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Sustained adrenergic signaling promotes cervical cancer progression

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SUSTAINED ADRENERGIC SIGNALING PROMOTES CERVICAL CANCER TUMOR PROGRESSION

by

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A

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SUSTAINED ADRENERGIC SIGNALING PROMOTES CERVICAL CANCER TUMOR PROGRESSION

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Background: Chronic stress and sustained adrenergic signaling are known to promote tumor progression. The underlying mechanisms behind this process are not well understood. We examined the effects of sustained adrenergic signaling on cervical cancer progression through increased expression of HPV oncogenes, E6 and E7.

Materials and Methods: ADRβ expression levels were examined in patient-derived cervical cancer samples. We used an orthotopic model of cervical cancer to investigate the effects of restraint stress on tumor growth and metastasis. We evaluated the *in vivo* effects of a β-blocker, propranolol, and HPV E6/E7 siRNA. *In vitro*, ADRβ positive cervical cancer cell were treated with norepinephrine (NE) or isoproterenol (ISO) to examine intracellular responses. Invasion and anoikis assays were performed to elucidate the biological effect of NE. Furthermore, the significance of secreted HPV was examined.

Results: Among tumor samples evaluated from cervical cancer patients, 61% had increased ADRβ2 expression, which correlates with decreased overall survival ($p=0.038$). In an orthotopic model of cervical cancer, chronic stress led to increased tumor weight and nodules. This effect was abrogated with propranolol. Further, treatment with HPV E6/E7 siRNA in the restraint stress model decreased tumor growth and metastases. *In vitro*, HPV E6/E7 mRNA was

elevated in cell lysates and corresponding supernatant after treatment with NE or ISO. NE exposure resulted in increased invasion and migration of SiHa cells, while E6/E7 siRNA abrogated these effects. After NE exposure, ME-180 cells showed a 45% reduction in anoikis compared to controls. Fibroblasts cultured with supernatant from SiHa cells had increased migration and elevated mRNA of pro-inflammatory genes CXCL2 and IL-8. Further, the fibroblasts took up cervical cancer cell derived exosomes that contain HPV E7. The conditioned fibroblasts demonstrated the ability to increase cervical cancer cell invasion when co-cultured.

Conclusion: This study shows increased adrenergic signaling promotes cervical cancer growth and progression. Disruption of this pathway with β-blockers could provide a novel complement to current therapies. In addition, we show HPV oncogenes E6 and E7 can be influenced by catecholamines. We also show exosomes as a potential mean of communication between cervical cancer cells and fibroblasts *in vitro*. The functional implications of this study need to be explored further.

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Background and Introduction

Cervical cancer

There are an estimated 12,000 new cases of cervical cancer in the US each year and about a third of the women affected die because of the disease. Globally, the disease causes around a 250,000 deaths a year [1]. The primary cause of cervical cancer is human papillomavirus (HPV); additional contributors include cigarette smoking, multiple sex partners, and immunosuppression [2]. The death rate from cervical cancer in developed countries has declined about 70% in the past 50 years because of increased use of the Papanicolaou (Pap) test [3]. The Pap test is performed by collecting cells from the outer opening of the cervix, which are then analyzed for abnormalities in aims of detecting early pre-cancerous lesions and cancer.

In early stages of the disease, conization or hysterectomy is used to remove cancerous lesions. Radiation and chemotherapy may be used for disease with regionalized spread [4]. Advanced cervical cancer can spread into the vagina and uterus, growing onto the muscular wall of the pelvis, and even can block one or both ureters, bladder, or rectum. The cancer can also metastasize to distant organs such as the liver and lungs. Larger early stage tumors typically require radical hysterectomy, including removal of local lymph nodes [5]. Palliative care is important in order to improve quality of life and relieve symptoms from treatment.

Human papillomavirus (HPV)

The primary cause of cervical cancer is the HPV. There are over 100 different strains of HPV; among these there are low risk types (6 and 11) which are most commonly associated with warts, and the high-risk types (HPV16, 18, 31, 33,45, 52, and 58) which are associated with cancer [6] [7]. High risk-HPV (HR-HPV) types 16 and 18 account for 70% of the HPV positive cervical cancer tumors [8]. The virus first infects the basal layer of the epithelium, typically entry is through an abrasion that exposes the basal cells [7]. The capability of the virus to directly infect stromal cells has not been previously described nor accepted in the virology field, HPV only infects basal cells of the skin and mucosa [9]. During the establishment of infection, the virus associates with heparan sulphate proteoglycans (HSPG) on the cell surface prior to being internalized [10]. The cell and virus replicate together in the proliferative layers of the epithelium. In the top layers of the epithelium, the virus is fully packaged and expression of all the viral proteins is high. The encapsidated virus can exit the cell as infectious virus particles at this point. Conversely, the virus can remain in a latent phase, in which viral replication has ceased [11]. In latency, viral gene expression is minimal and the virus is maintained episomally or integrated in host DNA. Persistent infection with HPV and additional aberrations in the cell ultimately lead to the tumor growth [12].

The virus is a small double stranded DNA virus (Figure 2). The genome has an upper regulatory region (URR), 6 early open reading frames (ORF)

(E1, E2, E4, E5, E6, E7), and 2 late ORFs (L1 and L2) [13]. After the host cell is infected early genes E1 and E2 are expressed which play roles in transcription and replication of the virus [14]. The E2 protein binds specific regions in the URR to negatively regulate the E6/E7 promoter. E6 and E7, the oncogenes of the virus, are transcribed bi-cistronically [15].The URR also contains other transcription factor binding sites that have been shown to modulate the promoter function as well, such as AP1, Oct-1, SP1, and glucocorticoid response elements [16]. The two late genes L1 and L2 encode the capsid protein coat of the virus [17].

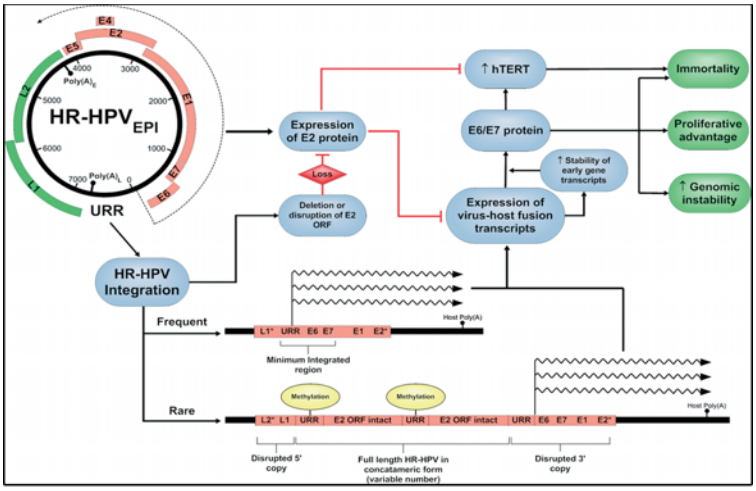

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Figure 1. Illustration of HPV in the cell. The HR-HPV episome contains all 8 genes and an URR, typically spanning 8,000bp. The most common integrated form of HPV contains an undisrupted URR, E6 and E7. Loss of E2 inhibitory effects plus stable expression of the oncogenes results in cellular immortality, increased proliferation, and genomic instability[12].

HPV oncogenes: E6 and E7

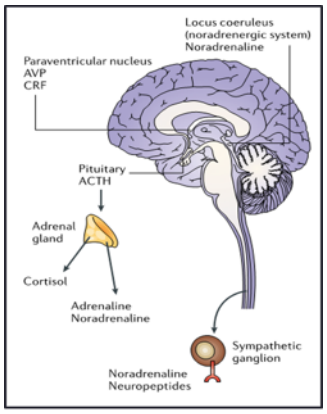
The E6 protein has many known interactions and functions in the cell. The most canonical oncogenic function is the ability to inactivate p53-dependent growth arrest and apoptosis [18]. Additional functions include the degradation of pro-apoptotic proteins BAX and BAK [19], and to promote immortalization E6 has a known role in up regulating the transcription of hTERT [20].

E7 proteins associate with the retinoblastoma protein to induce hyper proliferation. This interaction allows E2F6 mediated cell cycle progression through the S-phase, which for viral genome replication is favorable [21]. E7 has also been shown to deregulate the cell cycle by directly interacting with p21 and p27 [22].

Stress and cancer

Studies have shown a strong relationship between psychosocial factors, including depression, low social support, and hopelessness, and cancer progression [23]. Cervical cancer patients typically present with greater disruptions in quality of life (QOL) than in other cancers; factors that contribute to their psychosocial distress include poor coping skills, depression, anxiety, social isolation, and drug use [23]. Unfortunately, cervical cancer patients without social and clinical support service and the lack of access to care, often face a delay in diagnosis and have advanced disease at presentation [24]. For cervical cancer specifically, in one study patients were

given a stressful life events (SLE) assessment as a measure of psychosocial distress and those with higher scores were associated with higher risk for squamous intraepithelial lesions (SIL) [25]. In another study, women with negative coping styles were also more likely to have SIL when compared to those with positive coping styles [26]. High levels of pessimism, despair, anxiety, and uncontrollable stressors are all associated with higher risk for SIL [27]. Studies to date are correlative and future investigation of the mechanisms behind this relationship need to be explored.



Antoni M H et al. Nat Rev Cancer.
2006 Mar;6(3):240-8

Figure 2. The stress response. In response to a stimulus both the hypothalamic-pituitary-adrenal axis (HPA) and sympathetic nervous system (SNS) are activated to adapt physiologically to a threat. Through the HPA axis the hypothalamus secretes corticotrophin releasing factor (CRF) and arginine vasopressin (AVP), which activate the pituitary to produce hormones such as adrenocorticotropic hormone (ACTH). Circulating ACTH stimulates production of glucocorticoids. Additionally, when sympathetic nerves of the SNS are activated, they can also result in the release of epinephrine and norepinephrine.

Activation of the SNS or HPA prepares an individual to survive a threat and the physiological stress responses are considered adaptive (Figure 2) [28]. The acute stress response is commonly known as the “fight or flight” response and has a clear onset and offset with transient changes in hormones. However, under chronic stress the physiological systems are negatively affected by persistent elevation of stress hormone levels [29].

