facility at the University of California San Francisco, using the EpiTYPER assay (Sequenom, San Diego, CA) in conjunction
with the MassARRAY system. The target region was located at chr13:78492226-78493582 on the antisense strand of the human genome on the UCSC Genome Browser. This target region includes a CpG island of 77 CpG sites and spans from -792 to +451 relative to the EDNRB transcription start site (GenBank entry AY275463.1). At least 1 µg of DNA from each sample was treated with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA) and the converted DNA was amplified by PCR. Primer sequences were designed with EpiDesigner software and are listed in Table 2. The PCR product was treated with shrimp alkaline phosphate and served as the template for transcription according to manufacturer’s instructions. The samples were spotted on a 384-pad Spectro-CHIP and analyzed using a MassARRAY analyzer compact MALDI-TOF MS. Methylation calls were analyzed using EpiTyper software v1.0 to produce quantitative results for each CpG unit, which consists of a single CpG site or aggregate of adjacent CpG sites. Fully methylated DNA was used as positive control and water was used as negative control.

Recombinant Adenovirus and In Vitro Transduction

Subcloning and viral particle purification were completed through Viraquest (North Liberty, IA). Adenovirus containing only GFP was also obtained for use as a transduction control. The human head and neck cancer cell line derived from human tongue SCC, HSC-3 (ATCC, Manassas, VA), was transduced with recombinant adenovirus (Ad-EDRNB or Ad-GFP) at increasing multiplicities of infection (MOI; number of viral particles per cell), at 50, 100, and 200. Transduction was performed
in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, l-glutamine and sodium pyruvate, supplemented with 2% fetal bovine serum (FBS), 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin G. Twenty-four hours following transduction, cell media was changed to DMEM containing 10% FBS and the supplements mentioned above. mRNA quantification of transduced cells was performed using RT-PCR.

**Immunofluorescence**

Immunofluorescence was performed to evaluate the expression of the transgenes. HSC-3 cells were transduced with recombinant adenovirus at increasing multiplicities of infection. Twenty-four hours after transduction they were trypsinized and grown overnight at 37°C on 12-mm glass cover slips, stabilized in a 6-well plate with DMEM containing 10% fetal bovine serum and the aforementioned supplements. The cells were washed twice with PBS, fixed in ice-cold acetone for 5 minutes at room temperature (RT), permeabilized with 0.2% Triton X-100 for 15 minutes, washed three times in PBS then non-specifically blocked with 3% bovine serum albumin (BSA) for 2 hours. Incubation with primary rabbit polyclonal ET\textsubscript{B} receptor antibody (Abcam Inc., Cambridge, MA) diluted 1:500 in 3% BSA was performed at RT for 2 hours followed by incubation with goat anti-rabbit Texas Red-conjugated IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:200 in 3% BSA for 1 hour at RT. Nuclei were stained with 1:500 Hoechst stain (Invitrogen, Carlsbad, CA). Cover slips were washed and mounted on slides in Gel/Mount mounting medium (Biomedica Crop., Foster City, CA)
and visualized on the Zeiss AxioImager 2. Fluorescence quantification of captured images was performed with CellProfiler software. Data were manipulated according to the developer’s instructions.

**Quantitative Reverse Transcription PCR (RT-PCR) Analysis**

RNA extracted from fresh frozen paired oral SCC and normal tissues from the 20 patients was converted to cDNA. mRNA was reverse transcribed with Random Hexamers. An 8 ul cDNA aliquot was amplified in 25 ul of 2x TaqMan universal master mix and 2.5 ul of 20x 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 used for *EDNRB* was Hs00240747_m1 and does not detect residual genomic DNA. Human GUSB (product 4326320E) was used as the endogenous control (Applied Biosystems, Carlsbad, CA). Using *GUSB* as an internal locus control and the delta-delta Ct method, relative *EDNRB* transcript levels were obtained.

**ELISA Measurement of HSC-3 Supernatant**

ET-1 levels were quantified in the supernatant of nontransduced HSC-3 cells and Ad-EDNRB transduced HSC-3 cells. The samples were prepared as follows. Cells were grown to 60% confluence in 24-well plates and transduced in DMEM with 2% FBS. Twenty-four hours following transduction, transduction media was replaced with 1 ml DMEM containing 10% FBS and supplements. Control nontransduced
HSC-3 cells were treated similarly. Cells were incubated for 4 hours at 37°C. Cell supernatant was collected and 10 ul Halt Protease Inhibitor (Thermo Scientific, Rockford, IL) added. Samples were added to the ET-1 ELISA plate (Assay Designs, Plymouth Meeting, PA) and ELISA was performed according to manufacturer’s instructions. To perform the beta-endorphin ELISA, cells were incubated for 1 hour at 37°C. Cell supernatant was collected similarly and added to the beta-endorphin ELISA plate (MD Biosciences, St. Paul, MN). Samples were run in triplicate and measured using a spectrophotometer at recommended wavelengths.

**Cancer Pain Mouse Model**

The cancer pain mouse model was produced as previously described (Pickering et al. 2008). Experiments were performed on 4 week-old adult female BALB/c, athymic, immunocompromised mice weighing 16–20 g at the time of human oral SCC inoculation. The mice were housed in a temperature-controlled room on a 12:12 h light cycle (0700–1900 h light), with unrestricted access to food and water. All the procedures were approved by the University of California, San Francisco Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. The mice were divided into three inoculation groups: (1) Ad-EDNRB transduced in HSC-3 (n=6), (2) Ad-GFP transduced in HSC-3 (n=6), or (3) HSC-3 (n=5). Approximately 5 x 10⁶ cells from each group were suspended in a mixture of 35 ul of Matrigel (Becton Dickinson & Co., Franklin Lakes, NJ) and 15 µL DMEM and inoculated into the plantar surface of the right hind paw under isofluorane inhalational anesthesia.
Paw Withdrawal and Paw Volume Measurement

Paw withdrawal testing was performed as described previously (Pickering et al. 2008). Testing was performed by an observer blinded to the experimental groups between 0900 and 1200 h. Mice were placed in a plastic cage with a wire mesh floor which allowed access to the paws. Fifteen minutes were allowed for acclimation prior to testing. The probe was applied to the mid-plantar right hind paw. Paw withdrawal thresholds were 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 min intervals to allow resolution of previous stimuli. The results of the six values were averaged for each paw for that day. Paw volume measurements were performed with a plethysmometer (IITC Life Sciences, Woodland Hills). The paw was inserted into a water cell of which pressure is changed due to the immersion. The pressure change was calibrated in milliliters and shown on an electronic monitor. The measurements were accurate by .001 ml. Triplicate measurements were taken for each mouse. Paw withdrawal and paw volume measurements were made at -2, 0, 4, 7, 9, 11, 13, 15, 18 and 21 days relative to inoculation of HSC-3 cells.

RESULTS

Oral Dysplasia and SCC Patient Demographics
A total of 20 patients with biopsy-proven oral SCC were enrolled in the study. Patient demographics and tumor characteristics are described in Table 1. Nine men and eleven women were enrolled, with an age range of 49-93 and a median age 62. Pathology reports for all patients were reviewed to confirm the diagnosis of oral SCC. Tumor staging was also confirmed. None of the oral SCC patients had a cancer of another histologic type during the period of the study. A total of eight patients with biopsy-proven oral dysplasia were enrolled, with an age range of 50-89 and a median age of 69.5.

**Higher EDNRB Methylation in Human Oral SCC than Normal Tissue**

A CpG island of 77 CpG sites in the *EDNRB* promoter was identified on UCSC Genome Browser. Primers were designed using EpiDesigner software. The primer sets are detailed in Table 2. Taken together, the four primer pairs span the region -792 to 451 relative to the *EDNRB* transcription start site (GenBank entry AY275463.1). The PCR products of the second and third primer sets overlapped each other by 188 bases. Due to the proximity of many CpG sites, not all CpG sites could be quantified. The four primer sets had the ability to quantify methylation at 61 out of the total 77 CpG sites, resulting in a total coverage of about 80%. The location of quantified sites is detailed in Figure 1.

Methylation was quantified using the MassARRAY System. The result was a methylation value for a CpG unit, which consisted of a single CpG site or aggregate of adjacent CpG sites. Methylation values ranged from 0 to 1, with 1 signifying 100% methylation of the CpG unit in the sample. Figure 2 shows heat maps of methylation
values obtained for the CpG units within each EDNRB PCR product. Normal tissue has relatively low baseline methylation. Comparing the different EDNRB PCR products, EDNRB 4 has a higher baseline methylation level in normal tissues than any of the other products. EDNRB 4 is also the only product to be entirely located downstream of the transcription start site, in exon 1.