Studies of behavioral stress in animals have provided reliable models for dissecting stress effects on tumor progression [30]. Restraint stress, swim stress, social confrontation or isolation are models commonly used for inducing experimental stress [30]. These models accurately mimic physiological stress pathway activation which disrupts homeostasis as seen in patients [31]. Previous studies *in vivo* suggest chronic stress plays a role in tumor growth and metastasis. In an orthotopic murine model of breast cancer, chronic stress increased lung metastases significantly [32]. In another study, restraint stress led to increased production of vascular endothelial growth factor (VEGF), and enhanced angiogenesis and tumor burden in an orthotopic murine model of ovarian cancer [33]. *In vitro* effects of stress hormones parallel the results seen in animal models. Catecholamine exposure led to increased expression of metalloproteinase (MMP) 2 and 9, which increased ovarian cancer cell invasion compared to control [34]. Furthermore, ovarian cancer cells exposed to NE induced FAK-mediated

resistance to anoikis [35]. Adrenergic stimulation also has been shown to play a role in suppressing NK cell activity [36].

β-adrenergic signaling is the primary mediator of the SNS induced responses involved in the promotion of cancer [37]. Because this signaling pathway has shown to be critical in tumor progression, β antagonist/blockers provide an appealing adjuvant therapy strategy for abrogating the impact of stress. Studies done in patients with prostate, lung, ovarian, and breast cancers have shown higher survival and lower recurrence and metastasis rates when taking a β-blocker [38]. Current studies have not addressed β-blockers use in cervical cancer patients however; many clinical based assessments have supported the use of β-blockers in patient interventions [39].

Although the effects of stress and cancer are established, the role of stress in viral oncology is not well studied [40]. HPA or SNS can directly affect; viral replication, and activation of viral oncogenes, or indirectly bio-behavioral factors can cause evasion of immune response [41]. Neuroendocrine function has been shown to modulate tumor associated viruses such as; Hepatitis B and C virus, Kaposi's sarcoma-associated herpes virus (KSHV), Human immunodeficiency virus 1 (HIV1), and Epstein–Barr virus (EBV). Under chronic stress reactivation and increased expression of EBV and Hepatitis B and C viruses has been reported [42] [43]. β-adrenergic signaling induces a viral transcription factor in KSHV *via* a cAMP/PKA signaling pathway directly promoting lytic replication of the virus

[44]. Additionally, in cells infected with HIV1, replication was accelerated in response to norepinephrine through a PKA-dependent mechanism [45]. To our knowledge no relationship between catecholamine's and HPV has been shown.

Fibroblasts in the tumor microenvironment

Fibroblasts are a non-epithelial cell type in connective tissue primarily responsible for depositing extracellular matrix. Fibroblasts are often referred to as a scaffolding cell type, which have a well-studied role in wound healing [46], regulation of epithelial cell differentiation [47], and regulation of inflammation [48]. An activated fibroblast is referred to as a myofibroblast and this is associated with the type found in wound healing and more recently tumors [49]. The fibroblast associated with cancer cells and the tumor stroma are known as carcinoma-associated fibroblasts (CAFs) [50]. Studies of fibroblasts in the tumor microenvironment compared to normal fibroblasts have shown increases in migration, increases in pro-inflammatory genes, and increased growth factor secretion [51]. CAFs can be distinguished from normal fibroblast by their expression of fibroblast activating protein (FAP) [52] and increases in alpha-smooth muscle actin (α-SMA) protein [53]. How CAFs might contribute to the promotion of cancer is currently a very broad and popular topic of interest.

Exosomes

For intercellular communication, cells can release vesicles known as exosomes. Exosomes origin is from endosomes and their size ranges from 30-120 nm [54]. The most common pathways used for exosomal release are dependent upon SMPD2, Rab family proteins (Rab11,27,35), and ESCRT machinery proteins[55]. These vesicles can transfer biological information, such as membrane receptors, mRNAs, RNA, DNA, or proteins, between cells [56]. Exosomes content and proposed function can be different depending on the type of cell that release them (epithelial cells, macrophages, platelets) [57]. Extracellular vesicles content and specifically how to target these pathways in cancer are being studied extensively [55, 58].

Recent studies have shown tumor-associated viruses (HSV, HIV, and EBV) have release and uptake mechanisms similar to exosomes [59]. Further, virus containing cells can infect surrounding cells *via* microvesicles [60] [61]. Extracellular vesicles also provide a way for the viruses to evade and alter immune system to prevent their own destruction [62]. The understanding of the precise mechanism of communication between viral infected cells and their microenvironment paves new directions for viral oncology.

The impact of neuroendocrine modulation on HPV in cervical cancer cells and the biological consequences are the focus of this thesis. The possibility that cervical cancer derived exosomes could carry HPV oncogenes prompted us to specifically consider their role in manipulating the microenvironment to help promote cervical cancer progression.

Hypotheses and Specific Aims

The overall hypotheses of this project are:

1) Daily restraint stress promotes tumor growth in an orthotopic model of cervical cancer

2) Adrenergic activation will result in increased levels of HPV oncogenes E6 and E7 in cervical cancer cells

3) Secreted HPV can be transferred *via* exosomes to other stromal populations in the microenvironment which facilitates the invasion of cervical cancer cells

These hypotheses can be unified into a central hypothesis, which is modeled in Figure 3. HPV positive cervical cancer cells respond to catecholamines by increasing the oncogene's mRNA and protein levels. The three biological consequences of the sustained adrenergic signaling include 1) increased invasion and migration of cancer cells, 2) resistance to anoikis (in both cases an E6/E7- dependent mechanism is suspected), and 3) the virus is packaged and secreted *via* exosomes. Following the transfer of HPV to stromal populations, the fibroblast cells are altered for establishing more permissive conditions for tumor growth and metastasis. The following specific aims will test the hypotheses highlighted in this project:

Specific Aim 1: To determine the biological effect of chronic stress on cervical cancer growth and progression using orthotopic mouse models.

Specific Aim 2: To determine the functional consequences of catecholamine-mediated increases in HPV oncogenes E6 and E7 in cervical cancer cells.

Specific Aim 3: To evaluate the role of exosomal HPV oncogenes in cell-to-cell communication in the tumor microenvironment.

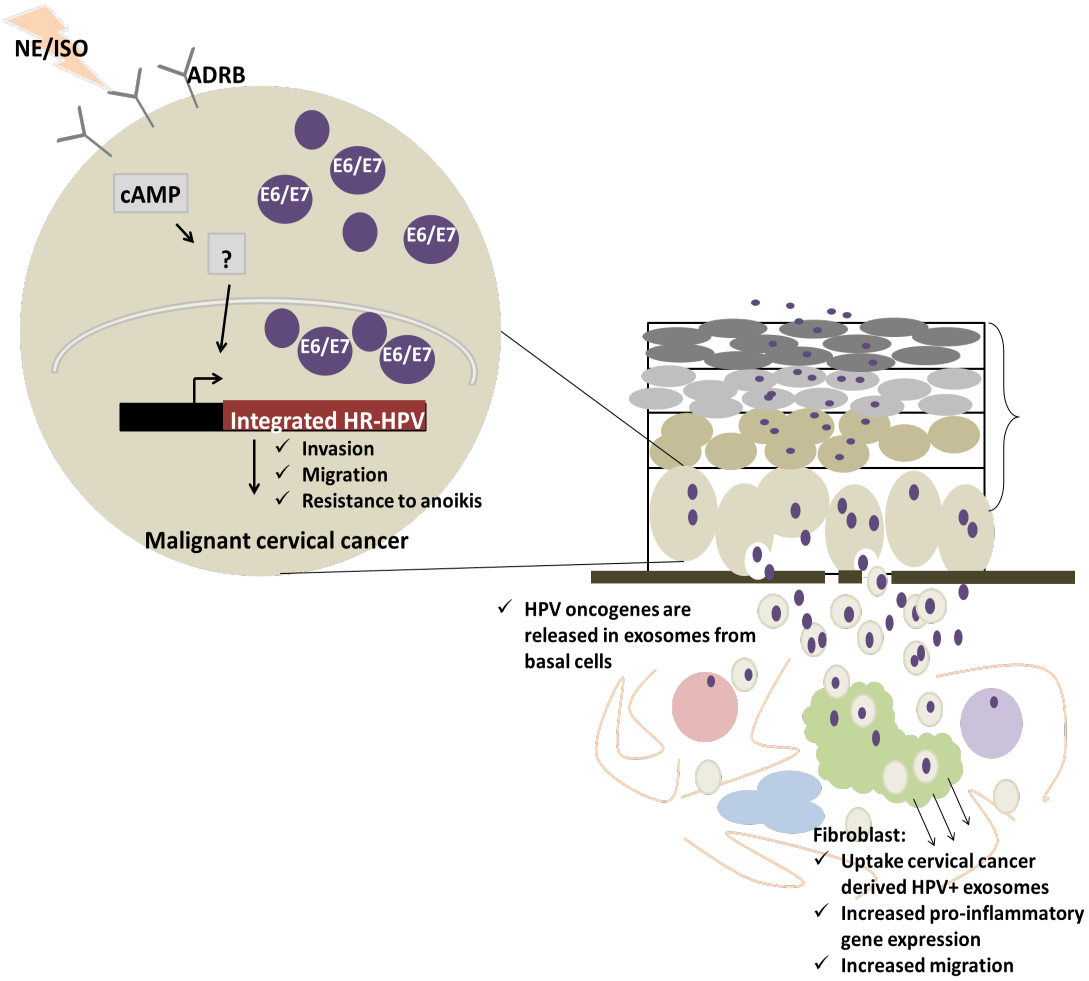


Figure 3. Central Hypothesis. HPV positive cervical cancer cells respond to stress hormones by increasing HPV oncogene expression. Biological consequences of stress in cervical cancer cells include increased invasion and migration and resistance to anoikis. The cervical cancer cells can secrete HPV in exosomes, which modulate the tumor microenvironment by being uptake by cell types such as, fibroblast. Increases in pro-inflammatory genes expression and increased proliferation and migration of fibroblasts promote a permissive environment for tumor progression of cervical cancer.

Methods

Cell lines and tissue culture

SiHa and CaSki cells were maintained in RPMI supplemented with 15% fetal bovine serum, and 0.1% gentamicin sulfate (Gemini Bioproducts, Calabasas, CA). ME-180 was maintained in McCoy's 5a Medium Modified with 10% fetal bovine serum, and 0.1% gentamicin sulfate. C4-1 cell line was maintained in Waymouth's MB 752/1 medium with 10% fetal bovine serum, and 0.1% gentamicin sulfate. The C33A cell line was cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum, and 0.1% gentamicin sulfate. The telomerase-immortalized human normal ovarian fibroblast (NOF) line NOF151-hTERTwere maintained in MCDB 105/199 medium (1:1) supplemented with 10% fetal bovine serum, 0.01% epidermal growth factor, and 0.01% Penicillin Streptomycin. All experiments were performed at 70-85% confluence and cell lines were routinely screened for mycoplasma.

Table 1. Cervical cancer cell lines and HPV status

Cervical cancer cell line	HPV status
SiHa	Positive, HPV 16
CaSki	Positive, HPV 16
ME-180	Positive, HPV 18
C4-1	Positive, HPV 18
C33A	Negative

Animal studies

All animal work was done in accordance with protocols approved by the MD Anderson Institutional Animal Care and Use Committee. Experiments

involving human cells lines were performed in 8-12 weeks-old female athymic nude obtained from Taconic Farms (Hudson, NY). All animals were cared for in the accordance to the guidelines set forth by the American Association for Accreditation of Laboratory Animal Care and the US Public Health Service policy on Human Care and Use.

In vivo model of cervical cancer and tissue processing

For all animal experiments, cells were harvested using trypsin-EDTA, neutralized with FBS-containing media, washed, and re-suspended to the appropriate cell number in HBSS prior to injection. SiHa and ME-180 cells ($1x10^6$) cells were transduced with lentivirus-encoding luciferase and expanded for injection. Nude mice were anesthetized using a ketamine-based anesthesia (100 mg/kg ketamine, 2.5 mg/kg xylazine, 2.5 mg/kg acepromazine) prior to surgery. After complete anesthesia, a small incision was made in the lower abdomen and the uterus was exposed where we then injected cells onto the top of the cervix where it meets the lower uterine segment. Mice were anesthetized with isoflourane and imaged once weekly for luminescent signals using Xenogen IVIS system. Tissue specimens used for all analyses were fixed in either optimal cutting temperature compound (OCT) (Miles, Elkhart, IN), formalin, or snap frozen in liquid nitrogen. Blood was collected immediately before necropsy, serum was allowed to clot for 20 minutes at room temperature. Samples were then centrifuged for 30 minutes at 2500 RPM. Supernatant was collected and stored at -80°C.

Restraint Stress Model

To experimentally induce stress, we use a daily restraint-stress procedure in which the animals are enclosed in a movement-restricted space for 2 hours daily for 21 days. Animals were given 7 days after laparotomy surgery to recover before restraint stress started. The nude mice were randomized (10 mice per treatment) into four groups: 1) control no stress, 2) control restraint stress, 3) propranolol no stress, and 4) propranolol restraint stress. All mice received daily intraperitoneal injection of PBS or propranolol, 2mg/kg. Animals were subsequently sacrificed seven days after stress was ceased. Researchers blinded to the groups noted distribution, mouse weight, tumor weight, and the number of nodules. The same model system was used for the E6/E7 siRNA experiment.

SiRNA preparation and treatment

HPV16 E6/E7 (GUCGAUGUAUGUCUUGUUGCAGAUC) and non-targeting control (UUCUCCGAACGUGUCACGU) sequence specific siRNA was obtained from Sigma Aldrich (Woodlands, TX). For *in vitro* studies, Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) was used according to manufacturer protocol to transiently transfect cells. For *in vivo* studies, siRNA was incorporated into 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC) nanoliposomes. For each treatment, 3.5 µg of siRNA was reconstituted in 200µl of sterile phosphate-buffered saline (PBS) and administered by intraperitoneal injection biweekly.

Quantitative real-time PCR

Total RNA from either tumor tissue or cell lines was extracted using Qiagen RNeasy Kit (Qiagen, Valencia, CA). Free-circulating RNA from serum or conditioned media was isolated using QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA). cDNA was generated using 1µg of the RNA using Verso cDNA kit (Thermo Scientific, Houston, TX) per the manufacturer's instructions. cDNA was subjected to amplification *via* real-time PCR using specific primer sequences (100ng/µl) which are specified in Table 1. Quantitative values were obtained for each sample that was normalized to its 18S content.

Conventional PCR

DNA was extracted from serum, conditioned media, or exosomes using QIAamp MiniElute Virus Spin Kit (Qiagen, Valencia, CA). 1 µl of extracted total DNA or cDNA was used as template. All PCRs were performed in a final volume of 25 µl under identical conditions on an Applied BioSystems 9500. PCR amplification was performed with 1µl of each primer (100ng/µl), 12 µl of PCR Master Mix (Promega, Madison, WI) and 9.5 µl dH2O. The PCR mixture was subjected to 30 cycles of amplification consisting of denaturation at 94°C for 1 min, annealing at 54°C for 45 s, and elongation at 72°C for 1 min. The first cycle was preceded by 3 min of denaturation at 94°C and the last cycle was followed by 10 min incubation at 72°C. The integrity of extracted mRNA was tested by amplification of Beta-Actin. Amplification products were analyzed by electrophoresis on 2-5% agarose gels and stained with Ethidium Bromide.

Table 2.PCR primer sequences.

Gene	Forward Sequence	Reverse Sequence
HPV16 E6/E7	AAGCAACAGTTACTGCGACGTG	GCCCATTAACAGGTCTTCCAAA
HPV 16 E6	AATGTTTCAGGACCCTACGG	TCAGGACACAGTGGCTTTTG
HPV 16 E7	TTTGCAACCAGAGACAACTGA	GCCCATTAACAGGTCTTCCA
HPV 16 E2	CGGAAATCCAGTGTATGAGC	GAGGCTGCTGTTATCCACAA
HPV16 L1	TTATTGCTGATGCAGGTGAC	AGCACGGATGAATATGTTGC
HPV 18 E7	GCATGGACCTAAGGCAACAT	TGTTGCTTACTGCTGGGATG
PTGS2	CCCTTGGGTGTCAAAGGTAA	GCCCTCGCTTATGATCTGTC
VEGF	AGCTGCGCTGATAGACATCC	CTACCTCCACCATGCCAAGT
IL6	GTCAGGGGTGGTTATTGCAT	AGTGAGGAACAAGCCAGAGC
IL8	AGCACTCCTTGGCAAAACTG	CAAGAGCCAGGAAGAAACCA
CXCL2	GCTTCCTCCTTCCTTCTGGT	GGGAGAAAGCTTGTCTCAA
ADRβ1	AAGGAACATCAGCAAGCCAC	GTGAACTCGAAGCCCACAAT
ADRβ2	TCCACCTGGCTAAGGTTCTG	TGTCCTTCTACGTTCCCCTG
ADRβ3	CTTCTTTCTGGCCAACGTG	CAGTAGATGAGCGGGTTGAAG
18S	CGCCGCTAGAGGTGAAATTC	TTGGCAAATGCTTTCGCTC

Immunoblotting

Lysates from cultured cells or tumor were prepared for immunoblotting using modified RIPA buffer (50mM Tris-hcl [pH 7.4], 150 mM NaCl, 1% Triton, 0.5% deoxycholate) plus 25 µg/ml leupeptin, 10 µg/ml aprotinin, 2mM EDTA, and 1mM sodium orthovanadate. The concentration of protein samples was determined using a BCA Protein Assay Reagent kit (Pierce Biotechnology, Rockford, IL). Lysates were loaded and separated on 8%-15% sodium dodecyl-sulfate-polyacrylamide gels, respective to the protein of interest size. Proteins were transferred to a nitrocellulose membrane by semidry electrophoresis (Bio-Rad Laboratories, Hercules, CA) overnight, blocked with 5% Bovine Serum Albumin for 1 hour and then incubated at 4°C with primary antibody overnight.

TBST was used to wash the membrane thoroughly before incubation with horseradish peroxidase (HRP)-conjugated secondary IgG (1:2000, GE Healthcare, UK) for 1 hour. An enhanced chemiluminescence detection kit (Pierce) was used to visualize HRP. To confirm equal loading of samples, the blots were probed for an antibody specific to beta-Actin (0.1 µg/ml; Sigma). ImageJ densitometry analysis was used for quantification.

Migration and Invasion assays

Both assays use a 24-well insert with 8uM pore size and polycarbonate membrane modified Boyden chamber (Coster, Boston, MA). For migration assays, the membrane was coated with 0.1% gelatin and an extracellular matrix component mixture composed of human laminin, type IV collagen and gelatin for invasion. Single-cell suspension of 75-100,000 tumor cells was seeded into the inserts in serum free media with serum containing media in the lower chamber to allow migration and invasion. Cells were incubated at 37°C in 5% CO_2 for 8 hours for migration and 24 hours for invasion. The membranes were fixed and stained at their respective time points, and counted in 5 random fields using light microscopy at 200x for quantification.

ELISA

Cell lysates were prepared as previously described in methods for immunoblotting. For both HPV16 E7 ELISA kit (MyBioSource, San Diego, CA) and cAMP assay kit (Cell Signaling, Danvers, MA), protein was used at the

concentration of 100µg and compared to standards provided in the kits. In addition, conditioned media was collected from cells then centrifuged at 4°C, 1200 RPM for 10 min. 100 µl of serum (methods for serum collection described in methods for tissue processing) or conditioned media were used for measurement of E7.

Cell Viability Assay

Cells were plated (1x 10^4 in 100µl) in a 96-well plate. 24-72 hours later, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT, was added to each well. Plate was incubated for 2 hours and then absorbance was read at 570 nm (Ceres UV 900C; Bio-Tek Instrument Inc, Winooski, VT).

Anoikis assay

Cells (5 x10^5 per well) were cultured on low attachment tissue culture plates for 24-96 hours at 37°C in 5%CO_2. After incubation, the cells in suspension were harvested and centrifuged at 1200 RPM for 5 min. Pellets were washed with cold PBS supplemented with 0.01% FBS twice. 5nM of SYTOX Red dead cell stain (Life Technologies, Grand Island, NY) was added to the cells in PBS/FBS solution, this suspension was then immediately analyzed by flow cytometry for viability (Gallios Cell Analyzer).

Proliferation assay

Cells were plated and treated with either siRNA or cultured in conditioned media for the duration of the experiment. At the respective time point, cells were incubated with EdU (5-ethynyl-2´-deoxyuridine) at a dilution of 1:100 for 2 hours before analysis by flow cytometry (Gallios Cell Analyzer). Using the Click-iT EdU Flow Cytometry Cell Proliferation Assay Kit (Life Technologies, Grand Island, NY), the EdU incorporation was determined.