Comparing the paired oral SCC to normal tissue, oral SCC tissue clearly has higher methylation than contralateral-matched normal tissue in all patients, at almost all CpG units. Out of the 61 CpG sites quantified, the average increase in methylation in oral SCC tissue compared to the corresponding normal tissue was at least 10% (methylation value change 0.1) in 57 sites (93% of sites). The four sites with a methylation value change of lower than 0.1 were CpG 1, 2, 3, and 6 in PCR product EDNRB 4, which starts 151 downstream of the transcription start site. Table 3 summarizes the mean methylation difference between oral SCC and normal tissue at each CpG unit, and the corresponding p-value from the Student’s t-test. Figure 3 shows box plots representing methylation difference values between oral SCC and normal tissue, for each of the four EDNRB PCR products. The difference values were averaged across all patients for each CpG site. The median, 10th, 25th, 75th and 90th percentiles were calculated for each group of CpG sites within a PCR product. PCR product EDNRB 2 had the highest methylation increase from normal to oral SCC tissue, with a median increase of .34 (34%). PCR products EDNRB 1 and 3 had a median increase in methylation of 0.25 (25%). Due to already higher baseline methylation levels of EDNRB 4 in normal tissues (Figure 2), the median increase of methylation in oral SCC tissue was 0.13 (13%). The Mann Whitney Rank Sum Test
demonstrated that there was a significant difference between methylation values of oral SCC tissue and the matched normal tissue. Methylation values for each sample were averaged across all CpG sites within each PCR product and oral SCC tissue was compared to normal tissue. There was a significant difference (p<.001) in methylation between cancer and normal tissue in all four PCR products, indicating significant increase in methylation spanning the whole EDNRB promoter in oral SCC.

These results show that there are consistently higher methylation levels in oral SCC tissue in 93% of the queried CpG sites in 100% of sample pairs. Furthermore, there is a larger increase in methylation in the first three PCR products, EDNRB 1, 2 and 3, which are located upstream of the transcription start site. There is a modest increase in methylation of CpG sites of EDNRB 4, which is located downstream of the transcription start site. These results suggest that increase in methylation upstream, and not downstream, of the EDNRB transcription start site, has a more significant contribution to carcinogenesis.

**Oral SCC Patients Demonstrate Higher EDNRB Methylation than Oral Dysplasia Patients**

Methylation quantification of EDNRB was performed on paraffin-embedded tissue of eight biopsy-proven oral dysplasia patients. Methylation results were then averaged and compared to the average methylation of oral SCC tissue. EDNRB 2 PCR product was too long and could not be reliably amplified using DNA purified from the paraffin embedded tissue of dysplasia patients. Oral SCC tissue
demonstrated higher methylation than oral dysplasia tissues at all queried CpG sites, and were on average 20% higher (range of 6-39%) (Figure 4). There was a significant difference between the methylation values at almost all CpG sites (Mann Whitney Rank Sum Test).

**EDNRB Promoter Methylation Levels are Higher in Patients with Nodal Metastasis**

We also classified our patients by their N staging in their TNM classification and compared *EDNRB* methylation among the different N stages. There were eleven patients with N staging of 0, three patients with N staging of 1, and six patients with N staging of 2. Figure 5 is a line and scatter plot graphing the difference in methylation between the oral SCC tissue of a patient and the normal tissue. The patients are categorized into the three possible N staging groups; each point on the graph represents the average of difference in methylation of oral SCC versus normal tissue, of all samples in the group. The plot shows average methylation difference in CpG sites comprised in PCR product EDNRB 1. These sites are the extreme 5’ end of the CpG island in the *EDNRB* promoter, and are located -792 to -425 upstream of the transcription start site. The plot shows that methylation difference is lowest in the N=0 group, and is highest in the N=2 group. There was a significant difference between the three groups in seven of the ten CpG units, equating to 11 or 16 CpG sites (ANOVA). Furthermore, the N=2 group as significantly different from the N=0 group in all seven CpG units, and the N=1 as significantly different from the N=0 group at one CpG unit (p < .05, Holm-Sidak).
EDNRB mRNA Expression is Lower in Oral SCC than Normal Tissue

The fold-change in expression of EDNRB in normal tissue compared to oral SCC tissue in the same patient was calculated. A fold change of more than 1 indicated that EDNRB expression was higher in the normal tissue than in the oral SCC tissue. Of the 20 patients, the average fold change of EDNRB expression was 10.9. Nineteen out of 20 patients (95%) had higher EDNRB expression in normal tissue than oral SCC tissue (range in fold change of 1.15-53.0), indicating downregulation of EDNRB in the oral SCC state. Figure 6 compares EDNRB expression in oral SCC to normal tissue. EDNRB expression in normal tissue is significantly higher than oral SCC tissue (p <0.001, Student's t-test).

Oral SCC Cells Transduced with Ad-EDNRB Express High Levels of EDNRB mRNA and ET\textsubscript{B} Receptor

The EDNRB promoter in human oral cancer tissue is heavily methylated, whereas the EDNRB promoter in normal and dysplastic oral tissues has low levels of methylation. Furthermore, EDNRB mRNA expression was considerably reduced in cancer tissue compared to normal tissues. We wanted to explore the biological role of silenced EDNRB expression in the cancer microenvironment. To do so, we used adenoviruses to overexpress EDNRB in vitro. To overexpress EDNRB we transduced oral SCC cells (HSC-3) with adenovirus containing the EDNRB gene. HSC-3 has nondetectable levels of EDNRB mRNA and, when methylation levels of the EDNRB promoter in this cell line were quantified using MassARRAY, an average
methylation level for all CpG sites was 85%. Our goal was to re-express EDNRB and determine the effect on the cancer microenvironment in vitro.

To confirm overexpression subsequent to transduction, immunofluorescence with EDNRB antibody was performed. ET-B receptor expression in oral SCC cells was minimal, while transduction of Ad-EDNRB resulted in a marked increase in ET-B receptor membrane expression. Figure 7 shows images from cells transduced at 200 MOI, as 50 MOI and 100 MOI show similar trends.

RT-PCR was performed on mRNA extracted from nontransduced oral SCC cells and oral SCC cells transduced with Ad-EDNRB at increasing MOI. RT-PCR results shown in Figure 8a illustrate a dose-dependent increase in EDNRB mRNA expression with increasing MOI. Oral SCC cells transduced with Ad-EDNRB at 200 MOI express 3 x 10^7 more mRNA than nontransduced oral SCC.

Figure 8b illustrates relative ET-B receptor expression in the three groups of oral SCC cells: nontransduced, Ad-GFP and Ad-EDNRB transduced at 200 MOI. ET-B receptor expression was quantified relative to nontransduced oral SCC cells, which have a relative expression of 1. While Ad-GFP transduced cells do not exhibit a difference in ET-B receptor expression, those transduced with Ad-EDNRB express 9.6 times more ET-B receptor than control oral SCC cells.

**Oral SCC Cells Transduced with Ad-EDNRB Secrete Lower Levels of ET-1 and Higher Levels of Beta-Endorphin**
We have previously shown that oral SCC cells secrete very high levels of ET-1 (Pickering et al. 2008). We wanted to determine the effect of re-expressing ETB receptor on ET-1 levels in oral SCC cells. We transduced oral SCC cells with Ad-EDNRB and then performed ELISA to measure ET-1 in the supernatant. Our results showed that oral SCC cells transduced with Ad-EDNRB secreted lower levels of ET-1 than nontransduced oral SCC. Supernatant of oral SCC cells transduced with Ad-EDNRB at 200 MOI had an average ET-1 concentration of 9.2 pg/l. Supernatant of control oral SCC cells, which were transduced with Ad-GFP, had an average ET-1 concentration of 37.3 pg/l.

Conversely, oral SCC cells transduced with Ad-EDNRB secreted more beta-endorphin than control. Supernatant beta-endorphin concentration of Ad-EDNRB-transduced oral SCC cells was 0.94 ng/ml. Control cells, which were transduced with Ad-GFP, secreted an average of 0.66 ng/ml.

**Transduction of Ad-EDNRB Decreases Nociception Without Affecting Tumor Size in a Cancer Mouse Model**

Human cancer cell proliferation and cancer-induced mechanical allodynia were assessed in the three orthotopic mouse model groups: (1) Ad-EDNRB-transduced HSC-3 tumors; (2) Ad-GFP-transduced HSC-3 tumors; and (3) non-transduced HSC-3 tumors. The results up to 21 days after inoculation of HSC-3 tumors showed that the three groups had similar paw volume, indicating that overexpressing EDNRB did not significantly alter tumor proliferation (Figure 9a). Overexpression of EDNRB did, however, affect cancer-induced mechanical
alldynia. Cancers expressing EDNRB displayed a significantly higher paw withdrawal threshold than either the nontransduced cancer or the cancer expressing GFP. Figure 9b shows that mice with cancers expressing EDNRB have paw withdrawal thresholds that stabilized at 48% below baseline, regardless of the fact that tumor size was increasing. The average paw withdrawal threshold of these mice on day 21 was 2.12 g, approximately 48% of the day 0 threshold of 4.09 g. The Ad-GFP transduced HSC-3 cancers and non-transduced HSC-3 cancer groups showed a 65% (day 0 average = 4.06g; day 21 average = 1.41 g) and 67% (day 0 average = 3.90g; day 21 average = 1.29g) reduction in paw withdrawal threshold, respectively. The baseline paw withdrawal of the three groups was not significantly different from each other. The paw withdrawal threshold of tumors overexpressing EDNRB was significantly higher than the control groups, indicating lower mechanical alldynia (Student’s t test, p-values as indicated in Figure 9b).

DISCUSSION

In this study we demonstrate that methylation of EDNRB is a novel mechanism generating cancer pain and expression of the ET$_B$ receptor reverses cancer pain. Evidence supporting this result includes our finding that EDNRB is heavily methylated in painful human oral cancer specimens compared to matched normal controls and oral dysplasia, which is typically not painful. In addition, oral cancer specimens express less EDNRB mRNA than matched normal controls, indicating a correlation between increased EDNRB methylation and decreased mRNA expression. Using a cancer pain mouse model we show that re-expression of
ETB receptors using adenoviruses leads to significant attenuation of cancer pain behavior. This study is the first to demonstrate that gene methylation could be a significant mechanism causing cancer-induced pain, and that reversing the gene silencing process with targeted therapy could produce analgesia.