Exosome isolation

Cells were treated and maintained in their normal media throughout all experiments, 24 hours prior to exosomal isolation their media was switched to media supplemented with Exosome-depleted FBS (System Biosciences, Mountain View, CA). Cultured media was collected and centrifuged at 4°C, 2000 x g for 30 minutes. The supernatant was removed carefully from pellet and debris at the bottom of the tube. Total Exosome Isolation Reagent (from cell culture media) (Invitrogen, Carlsbad, CA) was added 1:2; this suspension was placed on a rocker at 4°C overnight. Following incubation, samples were centrifuged at 10,000 x g for 1 hour at 4°C. The exosomes, in the pellet, were suspended in 1xPBS or Exosome Resuspension Buffer (Invitrogen, Carlsbad, CA) and used immediately or stored for further downstream analysis.

Labeling of exosomes

Exosomes were isolated as described in methods for Exosome isolation and resuspended in 100µl of PBS. 1µl of Bodipy-TR ceramide (Invitrogen, Carlsbad, CA) was added to exosome sample for 20 min at 37°C, protected from light. Excess unincorporated dye was removed following the standard protocol for the Exosome Spin Columns (Invitrogen, Carlsbad, CA). In short, column is rehydrated with PBS and exosome sample is applied directly to the center of the top of the column. After centrifugation, the elution in the bottom will contain just the labeled exosomes. Exosome tracing experiments were carried out immediately, excitation efficiency was analyzed for this dye at 594nm (LSRFortessa X-20 Cell Analyzer).

HPV E7 detection by flow cytometry

Cells were suspended in fixative solution (eBioscience, San Diego, CA) for 1h, RT, in the dark. 1x permeabilization washing buffer (eBioscience, San Diego, CA) was used to wash cells two times. Cells were stained with primary antibody (HPV16 E7 and HPV18 E7 1:200, Santa Cruz Biotechnology, Dallas, TX) in 100 µl permeabilization buffer for 30 min on ice. Cells were washed once with permeabilization buffer before secondary (Alexa-488 conjugated anti-goat for HPV16 E7 or anti-mouse for HPV18 E7, 1:800) was added. After 30 min in secondary on ice, suspension was washed once with FACS buffer (2% FBS in PBS). Flow analysis (LSRFortessa X-20 Cell Analyzer) was performed in 250µl of FACS buffer.

Conditioned media experiments

To prepare cultured media, cells (cancer cells, NOF, or conditioned-fibroblast) were incubated with minimal amount necessary, in their respective media supplemented with exosome-free FBS, for 24 hours at 37°. Conditioned media for NE-treated cells was collected in the same way, treatment with NE was 10μM at the beginning of the 24 our incubation period. Media was removed and centrifuged at 1200 RPM, 4°C, for 10 min to remove debris. Media was aliquoted to be stored at -20°C until use or directly placed onto the specific cell type to be conditioned. When placed in culture to condition, cells were incubated for 2.5 days before media was removed and replaced with a fresh aliquot of conditioned media or further analysis was performed at this point.

Statistical analysis

Microsoft Excel or GraphPad Prism (Microsoft, Redmond, WA) was used to analyze data. Continuous variables were compared using the Student *t* test or analysis of variance, and the Mann-Whitney test was used to compare differences. Using 2-way analysis of variance, we determined that a sample size of 10 animals per group would provide an effective size of 1.3 with 80% power at a significance level of $P = 0.05$. We considered $P < 0.05$ to be statistically significant. All statistical analyses were expressed as mean ± standard error of the mean. Kaplan-Meier analysis was used for survival analysis.

Results

ADRβ expression correlates with cervical cancer patient outcome

In light of previous studies showing the significance of adrenergic signaling in cancer progression, we chose to assess if ADRβ expression was correlated with patient survival. Among 166 tumor samples evaluated, 85% had high ADRβ1 and 61% had high ADRβ2 expression. Patients with high ADRβ2 expression had decreased overall survival (p=0.38, Figure 4).

Biological effect of chronic stress on cervical cancer growth and progression using orthotopic mouse models

To evaluate the role of stress in cervical cancer progression, we first established an orthotopic model of cervical cancer since there was no model where cells are directly injected into the cervix. First, we injected the cell lines SiHa, CaSki, ME-180, and C4-1 to evaluate their tumorigenicity (Figure 5). The route of injection we used was laparotomy in which cells are directly injected onto the cervix at the cervical-uterine junction. We observed tumors in SiHa, ME180, and C4-1 models. The cervical cancer tumor from an animal injected with SiHa cells was removed and recycled to create a cell line with high tumorigenicity and these were used in future *in vivo* experiments. Tumor growth was monitored weekly by IVIS imaging for the duration of the experiment. Bioluminescence imaging showed tumor localization at the cervix within the pelvic region (Figure 5). Further examination of tumor showed lympho-vascular invasion and spread into the parametrium and pelvic wall (Figure 5).

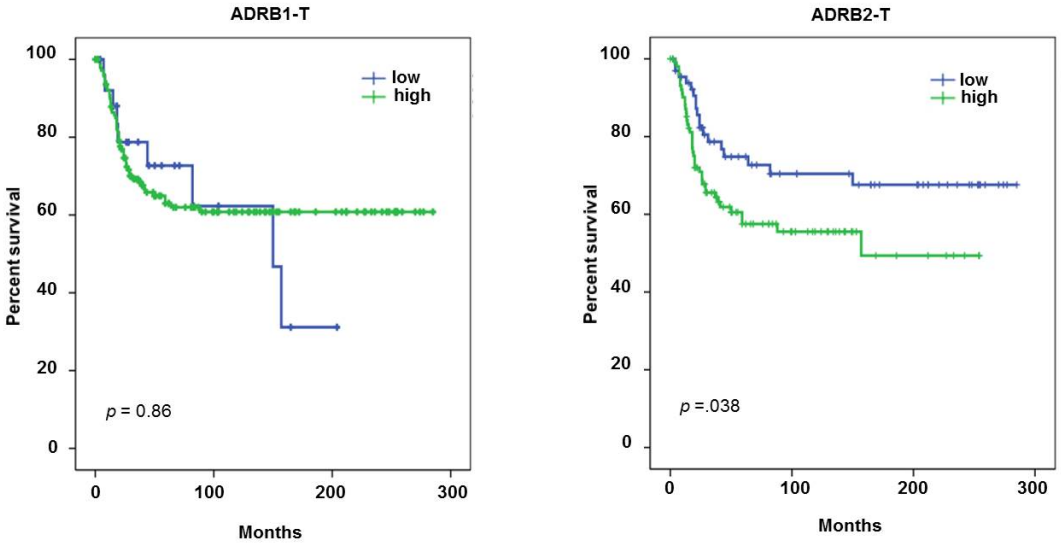
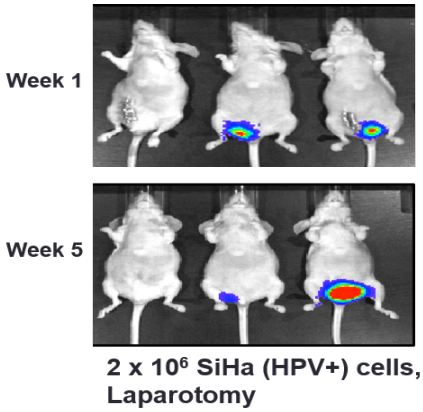


Figure 4. ADRβ expression and overall survival in cervical cancer patient tumors. ADRβ1 and ADRβ2 expression in cervical cancer tumor samples.

Cell line	Tumorigenic
SiHa	Yes (recycled)
CaSki	No
ME-180	Yes
C4-1	Yes



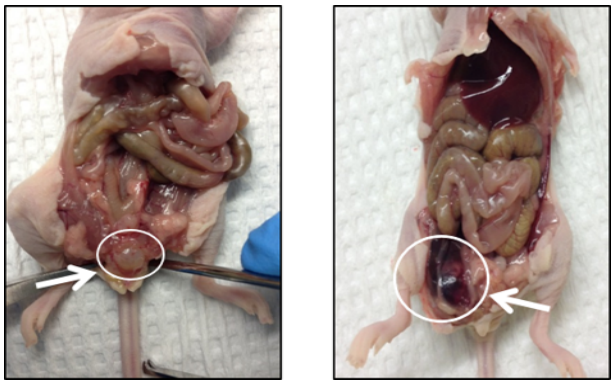


Figure 5. Establishment of an orthotopic model of cervical cancer.

Summary of cervical cancer cells tumorigenicity. Representative images of tumor growth over 5 weeks monitored by IVIS imaging, and established tumor in the animals at necropsy.

This model closely resembles the disease in the clinic and was adapted by our lab for further studies of studying cervical cancer progression.

To study the biological consequence of chronic stress on cervical cancer progression, we used a well-characterized restraint stress model and a non-specific β-blocker, propranolol. Animals submitted to daily restraint stress, had increased tumor weight and number of nodules while propranolol completely abrogated this effect ($p<0.01$ and $p<0.05$, Figure 6).

We investigated whether the HPV oncogene protein levels could be modulated by chronic stress in our orthotopic model of cervical cancer. Interestingly, we observed a trend for elevated E7 protein in tumors from mice exposed to daily restraint stress compared to controls (Figure 7a). Serum from tumor bearing mice was also evaluated for E7 protein, showing elevated E7 protein in restraint stress animals (124 vs. 177 ng/μl, Figure 7b). Further examination of the relative HPV E6/E7 DNA levels in the serum showed elevated levels in restraint stress animals (Figure 8). To dissect the effect of HPV E6 and E7 in our *in vivo* models, we silenced their expression by using DOPC nanoparticles. Daily restraint stress lead to increased tumor growth and nodules, while use of HPV16 E6/E7 DOPC siRNA nanoparticles completely blocked this effect ($p<0.0001$ and $p<0.001$, Figure 9). In addition, HPV16 E6/E7 siRNA-DOPC blocked restraint stress-induced increases in E7 protein in serum, shown by ELISA ($p<0.0001$, Figure 10).