Oral cancer is notoriously painful. The pain is consistent with mechanical allodynia and severely limits function (Connelly et al. 2004; Kolokythas et al. 2007). We used resected cancer specimens and anatomically matched normal oral tissue from the same patients to evaluate EDNRB methylation. This approach allows for an accurate analysis of methylation levels associated with carcinogenesis. We quantified methylation in the promoter region of EDNRB spanning 77 CpG sites and 1.2 kilobases, which to date represents the most comprehensive methylation panel of EDNRB in normal, dysplastic, and cancer tissue. Oral cancer patients demonstrated higher methylation of EDNRB than oral dysplasia patients at all queried CpG sites. In our recent study we quantified pain in our cohort of oral dysplasia and oral cancer patients using the validated UCSF Oral Cancer Pain Questionnaire (Lam et al. 2011). We demonstrated that only oral cancer patients, and not dysplasia patients, reported significant levels of spontaneous and function-related pain, with function-related pain being markedly pronounced. Additionally, when we compared methylation frequency in patients with and without nodal metastasis, patients with an N staging of 2 showed significantly higher methylation than patients with an N staging of 0 in the 5’ end of the EDNRB promoter. These results correlate with our previous finding that oral cancers that are metastatic are more painful (Connelly et al. 2004).
Next, we wanted to understand the biologic significance of \textit{EDNRB} expression on cancer-induced pain. Cancer pain has been hypothesized to result from either a tumor-mass effect or the activation of primary afferent nociceptors by mediators liberated by the cancer (Bjordal et al. 1995; Morton 1995; Diener 1996; Fujita et al. 1998; Mantyh et al. 2002). The tumor-mass effect is unlikely because even small carcinomas are painful (Connelly et al. 2004). We hypothesized that cancer pain results from an imbalance in pain-producing and pain-relieving mediators within the cancer microenvironment. The endothelin axis provides a compelling example of this imbalance. We have previously shown that for the human oral SCC cell line, the mRNA for ET-1 is nearly doubled, while mRNA for ET\textsubscript{B} receptors is down-regulated compared to normal oral keratinocytes (Quang et al. 2010). Similarly in lung cancer, ET\textsubscript{B} receptors are down-regulated via promoter methylation (Knight et al. 2009). The ET\textsubscript{B} receptors could mediate analgesia, attenuating the acute pain generated by ET-1 (Piovezan et al. 2000; Khodorova et al. 2002). This counterbalancing, pain-relieving effect could be lost in cancers because ET\textsubscript{B} receptors are not expressed. We therefore proposed that if the promoter for ET\textsubscript{B} receptors could be re-expressed then the receptor could be activated by ET-1. To test this hypothesis we developed a cancer mouse model in which ET\textsubscript{B} receptors were re-expressed. Since there is currently no reliable method to demethylate a specific gene and activate its transcription, adenovirus transduction was the most feasible method of re-expression. Our results showed that mice with oral SCC tumors expressing \textit{EDNRB} had significantly lower levels of mechanical allodynia than mice with oral SCC tumors that did not express \textit{EDNRB}. 
ET_B receptor expression leads to antinociception through two likely mechanisms: 1) activation of ET_B receptor, which activates an endogenous analgesic mechanism, and 2) reduced ET-1 secretion by the carcinoma. The endogenous analgesic mechanism by ET_B receptor activation was first demonstrated by Khodorova et al., who also showed that the analgesic cascade involved release of beta-endorphin (15). We demonstrated in this study that re-expression of ET_B receptor resulted in increased secretion of beta-endorphin into the oral SCC supernatant. These results correspond to our earlier results where treatment of oral SCC cells with an ET_B receptor agonist caused increased secretion of beta-endorphin compared to control cells (Quang et al. 2010). Support for the second analgesic mechanism comes from our finding that ET-1 levels in the cell supernatant were significantly lower in oral SCC re-expressing the ET_B receptor. ET_B receptors have a known role in ET-1 clearance and inhibiting ET-1 secretion in keratinocytes (Yohn et al. 1994; Nelson et al. 1997). Our previous studies in a mouse oral SCC model have shown that ET-1 protein and mRNA concentrations are markedly elevated in oral SCC tumors. These elevated ET-1 levels caused increased pain by activation of the ET_A receptor. Not only is ET-1 elevated in numerous cancer types, our studies on salivary biomarkers have shown that ET-1 is significantly elevated in saliva of patients with oral SCC compared to normal subjects (Pickering et al. 2007). High ET-1 concentration activates ET_A and downstream networks resulting in nociception. We also demonstrated that melanoma tumors of equal size to SCC tumors produced significantly less pain, since they had a lower ET-1 concentration. Therefore, cancer pain intensity depends
more on ET-1 concentration in that tumor than tumor size itself (Pickering et al. 2008). From these cumulative findings we propose that increased concentration of ET-1 activates $\text{ET}_A$ and subsequent nociceptive pathways, and re-expression of the $\text{ET}_B$ receptor produces an analgesic effect by counter-regulating ET-1/ $\text{ET}_A$ nociceptive mechanisms.

For most cancer patients, uncontrollable pain creates a poor quality of life (Hodder et al. 1997; Chaplin et al. 1999; Hammerlid et al. 2001; Connelly et al. 2004). Because improved chemotherapy and radiotherapy prolong survival, chronic cancer pain is a major public health problem. To date there is no effective treatment for chronic cancer pain. The role of gene methylation in cancer pain has not been previously demonstrated. The findings from this study establish gene methylation as a novel regulatory mechanism of cancer-induced pain. Furthermore, the findings suggest that targeted gene demethylation could serve as a novel analgesic therapy to chronic cancer pain. Such therapy targeted to the cancer microenvironment like we had demonstrated in this study could antagonize pain while minimizing systemic drug toxicity and solve the current public health problem of chronic cancer pain refractory to traditional therapeutics.

References


Chapter 3

OPRM1 Methylation is a Regulatory Mechanism of Cancer-Induced Pain in a Mouse Model

Abstract

Aberrant methylation of gene promoters has a well-established role in carcinogenesis. However its role in nociception is unknown. In this study we determine the effect of promoter methylation on cancer-induced nociception. We focus on OPRM1, the gene encoding the mu-opioid receptor, and demonstrate that it is heavily methylated in tumors of oral cancer patients. To establish the role of OPRM1 promoter methylation in cancer pain, we create an orthotopic oral cancer mouse model, where we re-express the OPRM1 gene using adenoviruses. Re-expression of OPRM1 results in mechanical and thermal antinociception in the mouse cancer pain model. Finally, we determine the antinociceptive potential of demethylating drugs zebularine and decitabine in the mouse cancer model. We show that combination treatment of these two drugs results in a rapid decrease in tumor size and cancer-induced nociception.
INTRODUCTION

DNA methylation is an early and common event during carcinogenesis. Transcriptional silencing by aberrant methylation of promoters of genes controlling cellular function is crucial to carcinogenesis. Promoter methylation involves carcinogenesis-related genes including tumor suppressor genes, DNA repair genes, cell-cycle regulatory genes, and apoptotic genes (Costello et al. 2001). Unlike genetic mutations, DNA methylation does not permanently change the DNA sequence itself, and is potentially reversible. It is therefore an attractive chemotherapeutic target. Therapeutic intervention with demethylating agents could reactivate key genes and restore normal cellular functions (Yoo et al. 2004; Lemaire et al. 2008; Yoo et al. 2008). Nucleoside analogs such as 5-Aza-2'-deoxycytidine and 5-azacytidine are effective demethylating drugs in vitro and have recently undergone clinical trials to treat acute leukemias (Lubbert 2000). Zebularine is another methylation inhibitor drug, but unlike the other methylation inhibitor drugs, it is very stable, making oral administration possible. It has effectively caused demethylation and reactivation of silenced and hypermethylated genes in several cancer models (Cheng et al. 2003; Yoo et al. 2004; Yoo et al. 2008).

While research on the role of DNA methylation in carcinogenesis has burgeoned within the past decade, the role of DNA methylation in cancer pain has gone entirely unnoticed. It is unknown whether DNA methylation contributes to cancer-induced pain. Uncontrollable pain is a common symptom in cancer patients that prevents them from functioning normally (Hodder et al. 1997; Chaplin et al.)
While little progress has been made in cancer pain treatment, DNA methylation involvement in cancer pain is an untapped area of research. In this study we hypothesize that epigenetic silencing by DNA methylation within the cancer itself holds the key to cancer pain. The key to inducing endogenous analgesia in the cancer is therefore reversal of epigenetic gene silencing within the cancer. Even though there are no studies on the effect of DNA methylation on cancer pain, genes mediating endogenous analgesia are structurally capable of epigenetic silencing through DNA methylation. To test this hypothesis we first quantified methylation of OPRM1, the gene for mu-opioid receptor, in squamous cell carcinoma (SCC) patients. We then assessed the antinociceptive potential of re-expressing OPRM1 in a mouse oral SCC model. We utilized two separate approaches to reverse epigenetic silencing and re-express OPRM1. Firstly, we performed adenovirus transduction of the OPRM1 into cancer cells in a mouse oral SCC model and determined whether re-expression of the OPRM1 gene had an antinociceptive effect. Secondly, we determined the antinociceptive potential of demethylating agents, which enhances re-expression of silenced genes, in a mouse oral SCC model.

MATERIALS AND METHODS

Tissue Collection

All procedures were approved by the University of California, San Francisco Committee on Human Research. We enrolled oral SCC patients with the following inclusion criteria: 1) biopsy-proven oral cavity SCC and 2) no history of prior surgical,
chemotherapeutic, or radiation treatment for head and neck SCC. We collected tissue at time of surgery from the primary cancer site and contralateral normal epithelium. Samples were flash frozen in liquid nitrogen and stored in -80°C. Demographic and health information were recorded for each patient including age, sex, and tumor characteristics. Cancer patients were staged according to the American Joint Commission on Cancer tumor-node-metastasis (TNM) staging system (Greene et al. 2002). Patient demographics and tumor characteristics are shown in Table 1.

**Recombinant Adenovirus and In Vitro Transduction**

Human cDNA of *OPRM1* containing a C-terminal GFP tag (OriGene, Rockville, MD) was subcloned into a pVQAd CMV K-NpA shuttle plasmid between Pmel and Acc65I sites. Recombinant adenovirus was transduced into HEK-293 cells. Subcloning and viral particle purification were completed through Viraquest (North Liberty, IA). Adenovirus containing only GFP was also obtained for use as a transduction control.

The human head and neck cancer cell line derived from human tongue SCC, HSC-3 (ATCC, Manassas, VA), was transduced with recombinant adenovirus (Ad-OPRM1 or Ad-GFP) at increasing multiplicities of infection (MOI; number of viral particles per cell), at 50, 100, and 200. Transduction was performed in Dulbecco’s Modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-glutamine and sodium pyruvate, supplemented with 2% fetal bovine serum (FBS), 25 μg/mL fungizone, 100 μg/mL streptomycin sulfate, and 100 U/mL penicillin G. Twenty-four hours
following transduction cell media was changed to DMEM containing 10% fetal bovine serum and the supplements mentioned above. Transduction efficiency was determined by visualization of GFP-expressing cells. mRNA quantification of transduced cells was performed using RT-PCR.

**Cancer Pain Mouse Model**

The cancer pain mouse model was produced as previously described (Pickering et al. 2008). Experiments were performed on 4 week-old 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 h light cycle (0700–1900 h light), with unrestricted access to food and water. All the procedures were approved by the University of California, San Francisco Committee on Animal Research. Researchers were trained under the Animal Welfare Assurance Program. The mice were divided into three inoculation groups and inoculated with the respective cell types: (1) HSC-3 (n=5), (2) Ad-OPRM1 transduced in HSC-3 (n=6), and (3) Ad-GFP transduced in HSC-3 (n=6). 5 x 10^6 cells from each group were suspended in a mixture of 35 µl of Matrigel (Becton Dickinson & Co., Franklin Lakes, NJ) and 15 µl DMEM and inoculated into the plantar surface of the right hind paw. 2–4% isofluorane inhalational anesthesia was used for inoculation. From our preliminary immunofluorescence experiments we had determined that HSC-3 cells and Ad-GFP-transduced HSC-3 cells did not express mu-opioid receptors, while Ad-OPRM1-transduced HSC-3 cells overexpressed mu-opioid receptors.
Zebularine and Decitabine Treatments

**Decitabine Treatment:** All mice received implantation of an osmotic pump (model 2001D, Alzet, Cupertino, CA) subcutaneously into their dorsum. The pump delivered either PBS (vehicle), or 2µg/g body weight of decitabine dissolved in PBS and sterilized by 0.2 µm filtration. Dosage was determined by a previous study on dose scheduling in BALB/c mice (Lemaire et al. 2008). Osmotic pumps were designed to administer the sham or drug treatment over the course of 24 hours. A total of four study groups were used. Groups 1 and 2 had pumps with vehicle PBS treatment. Groups 3 and 4 had pumps with decitabine. Osmotic pumps were implanted on post-inoculation day (PID) 4 because tumors were visible by this day.

**Zebularine Treatment:** Groups 1 and 3 received 3% sucrose water given *ad libitum*, which served as the vehicle treatment for zebularine. Groups 2 and 4 received 3% sucrose water mixed with 1mg/ml zebularine given *ad libitum*. Treatments began on PID 4.

Paw Withdrawal and Paw Volume Measurement for SCC Paw Model

Paw withdrawal testing was performed as described previously (Pickering et al. 2007). Testing was performed by an observer blinded to the experimental groups between 0900 and 1200 h. Mice were placed in a plastic cage with wire mesh floor which allowed access to the paws. Fifteen minutes were allowed for acclimation prior to testing. The investigator was blinded from the treatment each mouse received. The probe was applied to the mid-plantar right hind paw. Paw withdrawal
thresholds were 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 min intervals to allow resolution of previous stimuli. The results of the six values were averaged for each paw for that day. Paw volume measurements were performed with a plethysmometer (IITC Life Sciences, Woodland Hills). The paw was inserted into a water cell of which pressure is changed due to the immersion. The pressure change was calibrated in milliliters and shown on an electronic monitor. The measurements were accurate by .001 ml. Triplicate measurements were taken for each mouse. Paw withdrawal and paw volume measurements were made at -2, 0, 4, 7, 9, 11, 13, 15, 18 and 21 days relative to inoculation of HSC-3 cells. Each mouse was used as its own control, and relative changes in paw withdrawal and volume were calculated based on day 0 baseline.

**Thermal Hyperalgesia Measurement**

Thermal hyperalgesia of the hind paw was assessed on PID 20 according to the method previously described (Hargreaves et al. 1988). 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 real 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 both hind paws of each test animal. This stimulus was positioned under right footpad of each mouse. The time elapsed 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 5-minute intervals. An automatic 20 second cut-off was used to minimize tissue damage.

**Quantitative Methylation Analysis**

Quantitative methylation analysis of the mouse and human *OPRM1* promoters were performed through the Genome Analysis Core Facility at the University of California San Francisco, using the EpiTYPER assay (Sequenom, San Diego, CA) in conjunction with the MassARRAY system. The target region on the mouse promoter lies -304 to +71 relative to the transcription start site. The target region in the human promoter includes 20 CpG sites and spans from -232 to +109 relative to the transcription start site. Using the UCSC Genome Browser, a CpG island of 33 CpG sites was identified in the promoter region of *OPRM1*. Its DNA sequence was entered into the Sequenom’s EpiDesigner Software. Design settings were as follows: primer melting temperature 56-62°C, primer size 20-30 bases, product size 100-500 bases, and minimum of 4 product CpGs. A total of 21 primer pairs were designed on the sense and antisense strands. One primer pair was chosen from this list that spanned from -232 to 109 relative to the transcription start.
site. The PCR product from this primer pair allowed for quantification of 20 CpG sites within the promoter region.

At least 1 µg of DNA from each sample was treated with sodium bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA) and the converted DNA was amplified by PCR. Primer sequences were designed with EpiDesigner software (Sequenom). The forward primer was tagged with a 10mer (5'-AGGAAGAGAG-3') for mass load and each reverse primer was tagged with a T7-promoter (5'-CAGTAATACGACTCACTATAGGGAGAAGGCT’3’) for transcription of the PCR product. PCR was carried under the following conditions: hot start at 94°C for 15 minutes, 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, 72°C for 1 minute, and a final incubation at 72°C for 3 minutes. Excess dNTP was dephosphorylated with 0.3U shrimp alkaline phosphate (SAP; Sequenom, San Diego, CA). The reaction was incubated at 37°C for 40 minutes and SAP was inactivated at 85°C for 5 minutes. 2 µl of the SAP-treated PCR product served as the template for transcription according to manufacturer's instructions (Sequenom). The samples were spotted on a 384-pad Spectro-CHIP (Sequenom) and analyzed using a MassARRAY analyzer compact MALDI-TOF MS (Sequenom). Methylation calls were analyzed using EpiTyper software v1.0 (Sequenom) to produce quantitative results for each CpG unit, which consists of a single CpG site or aggregate of adjacent CpG sites. Fully methylated DNA was used as positive control and water was used as negative control.

**Quantitative Reverse Transcription PCR (RT-PCR) Analysis**
mRNA was reverse transcribed with Random Hexamers (Applied Biosystems, Foster City, CA). An 8 µl cDNA aliquot was amplified in 25 µl of 2x TaqMan universal master mix and 2.5 µl of 20x 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 primers and probes were designed and synthesized by Applied Biosystems. The Taqman gene expression assay used for OPRM1 does not detect residual genomic DNA. Human GUSB was used as the endogenous control.

Immunohistochemistry

Histological sections of skin tissue were heated and de-paraffinized and then pretreated with Target Retrieval Solution (Dako, Carpinteria, CA). Endogenous peroxidases were quenched by immersing sections in 3% hydrogen peroxide and Tris Buffered Saline (TBS) for 5 minutes, followed by placement in a TBS bath for 5 minutes. Sections were incubated for 2 hours with polyclonal mu-opioid receptor antibody (O5319, Sigma-Aldrich, Inc., Louis, MO) at a 1:200 dilution. The primary antibody was replaced with TBS for the negative controls. Following 45 minutes incubation with a goat anti-rabbit secondary antibody, Vector® NovaRED™ substrate was placed on the sections for 5 minutes, and the slides were then rinsed in water. After hematoxylin counterstaining, the sections were dipped in ammonia and then rinsed with water, dehydrated in alcohol, and cover slipped.