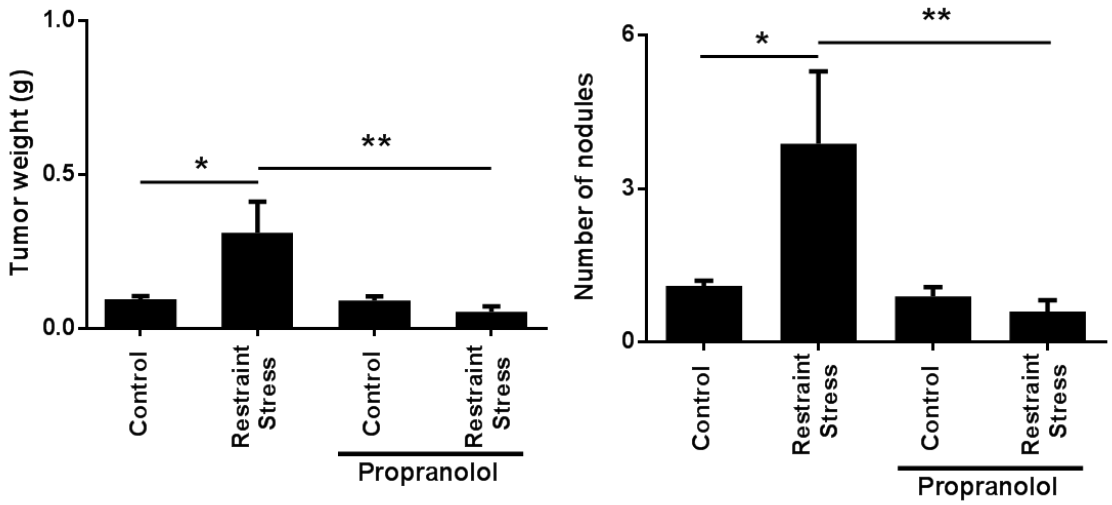


Figure 6. Effect of restraint stress on cervical cancer growth. Total tumor weight and number of nodules are shown in the SiHa cervical cancer restraint stress model following treatment with propranolol. **indicates $p<0.01$, *indicates $p<0.05$. (n= 10 animals per group)

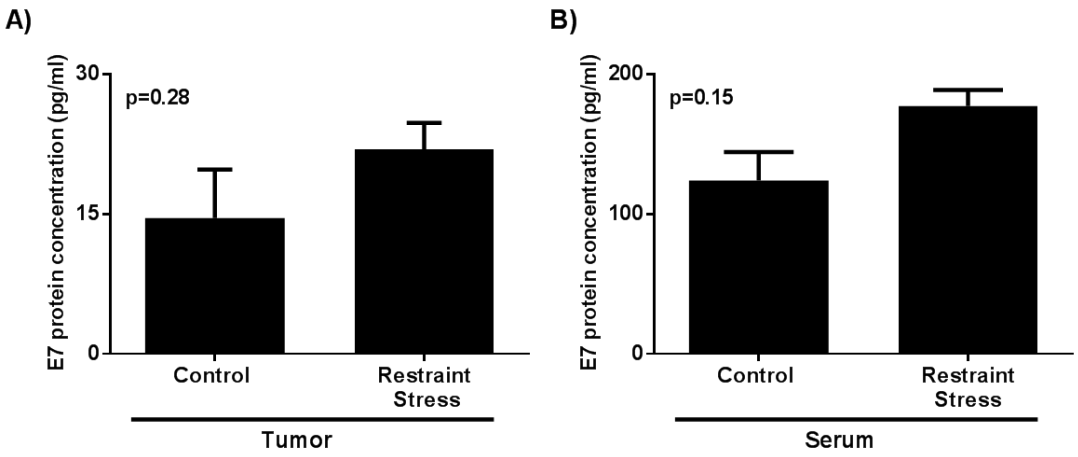


Figure 7. E7 protein expression in tumor and serum samples from restraint stress *in vivo*. E7 protein was measured by ELISA. Graphs represent E7 protein per 100µg of protein lysate (A), or 100µl of serum (B). (n= 3 animals per group)

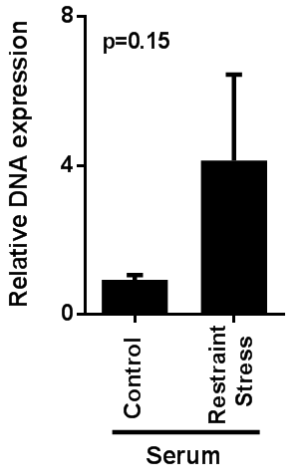


Figure 8. HPV16 E6/E7 DNA expression in serum from cervical cancer-bearing mice. DNA was isolated from 25µl of serum, relative E6/E7 DNA expression is shown above. (n= 3 animals per group)

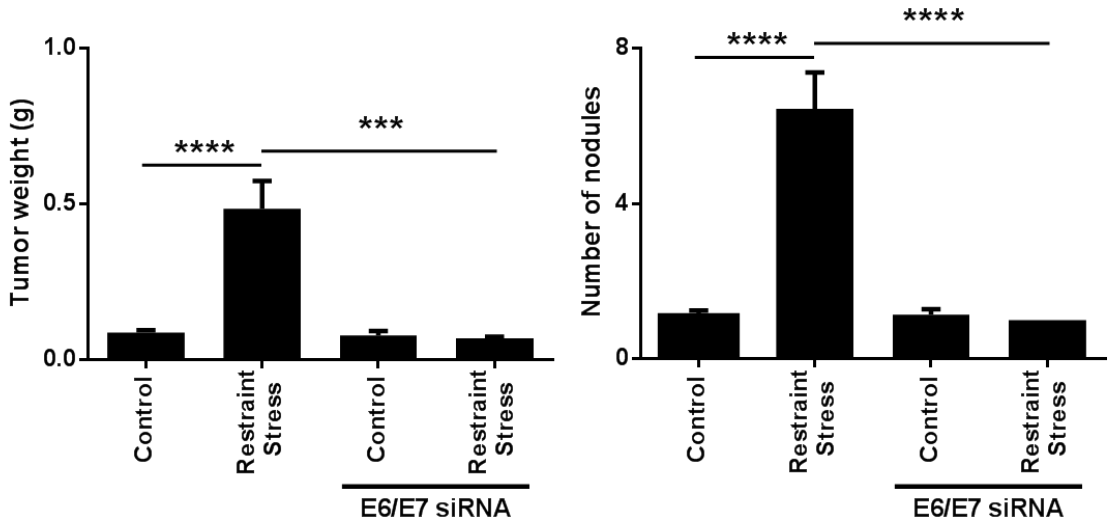


Figure 9. Effect of HPV16 E6/E7 siRNA in restraint stress model of cervical cancer. Total tumor weight and number of nodules are shown in SiHa cervical cancer model following treatment with E6/E7 siRNA-DOPC. ****indicates $p<0.0001$, ***indicates $p<0.001$. (n= 10 animals per group)

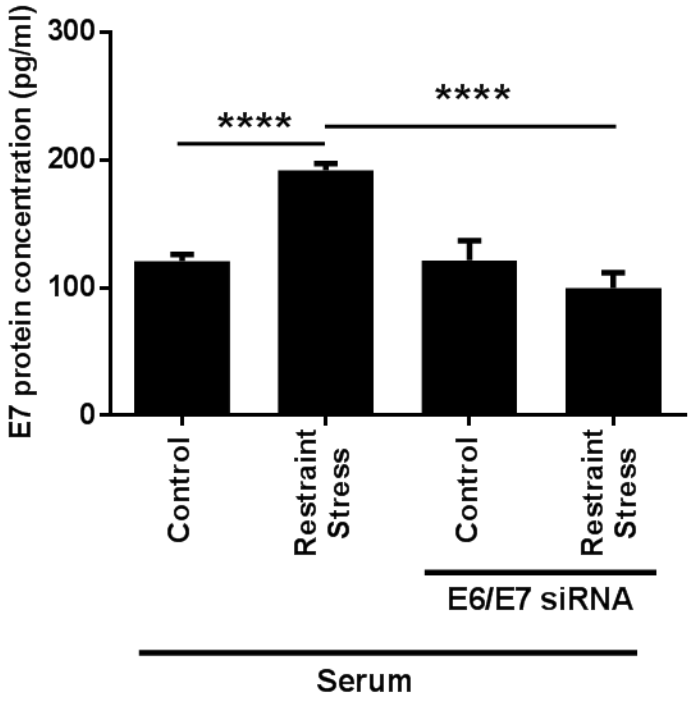


Figure 10. E7 protein expression in serum samples from cervical cancer restraint stress *in vivo* with HPV16 E6/E7 siRNA. E7 protein was measure by ELISA. Graph represents E7 protein per 40µl of serum. ****indicates $p<0.0001$. (n= 5 animals per group)

The functional consequences of catecholamine-mediated increases in HPV oncogenes E6 and E7 in cervical cancer cells

To determine the mechanism and cellular consequences of stress hormones in cervical cancer cells we first looked at ADRβ expression in a panel of cell lines by RT-PCR. CaSki, SiHa, ME-180, and C4-1 show expression of β1, β2, and β3 receptors (Figure 11a). C33A cervical cancer cells are null for all three of these receptors; therefore we introduced a β2 construct for future experiments requiring an HPV negative cell line to study the effect of stress hormones (Figure 11b). To determine if these cells could respond to adrenergic activation, we measured cAMP levels after exposure to NE or a non-specific β-receptor agonist, isoproterenol (ISO), for 30 min. A significant increase in cAMP levels was observed in NE and ISO treated ADRβ-positive cells CaSki and SiHa ($p<0.05$, Figure 12).

Our *in vivo* model shows HPV E6 and E7 protein levels were elevated in daily restraint stress animals tumor and serum, therefore we sought to investigate the underlying mechanism driving this increase. We first examined if catecholamines increased E6/E7 mRNA expression in CaSki, SiHa, and ME-180 cells. The quantitative PCR data shows NE or ISO exposure leads to increases in E6/E7 mRNA by three fold in CaSki ($p<0.001$, Figure 13). SiHa and ME-180 show similar increases in mRNA at 12 hours post-treatment with catecholamines ($p<0.001$ and $p<0.01$, Figure 13).

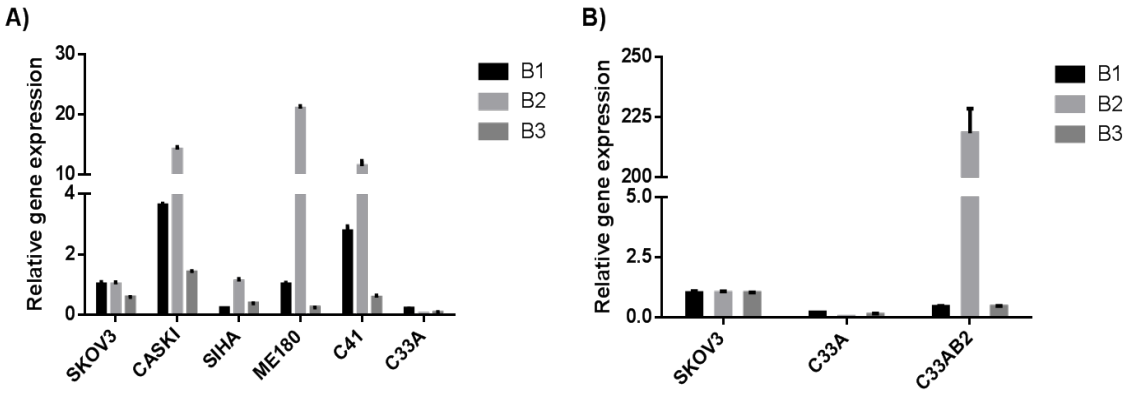


Figure 11. ADRβ expression in a panel of cervical cancer cell lines. ADRβ1, ADRβ2, and ADRβ3 expression were analyzed by qRT-PCR in CaSki, SiHa, ME-180, and C4-1 (A), C33A and C33A-B2 construct (B). Skov3 is an ovarian cancer cell line, used here as a positive control.