ELISA Measurement of Endogenous Opioids
To evaluate the effect of *OPRM1* re-expression and agonist or antagonist drugs on opioid production in HSC-3 cells, we used enzyme-linked immunosorbent assay (ELISA) to measure opioid levels. HSC-3 cells were seeded onto 24-well tissue culture plates with 1 mL of DMEM supplemented with 10% FBS and the aforementioned supplements. HSC-3 cells were cultured for 24 h until the wells reached 60% confluence. Wells were divided into one of the different groups and transduced accordingly: (1) Ad-OPRM1 transduced at 200 MOI, (2) Ad-OPRM1 transduced at 100 MOI, (3) no transduction. Transduction was carried out in DMEM with 2% FBS and supplements. After 24 hours the wells were replaced with DMEM with 10% FBS and supplements. The culture media were collected and treated with 10 µl HALT Protease Inhibitor Cocktail (Pierce, Rockford, IL) before performing ELISA to detect the levels of beta-endorphin (MD Biosciences, St. Paul, MN).

**RESULTS**

*OPRM1* Promoter is Hypermethylated in Oral SCC Tissue Compared to Contralateral Normal Tissue

The result obtained from the MassARRAY system was one methylation value for each CpG unit, which consisted of a single CpG site or aggregate of adjacent CpG sites. Methylation values ranged from 0 to 1, with 1 signifying 100% methylation of the CpG unit in the sample. Figure 10 shows a heat map of methylation values obtained for each CpG unit. CpG units with poor resolution were omitted. Normal contralateral tissue has relatively low baseline methylation. Methylation levels for all CpG units were significantly higher in cancer tissue than
contralateral normal tissue (Student’s t-test, p < .001). The average difference in methylation levels was 24.6% (range 8.1 – 35.1%). Patient demographics are shown in Table 1.

**Immunohistochemistry of Hind Paw Tumors Show Re-expression of Mu-Opioid Receptor in Ad-OPRM1-Transduced Mice**

Immunohistochemistry using mu-opioid receptor antibody was performed on deparaffinized hind paw tumors. Figure 11 shows captured images from immunohistochemistry at 20X magnification. All paw samples from the three treatment groups were stained. The expression pattern in Figure 11 is representative of tissues in each respective group. Ad-GFP and nontransduced groups were relatively absent in mu-opioid receptor expression, meaning that oral SCC cells (HSC-3) do not express mu-opioid receptor. Ad-OPRM1 transduced tumors, however, showed markedly high expression of mu-opioid receptor. These results confirm expression of mu-opioid receptor in Ad-OPRM1-transduced cells.

**Oral SCC Tumors Re-expressing Mu-Opioid Receptor Produce Lower Levels of Allodynia than Control Tumors in an Orthotopic Mouse Model**

The results up to PID 21 of orthotopic oral SCC tumors showed that the three groups, (1) nontransduced oral SCC (HSC-3) tumors (2) Ad-OPRM1-transduced oral SCC tumors, and (3) Ad-GFP-transduced oral SCC tumors, had paw volumes that were not significantly different from each other (One Way ANOVA). Average paw volumes at baseline were 0.18 ml, 0.17 ml, and 0.17 ml, for groups 1, 2, and 3, respectively. Average paw volumes at PID 21 were 0.29 ml, 0.29 ml, and 0.29 ml,
respectively. Therefore, re-expressing mu-opioid receptor did not significantly alter tumor proliferation. Re-expression of mu-opioid receptor did, however, affect mechanical allodynia. Tumors re-expressing mu-opioid receptor showed a significantly higher paw withdrawal threshold than either the nontransduced tumor or the tumor expressing GFP. Figure 12 shows that mice with tumors overexpressing mu-opioid receptor had paw withdrawal thresholds that stabilized at 36% below baseline (PID 0 = 3.9 g; PID 21 = 2.5 g), despite paw volume increase that was comparable to the other two groups. Control tumors, on the other hand, demonstrated much more reduction in paw withdrawal thresholds. Ad-GFP-transduced tumors displayed at 60% decrease from 4.0 g on day 0 to 1.6 g on PID 21. Nontransduced tumors demonstrated a 69% decrease from 4.2 g on day 0 to 1.3 g on PID 21. Paw withdrawal threshold of mice with tumors overexpressing mu-opioid receptor was significantly higher than the nontransduced control group indicating lower mechanical allodynia (One Way ANOVA, Holm Sidak Test, p values as indicated in Figure 12). The Ad-GFP group had no significant difference with the nontransduced control group.

**Oral SCC Tumors Overexpressing Mu-opioid Receptor Demonstrate Lower Levels of Thermal Hyperalgesia than Control Tumors in an Orthotopic Mouse Model**

We used the Hargreaves' test (Hargreaves et al. 1988) as a measure for thermal hyperalgesia. The results indicated that mice with tumors overexpressing mu-opioid receptor experienced a significantly longer latency period before paw withdrawal
than the control groups (ANOVA on Ranks, Dunn’s Test, p < .05). These mice had an average latency of 8.2 seconds, whereas mice with Ad-GFP tumors had an average latency of 5.3 seconds and mice with nontransduced tumors had a latency of 4.0 seconds. Mu-opioid receptor overexpression, therefore, correlated with significantly increased threshold for thermal hyperalgesia (Figure 13).

**Beta-endorphin Secretion Levels is Increased in Oral SCC Cells Overexpressing Mu-opioid Receptor**

We measured beta-endorphin levels using ELISA in oral SCC cells transduced with Ad-OPRM1 at different MOI. We showed a dose-dependent increase in beta-endorphin secretion in the cell supernatant with increasing MOI (Figure 14). While control oral SCC supernatant contained almost no beta-endorphin, oral SCC cells transduced with Ad-OPRM1 at 200 MOI secreted an average of 0.4 ng/ml beta-endorphin, which was significantly higher than non-transduced cells (Student’s t-test, p < .05). As a control for transduction, beta-endorphin level was also measured in cells transduced with Ad-GFP. These cells showed very low secretion, which was not significantly higher than nontransduced cells.

**Combination Treatment of Zebularine and Decitabine Produces a Stronger Antiproliferative Effect in an Orthotopic Oral SCC Mouse Model than either Drug Alone**

The four drug treatment groups are detailed in Table 4. Mice from all four groups developed visible tumors by PID 4. Therefore drug treatment began on PID 4.
Relative change in paw volume was calculated based on day 0 measurements. Control mice displayed a steady increase in paw volume, totaling to a 97% increase in paw volume on PID 21. The average paw volume was 0.18 ml on day 0 and 0.36 ml on PID 21. Decitabine only treatment showed little effect on tumor growth, and there was not a significant difference between the control and decitabine groups until PID 18. The decitabine group had a total increase of 39% in paw volume by PID 21, from 0.17 ml on day 0 to 0.29 ml on PID 21. Zebularine treatment resulted in a more significant reduction in tumor growth, and a significant difference between the zebularine and control groups were observed on PID 9. The average paw volume was 0.18 ml on day 0 and 0.21 ml on PID 21. Combination treatment of zebularine and decitabine, however, showed the strongest anti-tumor effect. This therapeutic intervention caused a reduction in paw volume from 58% above baseline on PID 4 back to baseline on PID 7. The paw volume average was 0.17 ml on day 0, increased to 0.27 ml on PID 4, and decreased back to 0.19 ml by PID 7. Paw volumes were maintained at baseline in this treatment group for the remainder of the experiment and was 0.18 ml on PID 21, signifying no further tumor growth (Figure 15a; ANOVA, ANOVA on Ranks, Holm-Sidak, Dunn’s test as appropriate).

**Combination Treatment with Decitabine and Zebularine Produces a Stronger Antinociceptive Effect in an Orthotopic Oral SCC Mouse Model than either Drug Alone**

By PID 4, all four groups exhibited a uniform decrease in paw withdrawal threshold, indicating increase in mechanical allodynia. The control group decreased from an
average of 3.5 g to 2.0 g, totaling a 43% change. The zebularine group decreased from 3.3 g to 1.0 g, averaging a 43% change. The decitabine group decreased from 3.9 g to 2.1 g, totaling a 46% decrease. The combination treatment group decreased from 3.8 g to 1.9 g, totaling a 52% change. Drug treatment was initiated on PID 4. Control mice showed a steady decrease in paw withdrawal, indicating increasing allodynia. The average withdrawal threshold on PID 0 of 3.3 g, and this decreased to 1.0 g on PID 21. Statistical differences were calculated between the control group and all other treatment groups (Figure 15b; ANOVA, ANOVA on Ranks, Holm-Sidak, Dunn’s test as appropriate). Mice treated with decitabine showed a similar decrease in paw withdrawal, and it was not until PID 21 that there was a significant difference between this group and the control group. While the zebularine group gradually showed an increase back to baseline by PID 13, the combination treatment group showed an immediate increase to baseline by PID 7. The average paw withdrawal threshold for the combination group remained at baseline for the remainder of the experiment. The average withdrawal threshold was 2.9 g on PID 7 and 3.9 on PID 21. This result indicated that the combination treatment had a stronger antinociceptive effect in the orthotopic oral SCC mouse model, compared to treatment with either drug alone.