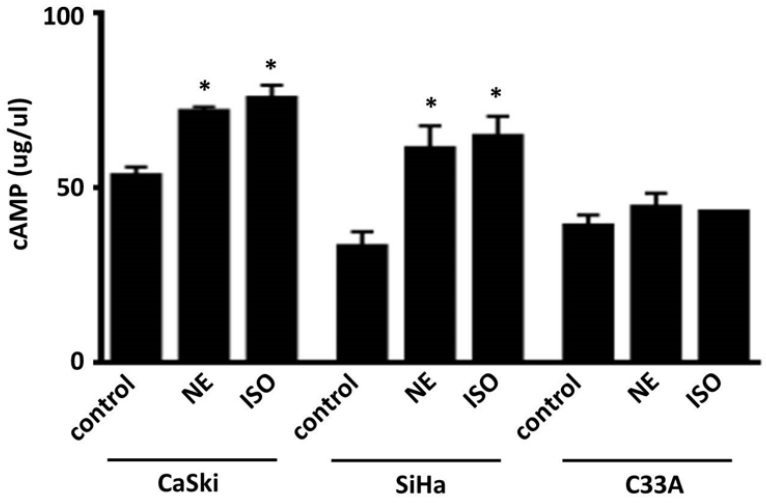


Figure 12. Intracellular cAMP levels after adrenergic stimulation. Cervical cancer cells were exposed to NE or ISO and intracellular cAMP levels were measure by ELISA. *indicates $p<0.05$.

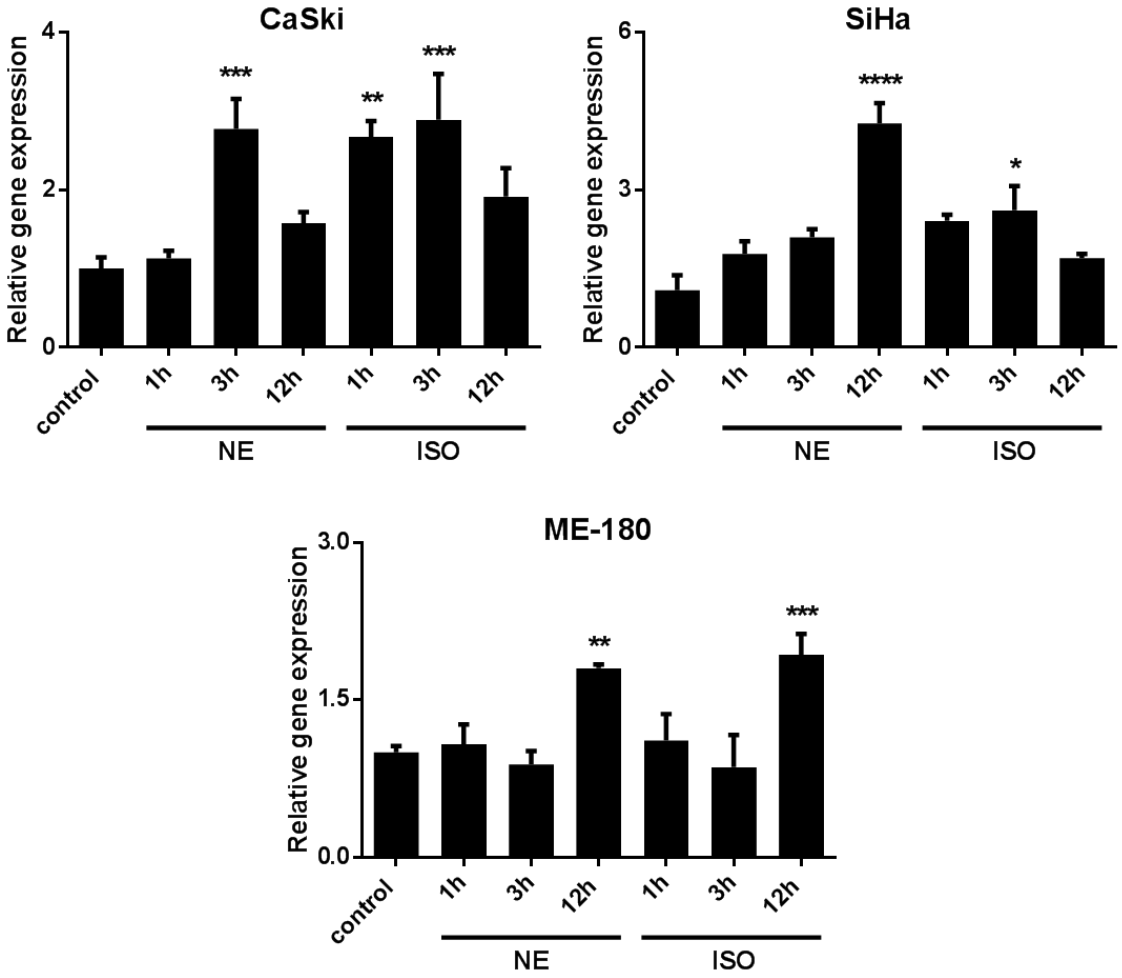


Figure 13. Relative HPV oncogene mRNA expression after catecholamine exposure. Following treatment with NE or ISO, cervical cancer cells relative mRNA expression for HPV16 E6/E7 (CaSki and SiHa), and HPV18 E7 (ME-180) were analyzed by qRT-PCR.****indicates $p<0.0001$, ***indicates $p<0.001$, **indicates $p<0.01$, *indicates $p<0.05$.

Since HPV was detected in the serum from tumor-bearing mice, we analyzed the corresponding supernatant from these treated cells for E7 protein by ELISA. There was an increase in E7 after 6 hours with NE or ISO (Figure 14). Next, we analyzed viral DNA isolated from the supernatant samples. qRT-PCR analyses show 9-fold increases in E6/E7 DNA in the supernatant from samples 3 hours post-treatment with NE and ISO (Figure 15). This data provides evidence that catecholamines can increase the production and secretion of HPV E6 and E7.

To examine the cellular response and consequences of stress hormones in cervical cancer cells we first investigated invasion and migration of cervical cancer cells treated with NE compared to control. SiHa and ME-180 cervical cancer cells were treated with NE, and allowed to invade or migrate through a defined matrix or gelatin coated membrane for 6 hours or 24 hours, respectively. NE increased cervical cancer invasion (SiHa 127 vs. 156 cells per HPF, $p=0.01$ and ME-180 97 vs. 138 cells per HPF, $p=0.03$, Figure 16a, b) and migration (SiHa 25 vs.42 cells per HPF, $p=0.03$, Figure 17). To investigate if HPV E6/E7 aids cervical cancer invasion, we treated cells with E6/E7 siRNA prior to the assay. Transfection with siRNA did not lead to changes in cell proliferation or viability (data not shown). Cells treated with siRNA showed reduction in invasion when compared to control ($p<0.0001$, Figure 18). Further, while NE increased tumor cell invasion, E6/E7 siRNA completely abrogated this effect ($p<0.05$ and $p<0.0001$, respectively, in Figure 18).

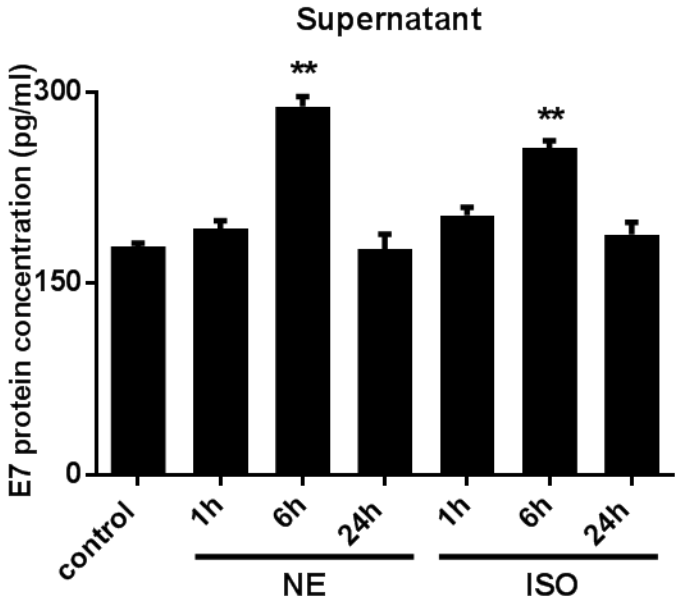


Figure 14. HPV16 E7 protein expression in supernatant from SiHa cells exposed to catecholamines. Following treatment with NE or ISO cervical cancer cells E7 protein expression was measured by ELISA in supernatant. **indicates $p<0.01$.

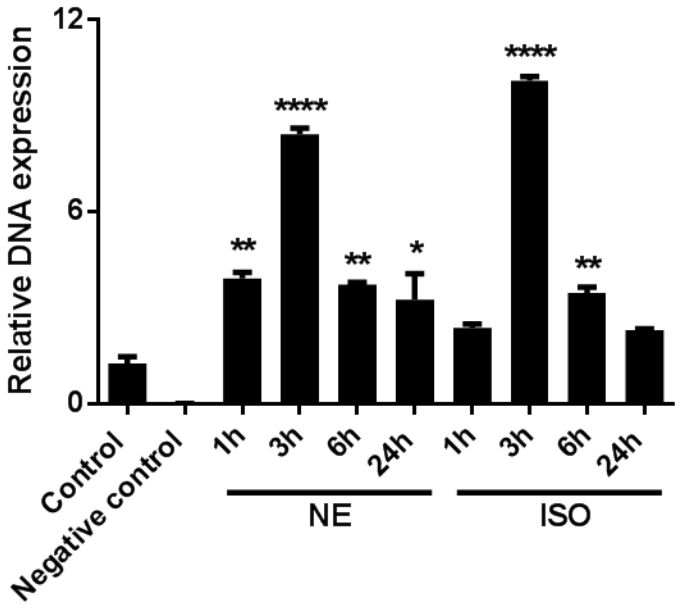


Figure 15. HPV16 E6/E7 DNA expression in supernatant from SiHa cells exposed to catecholamines. DNA was isolated from supernatant, relative E6/E7 DNA expression is shown above. ****indicates p<0.0001, **indicates<0.01, *indicates p<0.05.

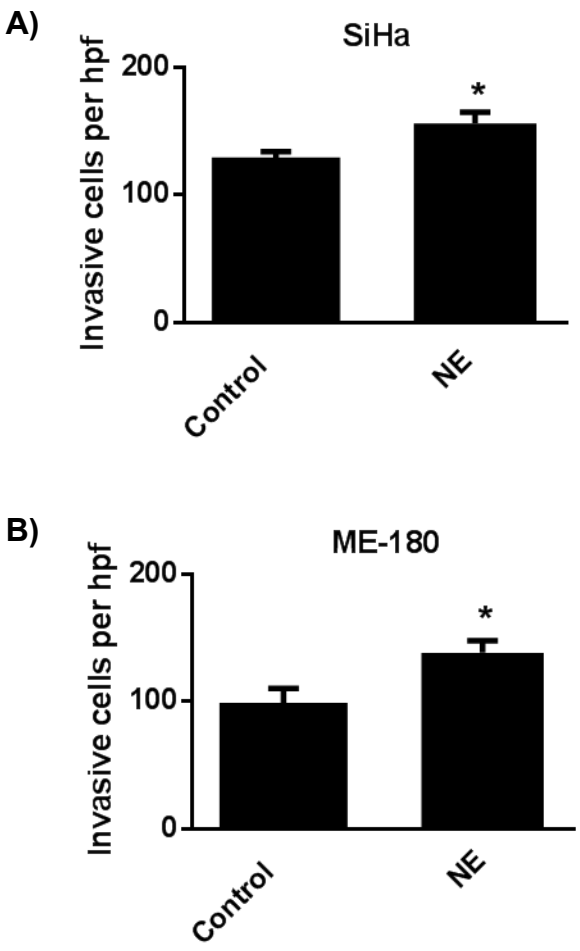


Figure 16. Invasion of cervical cancer cells treated with NE. Cervical cancer cells were treated with NE and then plated into modified Boyden chambers to assess invasion (24 hours). Cell numbers per high power field (HPF, 200X) were then counted. *indicates $p<0.05$.

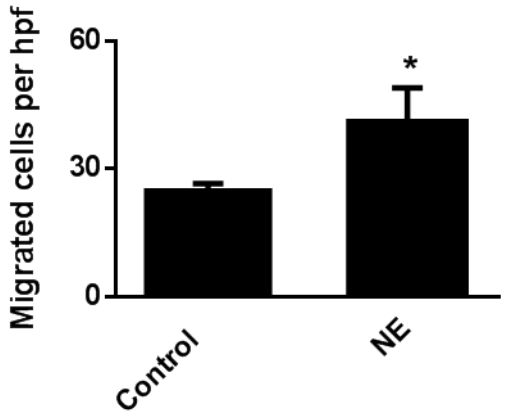


Figure 17. Migration of cervical cancer cells treated with NE. Cervical cancer cells were treated with NE and then plated into modified Boyden chambers to assess migration (6 hours). Cell numbers per high power field (HPF, 200X) were then counted. *indicates $p<0.05$.

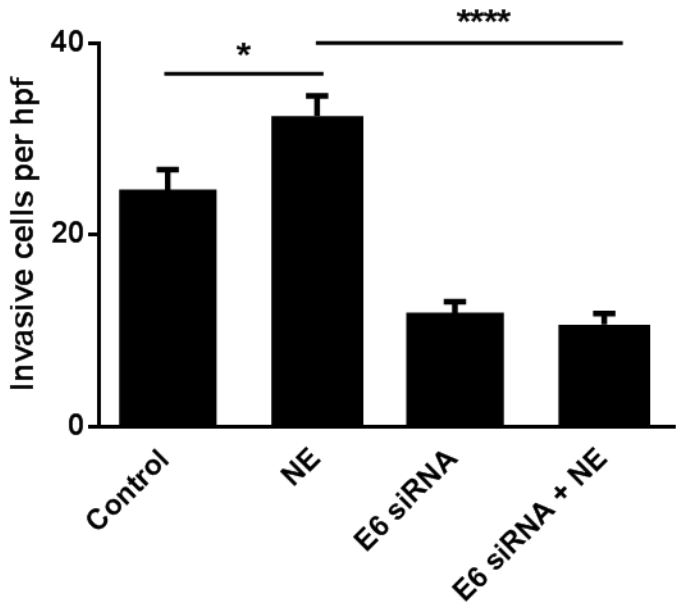


Figure 18. Invasion of cervical cancer cells with E6/E7 siRNA. Cervical cancer cells were treated with HPV16 E6/E7 siRNA 36 hours prior to treatment with NE and plated into modified Boyden chambers to assess invasion (24 hours). Cell numbers per high power field (HPF, 200X) were then counted. ****indicates $p<0.0001$, *indicates $p<0.05$.

To further elucidate the mechanism by which the stress-induced increases in HPV oncogenes can promote cervical cancer growth and metastasis we examined their role in anoikis. Anoikis is a process by which cells enter apoptosis when detached from the extracellular matrix and neighboring cells. We assessed ME-180 cancer cells maintained in low attachment tissue culture plates for basal anoikis rates every 24 hours; cells were analyzed by flow cytometry for SYTOX Red dead cell stain to determine viability. After 48 hours in these conditions, 50% of the cells went through anoikis and at 72 hours 75% went through anoikis (Figure 19). Exposure to NE resulted in significant inhibition of anoikis in ME-180 cells ($p<0.0001$, Figure 20).

The role of exosomal HPV oncogenes in cell-to-cell communication in the tumor microenvironment

We sought to investigate the biological significance of secreted HPV. Specifically, we wanted to know if cancer cells and the extracellular HPV can condition their microenvironment into one that is more supportive for tumor growth. In this case, we looked at fibroblasts because they have been shown to be modulated directly by cancer cells [50]. Normal fibroblast cell lines 151 (NOF) were exposed to supernatant from C33A or SiHa for two days. In the study done by, Hanahan *et. al*, genes such as CXCL2, VEGF, IL-6 and IL-8 were said to be part of the pro-inflammatory gene signature observed in cancer-associated fibroblast [63]. In our experiment, NOF showed an up-regulated cancer associated pro-inflammatory gene signature within two days of culture with SiHa

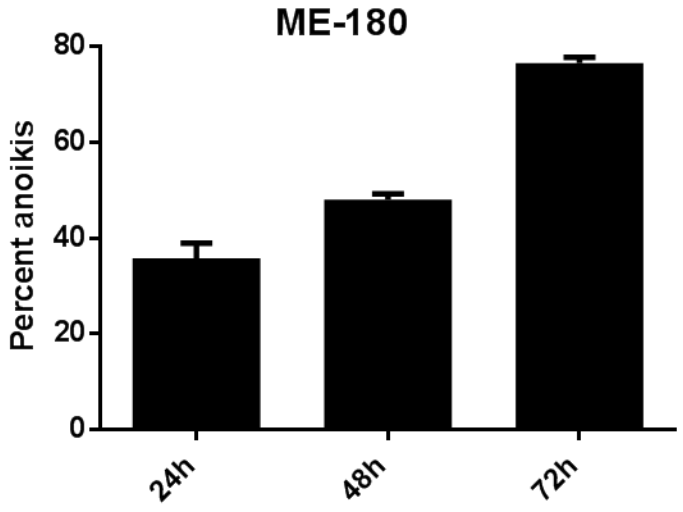


Figure 19. Basal anoikis rates of ME-180 over 72 hours. Percent of cells stained as positive for SYTOX Red are graphed here as percent anoikis over 72h.

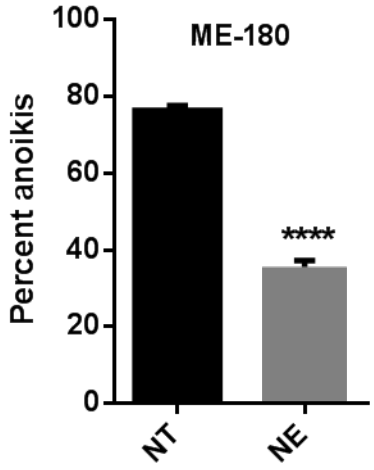


Figure 20. NE-induced resistance to anoikis. Cells treated daily with NE are represented here, compared to control. Percent of cells stained positive for SYTOX Red are graphed here as percent anoikis. ****indicates p<0.0001.

(HPV+) conditioned media when compared to C33A (HPV-) conditioned media (Figure 21a). Furthermore this effect was amplified when fibroblast were exposed to media from cells treated with NE (Figure 21b). Since we previously showed HPV in the supernatant, we wanted to see if the NOF became HPV positive after incubation with the cultured media from SiHa cells. Fibroblasts that were cultured with conditioned media from SiHa for two days had detectable HPV E6/E7 mRNA after just 24 hours ($p< 0.0001$, Figure 22). Proliferation of fibroblast has been shown to increase when co-cultured with breast cancer cells [64]. To assess if there was a functional consequence of the CAF conversion by cervical cancer cells, we analyzed proliferation of the fibroblast conditioned with SiHa cultured media, with or without NE. There was an increase in fibroblast proliferation, seen by percent of EdU incorporation after 2 days of co-culture (Figure 23). Since this was not a robust biological effect, we looked into other functional read outs, which could be the main driver in how NOF to CAF conversion can promote progression of cervical cancer.

A change in migration of fibroblasts is a signature characteristic of transition from normal to a cancer-associated phenotype. To assay the ability of fibroblast to migrate following exposure to conditioned media, fibroblasts were cultured for five days and then were plated into gelatin coated modified Boyden chambers for 8 hours. As expected, NOF fibroblasts cultured with conditioned media from SiHa cervical cancer cells had increased migration, this effect was amplified with conditioned media from cervical cancer cells treated with NE (24 vs. 93 and 24 vs. 164, $p<0.01$ and $p<0.001$, Figure 24).