**Mice Treated with Demethylating Drugs Show Lower Methylation of the**

**OPRM1** **gene, Higher mRNA Expression, and Increased Mu-opioid Receptor Expression**
We next determined whether the increased expression of mu-opioid receptor in the treatment groups was a result of decreased methylation of \textit{OPRM1}. We used the EpiTYPER assay to quantify methylation of the \textit{OPRM1} promoter region, -304 to +71 relative to transcription start site. Using the Student’s t test, zebularine-treated mice had significantly lower methylation levels of \textit{OPRM1} in their tumors than control mice (Figure 16). OPRM1 mRNA expression levels were correspondingly 51 times higher in zebularine-treated mice than non-treated mice (Mann-Whitney test, \( p = 0.008 \)). We performed immunohistochemistry to stain for the mu-opioid receptor in the hind paws of mice treated with zebularine and those of control mice. We were primarily concerned with mu-opioid receptor expression on cancer cells. Our results demonstrated that mu-opioid receptor expression was relatively absent on control, non-treated oral SCC tumors. Tumors that were treated with zebularine, however, showed re-expression of the mu-opioid receptor, particularly in cancer cells surrounding peripheral neurons (Figure 17).

\textbf{DISCUSSION}

In this study we demonstrated that promoter methylation of \textit{OPRM1}, the gene for a receptor that mediates endogenous analgesic mechanisms, regulates cancer pain. Evidence for the likely role of methylation in cancer pain was our finding that the \textit{OPRM1} promoter is hypermethylated in human oral SCC specimens and an oral SCC cell line. To determine the role of \textit{OPRM1} promoter methylation in cancer pain we created a mouse cancer pain model using an oral SCC cell line that overexpressed mu-opioid receptor through adenoviral transfection. The oral SCC
cell line was used because oral SCC produces significant mechanical allodynia in patients and in mouse oral SCC models (Connelly et al. 2004) (Kolokythas et al. 2007; Pickering et al. 2008). Re-expression of OPRM1 in the oral SCC cell line had significant mechanical and thermal antinociceptive effects in orthotopic oral SCC tumors, without affecting tumor proliferation. While it is well established that mu-opioid receptor expression on peripheral neurons contributes to attenuation of cancer pain, and mu-opioid receptors are present on cancer cells including breast and colon, the finding that mu-opioid receptor expression on the cancer cells could moderate anti-nociception in a cancer pain model has not been demonstrated (Farooqui et al. 2006; Nylund et al. 2008; Yamamoto et al. 2008; Gach et al. 2009; Bedini et al. 2010; Joseph et al. 2010; Taguchi et al. 2010).

To explore the potential mechanism behind the antinociceptive effect of OPRM1 re-expression we measured beta-endorphin levels in the supernatant of oral SCC cells that were transduced with Ad-OPRM1. Our results showed that beta-endorphin levels in Ad-OPRM1-transduced cells were higher than non-transduced control cells. These findings are consistent with work demonstrating that increased endogenous beta-endorphin levels provides thermal antinociception, and that administration of anti-beta-endorphin antibody suppresses this antinociceptive effect in a murine cancer pain model (Baamonde et al. 2006). Moreover, similar to our findings in mice, in human patients with either intractable bone or abdominal visceral cancer pain there is a significant increase in serum beta-endorphin following treatment with different pain relief modalities (Mystakidou et al. 1999; Befon et al. 2000; El-Sheikh et al. 2004). It has been shown that functional mu-opioid receptors are expressed on
keratinocytes aside from being expressed on peripheral unmyelinated nerve fibers in human skin. Furthermore keratinocytes specifically bind and also produce beta-endorphin, which acts on the peripheral unmyelinated nerve fibers to produce analgesia (Bigliardi et al. 1998; Bigliardi-Qi et al. 1999; Bigliardi-Qi et al. 2004). Conversely, beta-endorphin expression downregulates mu-opioid receptor expression in epidermis of culture human skin (Bigliardi-Qi et al. 2000). Our finding that beta-endorphin expression is upregulated in oral SCC cells that overexpress mu-opioid receptor could be due to a negative feedback mechanism, whereby mu-opioid receptor overexpression causes a regulatory increase in beta-endorphin production to result in overall inhibition of further mu-opoid receptor expression. This compensatory upregulation of beta-endorphin, however, could act on peripheral nerve receptors and produce antinociception in the mouse cancer model.

Having established promoter methylation of OPRM1 as a significant contributor to cancer-induced pain, we tested the analgesic potential of two demethylating drugs, decitabine and zebularine. The anti-proliferative effect of these agents had been established in many in vivo studies of mouse cancer models (Iliopoulos et al. 2007; Lemaire et al. 2008; Yoo et al. 2008). However, the effect of these chemotherapeutic drugs on oral SCC pain and proliferation has not been tested. We demonstrated that decitabine and zebularine have an antiproliferative and antinociceptive effect in a cancer pain mouse model. In addition, we showed that combination treatment with both decitabine and zebularine produced a more rapid decrease and effective maintenance of tumor size and tumor-induced nociception than either drug alone. Not only was this study the first demonstration of effective treatment of oral SCC
with demethylating agents, it also revealed a novel analgesic effect of these drugs.
The analgesic effect could have been due to demethylation of the \textit{OPRM1} promoter.
\textit{OPRM1} promoter methylation was significantly lower in tumors treated with
zebularine compared to control tumors. \textit{OPRM1} mRNA was correspondingly 51
times higher in zebularine-treated tumors. Furthermore, immunohistochemistry of
hind paw tumors showed higher expression of mu-opioid receptor in the cancer
microenvironment of mice treated with zebularine than with untreated tumors.
Ultimately, successful demethylation therapy that targets the cancer
microenvironment might thwart cancer pain-producing mechanisms at the source,
thus freeing patients of systemic drug toxicity.

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Chapter 4

Conclusion

Methylation as a Novel Regulatory Mechanism of Cancer Pain

In this dissertation we determined that genes intimately involved in endogenous analgesia, *EDNRB* and *OPRM1*, are capable of epigenetic silencing during cancer, which could result in dysregulation of the endogenous analgesic system.

Hypermethylation of these genes results in a biologically significant reduction in expression in oral squamous cell carcinoma (SCC) patients. Oral dysplasia patients, however, do not exhibit such hypermethylation in the *EDNRB* gene. Unlike oral SCC, where functional intensity, functional sharpness, and functional aching are associated with restricted speech, swallowing, eating, and drinking (Connelly et al. 2004; Kolokythas et al. 2007), oral dysplasia is not painful on function (Lam et al. 2011). Being that the main differences in oral SCC and oral dysplasia are that oral SCC is locally invasive, proliferative, and painful, whereas oral dysplasia is not, we hypothesized that hypermethylation of the two genes could contribute to these differences.

To determine whether *EDNRB* and *OPRM1* contribute to proliferation or pain, or both, we decided to reverse the epigenetic silencing process and analyze the effect *in vivo*. Since there is currently no reliable technique to demethylate a single gene, we re-expressed the genes by transducing oral SCC cells with adenoviruses containing plasmids with the cDNA sequence of the genes. We created mouse oral
SCC models from cancer cells transduced with either \textit{EDNRB} or \textit{OPRM1}. By quantifying tumor proliferation, mechanical allodynia and thermal hyperalgesia levels in the mouse oral SCC model over 21 days, we concluded that \textit{EDNRB} and \textit{OPRM1} gene expression have no significant effect on tumor proliferation. Their re-expression did, however, result in a significant analgesic effect to nociceptive stimuli.

Our results designate DNA methylation as a novel regulatory mechanism for genes mediating nociception and analgesia in patients with cancer. Epigenetic modification, therefore, serves as the key to unlocking endogenous analgesia as a new treatment modality for cancer-induced pain. The interventional therapeutic potential lies in the reversible nature of epigenetic silencing.

**An Emerging Role for Endogenous Analgesia in Cancer Pain Treatment**

Furthermore, we established that the mechanism by which gene re-expression produced analgesia was by restoring the balance of nociceptive and analgesic mediators in the cancer microenvironment.

We had discussed in the Introduction the clear role of endothelin-1 (ET-1) in inducing nociception (Wacnik et al. 2001; Peters et al. 2004; Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010; Quang et al. 2010). Activation of ET$_A$ receptors on the primary afferent nerves within the cancer microenvironment leads to significant pain (Pickering et al. 2007; Schmidt et al. 2007; Quang et al. 2010). The results of this dissertation elucidate the actions of the ET$_B$ receptor in cancer pain. Re-expression of the ET$_B$ receptor in a mouse oral SCC model effects significant analgesia. From our \textit{in vitro} experiments we determined that re-
expression of ET_B causes a reduction in ET-1 production. This effect could be due to its known role in ET-1 clearance and inhibiting ET-1 secretion in keratinocytes (Yohn et al. 1994; Nelson et al. 1997). In cancer cells where EDNRB is methylated and ET_B is absent, this feedback mechanism is lost and ET-1 is produced in high concentrations to activate ETA receptors, resulting in nociception. EDNRB re-expression, therefore, reduces ET-1 levels in the cancer microenvironment to tip the balance toward an analgesic effect.

In addition, EDNRB re-expression increases levels of beta-endorphin, which produces analgesia. We had shown in our previous studies that activation of ET_B receptors in a mouse oral SCC model leads to the production and release of beta-endorphin, resulting in analgesia. This effect is reversed by naloxone, a competitive antagonist with highest affinity for the mu-opioid receptor (Quang et al. 2010). Our current results indicate that re-expression of the ET_B receptor in oral SCC cells leads to significantly higher levels of beta-endorphin secretion into the cancer microenvironment. Re-expression of the mu-opioid receptor on cancer cells also has the same effect. Increase in beta-endorphin levels further favors analgesia over nociception.

While current management of cancer pain involves traditional therapeutics that deliver exogenous opiates systemically, escalating doses are required, and cancer patients develop opiate tolerance. Systemic overdose causes nausea, vomiting, constipation, and respiratory depression. From our results we propose an innovative molecular approach to cancer pain treatment: manipulating the local cancer microenvironment by reversing epigenetic silencing, to harness its
endogenous analgesic potential at the site of nociceptive stimuli. Tolerance and systemic toxicity could therefore be avoided.

**Novel Therapeutics Target the Cancer Microenvironment**

In this dissertation we have introduced two unconventional analgesic therapies that target the cancer microenvironment: 1) demethylating agents decitabine and zebularine and 2) virus-mediated gene delivery. For both approaches the end goal is reversal of epigenetic silencing and re-expression of analgesic mediators.

We demonstrate for the first time the analgesic potential of demethylating agents decitabine and zebularine. While they have clear antineoplastic effects in hematological and solid malignancies with minimal systemic toxicity (Blum et al.; Cashen et al.; Daskalakis et al.) (Cheng et al. 2003; Yoo et al. 2004; Yoo et al. 2008), they have no known role in analgesia and have not been used to treat painful conditions. We showed through our behavioral experiments that combination treatment of both decitabine and zebularine produced significant mechanical analgesia in the mouse oral SCC model. We determined that the potential mechanism for analgesia was by demethylation of the \textit{OPRM1} gene and subsequent re-expression of the mu-opioid receptor in the mouse oral SCC tumor environment.

Virus-mediated gene delivery has previously been explored to treat chronic pain. An HSV-based vector expressing inhibitory neurotransmitters or anti-inflammatory mediators were delivered to peripheral sensory neurons, and resulted
in reduction of pain related responses in rodent models of inflammatory pain, neuropathic pain, and bone cancer-induced pain. The expressed factors were either delta opioid agonist peptide enkephalin, mu opioid agonist peptide endomorphin-2, glutamic acid decarboxylase (GAD) to effect the release of gamma amino butyric acid (GABA), interleukin-4, interleukin-10, vascular endothelial growth factor, or tumor necrosis factor alpha (Mata et al. 2003; Chattopadhyay et al. 2005; Hao et al. 2006; Lee et al. 2006) (Liu et al. 2004; Hao et al. 2005; Hao et al. 2007; Wolfe et al. 2007; Zhou et al. 2008; Hao et al. 2009).

Our results showed an analgesic effect through adenovirus-based delivery of \textit{EDNRE} and \textit{OPRM1} to cancer cells. Re-expression of either the \textit{ETB} or mu-opioid receptor resulted in reduction of mechanical allodynia. Mu-opioid receptor re-expression additionally lowered thermal hyperalgesic responses. The main difference between our technique and HSV-based gene transfer lies in the target cell. While HSV preferentially acts on peripheral neurons, our adenoviruses were transduced in oral SCC cells. Our result that re-expression of the \textit{ETB} or mu-opioid receptor in the cancer cell produces analgesia is consistent with our hypothesis of cancer-mediated endogenous analgesia. We showed that cancer cells themselves can control nociception, and that epigenetic silencing plays an important role in this process.

**Future Directions**

Future experiments could further elucidate the role of the cancer cell in pain modulation. Specifically, the effect of mediators secreted by the cancer cell on
peripheral neurons could be explored. It is known that mediators secreted by cancer cells could transiently affect neuronal activity by binding to receptors on the neuron. The more salient question, however, is whether the cancer cell could effect a stable and long-term change in peripheral neurons of the cancer microenvironment, such that there is a permanent shift in neuron behavior. For example, could there be epigenetic modifications within neurons in response to carcinogenesis. The answer could reveal an intimate relationship between cancer cells and their peripheral neurons, which will ultimately lead to development of more effective cancer pain therapy.

References


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Table 3. Mean Methylation Difference Between Paired Cancer and Normal Tissue of *EDNRB* CpG Sites

<table>
<thead>
<tr>
<th>EDNRB PCR Product</th>
<th>CpG Site</th>
<th>Mean Methylation Difference</th>
<th>p-value of Student t test</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDNRB 1</td>
<td>CpG 4</td>
<td>0.15 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 5</td>
<td>0.24 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 6-7</td>
<td>0.30 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 8-9</td>
<td>0.29 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 10-11-12</td>
<td>0.29 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 13</td>
<td>0.24 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 14-15</td>
<td>0.27 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 16</td>
<td>0.17 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 17-18</td>
<td>0.20 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 19</td>
<td>0.30 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 1-2</td>
<td>0.22 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 3-4</td>
<td>0.51 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 5</td>
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<tr>
<td></td>
<td>CpG 8</td>
<td>0.34 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 9-10</td>
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<tr>
<td></td>
<td>CpG 13-14-15</td>
<td>0.36 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 16</td>
<td>0.32 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 17</td>
<td>0.51 &lt;.0001</td>
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<tr>
<td></td>
<td>CpG 18-19</td>
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<tr>
<td></td>
<td>CpG 20</td>
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<td></td>
<td>CpG 21</td>
<td>0.56 &lt;.0001</td>
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<tr>
<td></td>
<td>CpG 22-23</td>
<td>0.43 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 24</td>
<td>0.36 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 25</td>
<td>0.39 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 26</td>
<td>0.39 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 1</td>
<td>0.23 .0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 2-3-4</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>CpG 5</td>
<td>0.21 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 6-7</td>
<td>0.37 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 8</td>
<td>0.32 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 9</td>
<td>0.26 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 10-11</td>
<td>0.33 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 12</td>
<td>0.28 &lt;.0001</td>
<td></td>
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<tr>
<td></td>
<td>CpG 14</td>
<td>0.14 .0002</td>
<td></td>
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<tr>
<td></td>
<td>CpG 15</td>
<td>0.26 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 16</td>
<td>0.26 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 18</td>
<td>0.19 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 19</td>
<td>0.22 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 20</td>
<td>0.32 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 27</td>
<td>0.19 &lt;.0001</td>
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<tr>
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<td>CpG 28</td>
<td>0.31 &lt;.0001</td>
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<tr>
<td></td>
<td>CpG 1</td>
<td>-0.03 .4720</td>
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<tr>
<td></td>
<td>CpG 2-3</td>
<td>0.02 .3934</td>
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<tr>
<td></td>
<td>CpG 4</td>
<td>0.10 .0557</td>
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<td></td>
<td>CpG 6</td>
<td>0.16 .0011</td>
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<td>CpG 7</td>
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<td>CpG 8</td>
<td>0.10 .0082</td>
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<td>CpG 9-10</td>
<td>0.27 &lt;.0001</td>
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<td>CpG 12</td>
<td>0.32 &lt;.0001</td>
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<td>CpG 14-15</td>
<td>0.26 &lt;.0001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CpG 16</td>
<td>0.20 .0003</td>
<td></td>
</tr>
</tbody>
</table>
**Table 4. Demethylating Drug Treatment Scheme**

<table>
<thead>
<tr>
<th>Group</th>
<th>3% sucrose water <em>ad libitum</em> (vehicle)</th>
<th>3% sucrose water + zebularine <em>ad libitum</em></th>
<th>Osmotic pump delivery of PBS (vehicle)</th>
<th>Osmotic pump delivery of decitabine dissolved in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Oral SCC zebularine only</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>2) Oral SCC vehicle treatment</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>3) Oral SCC decitabine + zebularine</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>4) Oral SCC decitabine only</td>
<td></td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
</tbody>
</table>
Figure 1. *EDNRB* PCR products. The *EDNRB* promoter region and exon 1 is shown at the top. This region contains a CpG island with 77 CpG sites. The methylation frequency of each CpG site could be quantified using MassARRAY technology. The queried region is analyzed by creating four primer sets that yield four PCR products, which together survey the entire CpG island. Start and end bases for each product are noted; bases are numbered relative to the transcription start site (TSS). PCR product EDNRB 1 surveys the extreme 5' end of the CpG island. EDNRB 2 and 3 are located proximal to the TSS. They overlap each other by 188 bases. EDNRB 4 is located in exon 1 of the gene. Green circles denote the 61 out of 77 CpG sites whose methylation frequencies were quantified. Grey circles denote the 16 CpG sites that were not quantified.
Figure 2. Heat map of methylation values in paired oral SCC and normal tissue. The heat map was created with methylation values obtained using the MassARRAY system. Each column represents a CpG unit, which could be a single or an aggregate of CpG sites. Each row represents a patient enrolled into the study. Within each cell is the methylation value of a patient sample at a particular CpG unit. Samples for which the methylation value is not reliable are left blank. The values are color coded using a green-red scale, where green is low methylation and red is high methylation. The top heat map shows methylation values of oral SCC tissue. The bottom heat map shows methylation values of contralateral normal tissue. The EDNRB PCR product in which the CpG unit is located is indicated.
Figure 3. CpG sites have increased methylation in oral SCC tissue compared to normal tissue. The box plots represent methylation difference values between oral SCC and normal tissue from the same patient. The difference values are averaged from all patients within each CpG site. All CpG sites within a PCR product are grouped together; the median, 10th, 25th, 75th, and 90th percentiles are shown for each group. PCR product EDNRB 2 has the highest increase in methylation in oral SCC tissue (median = 0.34, 34%). EDNRB 1 and 3 have a median increase of 0.25 (25%). EDNRB 4 has the smallest increase of 0.13 (13%). Methylation values for each sample are averaged across all CpG sites within each PCR product and oral SCC tissue is compared to normal tissue using the Mann Whitney Rank Sum Test. The methylation values of the tissues are significantly different with p < .001.
Figure 4. Oral SCC patients demonstrate higher EDNRB methylation than oral dysplasia patients. The three plots show methylation values of CpG sites from PCR products EDNRB 1, 3, and 4, respectively, in oral SCC and oral dysplasia tissues. Oral SCC tissues exhibit an average of 20% higher methylation than oral dysplasia tissues at all CpG sites. The Mann Whitney Rank Sum Test is used to demonstrate significant differences in methylation between oral SCC and dysplasia tissues (\(^*p < .05\), \(^{**}p < .01\), \(^{***}p < .001\)). Standard error bars are shown.
Figure 5. *EDNRB* promoter methylation levels are higher in patients with nodal metastasis. The graph shows three plots of different nodal metastasis levels. Patients were classified by their TNM staging as N=0, N=1, or N=2. The N=0 group had 11 patients, the N=1 group had 3 patients, and the N=2 group had 6 patients. The plots represent methylation difference values (methylation value of normal tissue subtracted from that of oral SCC tissue) at each CpG unit in the *EDNRB* 1 product, which is the extreme 5’ end of the CpG island in the *EDNRB* promoter. The N=0 group has the lowest methylation value differences out of the three groups. The N=1 has intermediate differences, and the N=2 group has the highest methylation value differences. Using ANOVA and the Holm-Sidak test, we showed that the N=2 group is significantly higher than the N=0 group at 7 of 10 CpG units, and that the N=1 group is significantly different from the N=0 group at one CpG unit (*p < .05, **p < .01). Standard error bars are shown.
**Figure 6.** *EDNRB* mRNA expression is lower in oral SCC than normal tissue. *EDNRB* mRNA expression in normal and oral SCC tissue was quantified using RT-PCR. *GUSB* was used as the internal locus control. The relative *EDNRB* mRNA levels was obtained using delta-delta Ct. When compared to oral SCC tissue, normal contralateral tissue has on average 10.9 times more *EDNRB* mRNA than oral SCC tissue (Student's t test, ***p > .001).
Figure 7. Immunofluorescence images of Ad-EDNRB transduced and nontransduced oral SCC cells. Images are shown at 20X magnification. Images are taken from a single exposure setting. The top row of images shows Texas Red and Hoechst staining. The bottom row of images shows GFP and Hoechst staining. ET₉ receptor antibody was secondarily tagged with Texas Red and cell nuclei were counterstained with Hoechst. Nontransduced and Ad-GFP transduced HSC-3 cells display minimal ET₉ receptor expression, whereas Ad-EDNRB transduced HSC-3 cells exhibit strong ET₉ receptor expression. Ad-GFP transduced HSC-3 cells serve as the positive control for transduction. Ad-GFP transduced HSC-3 cells show strong GFP expression. Ad-EDNRB transduced HSC-3 cells also show GFP expression due to a C-terminal GFP tag on the ET₉ receptor protein. GFP and Texas Red colocalize in Ad-EDNRB transduced cells as both tag ET₉ receptor expression.
Figure 8. (a) Ad-EDNRB transduced oral SCC cells express high levels of ET$_B$ receptor compared to control oral SCC cells. Immunofluorescence images are quantified using Cell Profiler software. ET$_B$ receptor expression is quantified, and relative expression is calculated based on nontransduced HSC-3 expression. Cells transduced with Ad-GFP do not express high levels of ET$_B$ receptor. However, cells transduced with Ad-EDRNB express 9.6 times more ET$_B$ receptor than control, nontransduced cells. (b) Oral SCC cells show dose-dependent increase in EDNRB mRNA expression in response to transduction with Ad-EDNRB. HSC-3 cells are transduced at 12.5, 25, 50, 100, and 200 MOI. RT-PCR performed on each group reveals that there is a dose-dependent increase in EDNRB mRNA expression with increasing MOI. Fold change is calculated relative to nontransduced HSC-3.
Figure 9. (a) Ad-EDNRB transduction does not alter oral SCC tumor growth. The graph shows change in paw volume, which is a measure of tumor growth. The X axis shows the days of the experiment, and the Y axis shows the average change in paw volume. All three study groups show a similar increase in paw volume as the experiment progresses. There is no statistically significant difference in paw volumes among the study groups. Standard error bars are shown. (b) Ad-EDNRB transduction decreases mechanical alldynia. The graph shows change in paw withdrawal threshold, which is a measure of mechanical alldynia. Mice with tumors that have been transduced with Ad-EDNRB exhibit significantly higher paw withdrawal thresholds, indicating lower mechanical alldynia. ANOVA and Holm-Sidak tests are used (*p < .05, **p < .01, ***p < .001). Standard error bars are shown.
Figure 10. Heat map of OPRM1 promoter region. Analyzed region is -232 to +109 relative to transcription start site. The heat map compares the methylation status between DNA extracted from frozen oral SCC tumor versus contralateral normal tissue of 19 patients with biopsy-proven oral SCC in the EDNRB promoter region. The results for 20 CpG sites are shown; the percent of methylation at each site was correlated to a color, with green representing 0% methylation and red representing 100% methylation.
Figure 11. Immunohistochemistry of mouse hind paw tumors after adenovirus transduction. Orthotropic oral SCC tumors were created in the right hind paw. Immunohistochemistry with mu-opioid receptor antibody showed that tumors transduced with Ad-OPRM1 expressed high levels of mu-opioid receptor. Control tumors, which were either not transduced, or were transduced with Ad-GFP, showed an absence of mu-opioid receptor expression.
Figure 12. Mice with tumors re-expressing mu-opioid receptor demonstrate lower mechanical allodynia than control. Graph shows percent change in mean paw withdrawal threshold from baseline. Based on the One-Way ANOVA and Holm-Sidak methods, paw withdrawal threshold of tumors re-expressing mu-opioid receptor was significantly higher than the control groups, indicating lower mechanical allodynia (** p < .01, *** p < .001).
Figure 13. Mice with tumors re-expressing mu-opioid receptor demonstrate lower thermal hyperalgesia than control. Box plot represents the median, 10th, 25th, 75th, and 90th percentiles. Mice with tumors re-expressing mu-opioid receptor had significantly longer latency than control mice, which had tumors that do not express mu-opioid receptor (Student’s t-test, *p < .05).
Figure 14. Oral SCC cells re-expressing mu-opioid receptors produce more beta-endorphin than control oral SCC cells. Graph compares beta-endorphin levels in the supernatant of oral SCC cells re-expressing mu-opioid receptor and control oral SCC cells. While control oral SCC cell supernatant contains almost no beta-endorphin, cells transduced with Ad-OPRM1 at 200 MOI secrete an average of 0.4 ng/ml beta-endorphin, which was significantly higher than non-transduced cells (Student’s t-test, *p < .05). Oral SCC cells are transduced with Ad-GFP as a control for transduction. These cells showed very low secretion, which was not significantly higher than nontransduced cells. Standard error bars are shown.
Figure 15. Combination Therapy with Decitabine and Zebularine has an Antiproliferative and Analgesic Effect in a Mouse Model (a) Combination treatment of zebularine and decitabine produce a stronger antiproliferative effect in an orthotopic oral SCC mouse model than either drug alone. Paw volume measurements for Groups 1-4 from PID -2 to 21 are shown. Treatment with either decitabine or zebularine alone produces a slow reduction in paw volume. Combination treatment of the two drugs, however, causes a rapid decrease in paw volume between days 4 and 7, and subsequent maintenance of paw volume at baseline for the remainder of the experiment. (b) Combination treatment with decitabine and zebularine produces a stronger analgesic effect in an orthotopic oral SCC mouse model than either drug alone. (ANOVA, ANOVA on Ranks, Holm-Sidak, Dunn’s test as appropriate, *p < .05, **p < .01, ***p < .001).
Figure 16. Mice treated with demethylating drugs show lower methylation levels of the *OPRM1* gene. The MassARRAY EpiTYPER assay (Sequenom) was used to quantify methylation of each CpG site in the mouse promoter region of *OPRM1* (-304 to +71 relative to transcription start site; antisense strand was analyzed). Mice treated with zebularine have significantly lower methylation levels of the *OPRM1* gene (Student’s t test, *p* < .05, **p** < .01). Standard error bars are shown.
Figure 17. Immunohistochemistry of mouse hind paw tumors after demethylating drug treatment. Immunohistochemical stain with mu-opioid receptor antibody and corresponding hematoxylin and eosin staining are shown. Tumors from mice that underwent demethylating drug treatment (with zebularine) show a re-expression of the mu-opioid receptor on cancer cells. Mice not treated with demethylating drugs have an absence of mu-opioid receptor expression in their tumors.
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