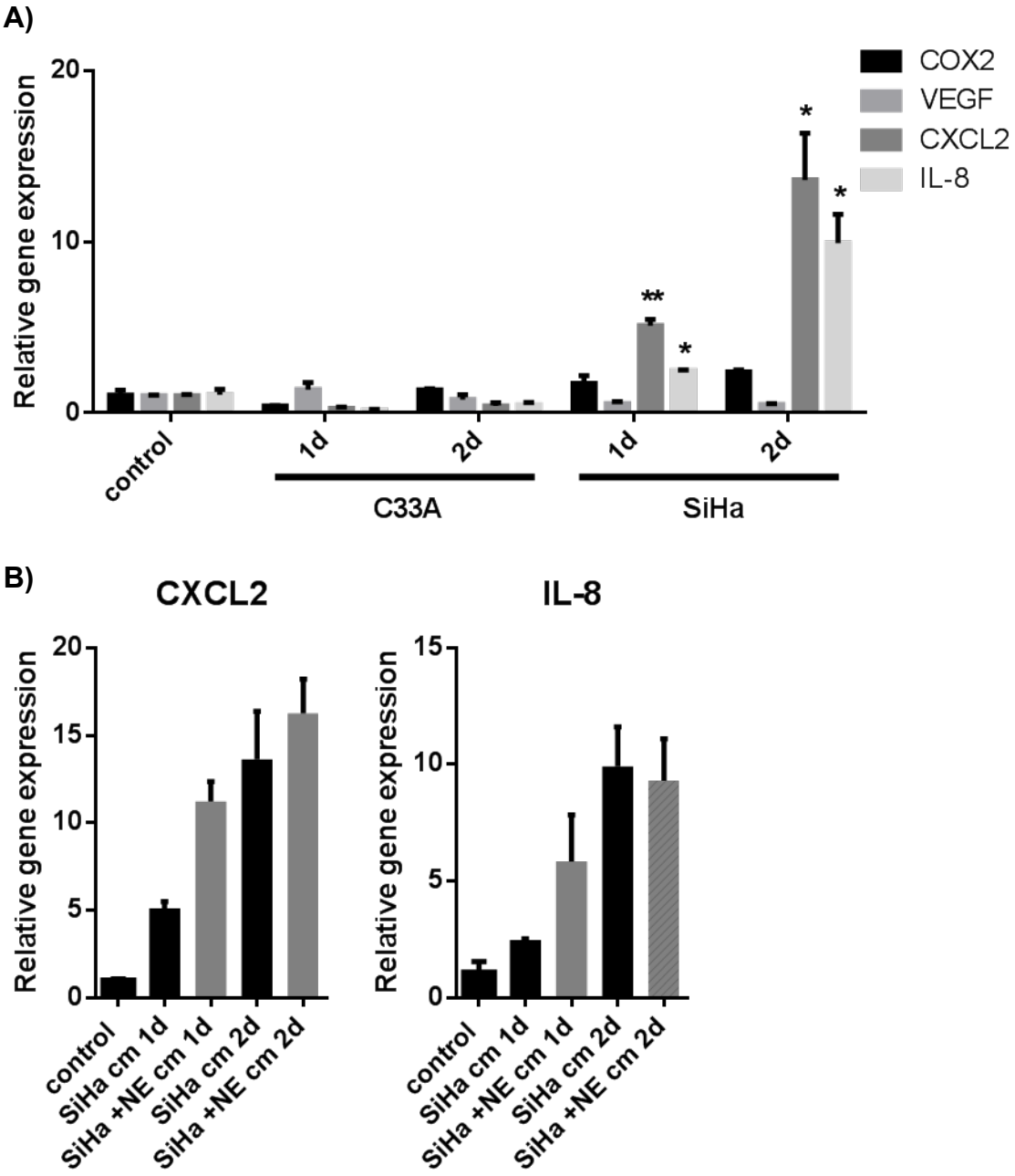


Figure 21. Pro-inflammatory genes expression in fibroblast conditioned with cervical cancer cell supernatant. mRNA expression changes were analyzed by qRT-PCR in fibroblast cultured with SiHa and C33A conditioned media over 2 days. *indicates $p<0.05$.

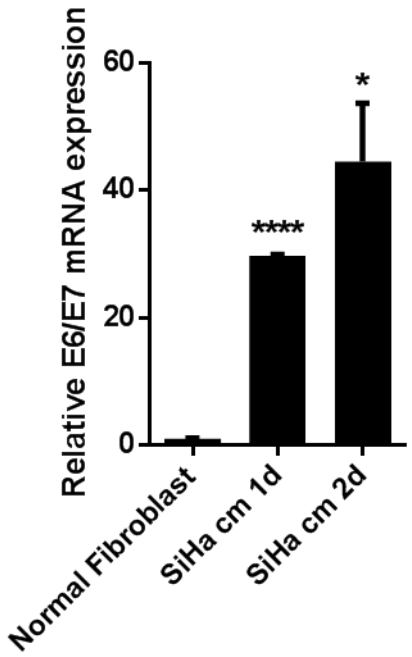


Figure 22. HPV mRNA expression in fibroblast conditioned with SiHa cell supernatant. Analysis of HPV16 E6/E7 mRNA expression by qRT-PCR in fibroblast after culture with supernatant from SiHa cells.****indicates $p<0.0001$. *indicates $p<0.05$.

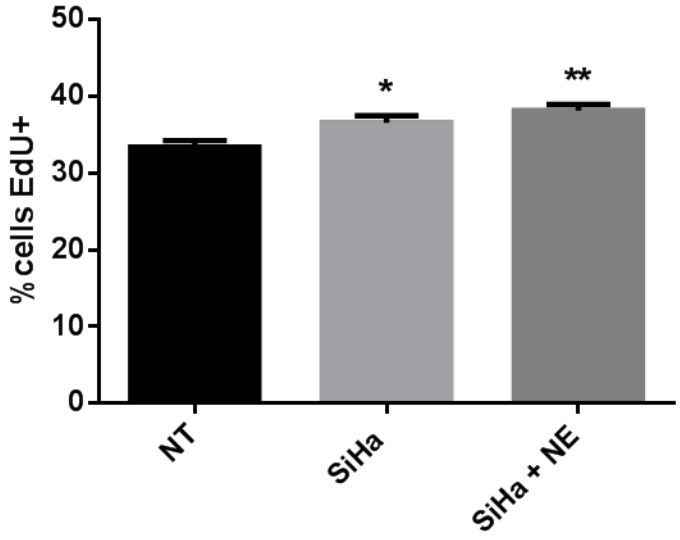


Figure 23. Effect of SiHa cell conditioned media on fibroblast proliferation. Proliferation graphed as percent of EdU incorporation in fibroblast over 2 hours, 48 hours after placed in SiHa cultured media.**indicates $p<0.01$.*indicates $p<0.05$

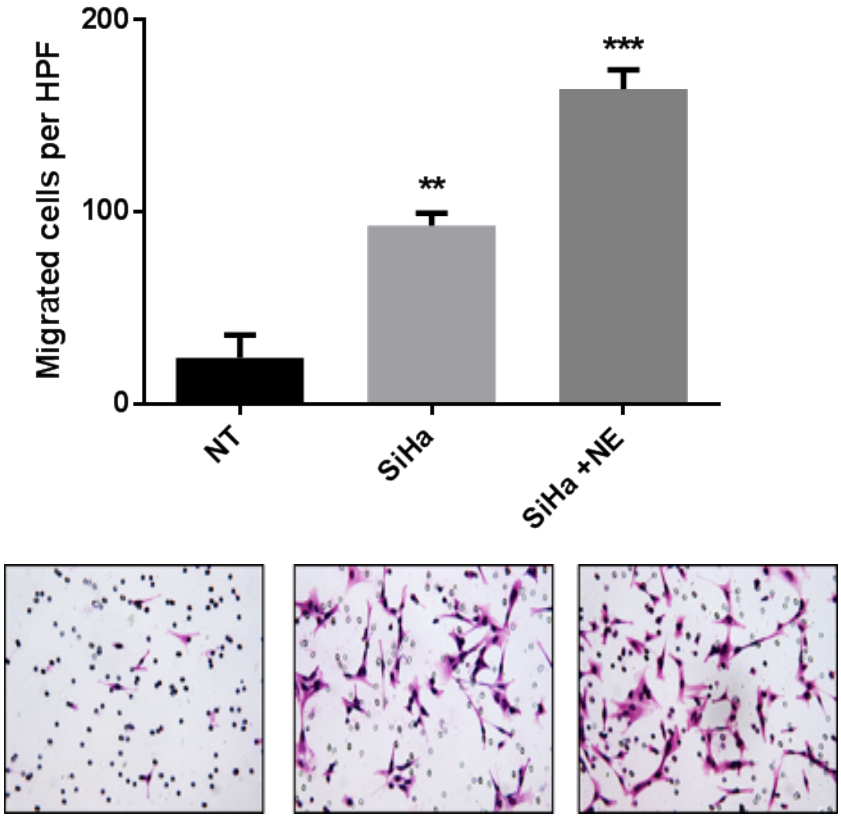


Figure 24. Migration assay for fibroblast with conditioned media from SiHa cervical cancer cells. Fibroblasts were plated into modified Boyden chambers to assess migration (8 hours) to conditioned media from cervical cancer cells. Cell numbers per high power field (HPF, 200X) were then counted. ***indicates $p<0.001$. **indicates $p<0.01$.

It has been suggested that HPV can only infect stratified epithelium. Based on our previous finding that HPV E6/E7 mRNA was detectable in NOF cultured with supernatant from SiHa cells, we wanted to determine how the virus can infect other cell types. Therefore we hypothesize that HPV viruses can be packaged into exosomes and transferred to nearby fibroblasts. First, we isolated exosomes from cervical cancer cells and validated the extraction by determining the presence of hsc70 on these samples (Figure 25).

The interaction of the cancer cells and the stromal populations, as well as the ability to penetrate and breakdown the basement membrane represent critical steps in the metastatic process [65]. To see if the exosomes themselves were capable of invading a defined matrix that mimics the basement membrane, exosomes labeled with a red fluorescent dye, Bodipy-TR ceramide, were placed in an insert coated with defined matrix and allowed to invade for 24 hours. Fluorescence imaging shows that exosomes were able to penetrate through the matrix and further they can be visualized amongst cancer cells that were plated below the insert (Figure 26).

To delineate a mechanism by which normal fibroblasts acquire viral oncogene expression, we specifically looked at the ability of normal fibroblast to uptake cervical cancer derived exosomes. Fibroblasts were incubated with Bodipy-TR ceramide labeled exosomes for 24 hours and analyzed by flow cytometry. These analyses demonstrate that fibroblast can uptake labeled exosomes (Figure 27).

Figure 25. Hsc70 expression by western blot in exosomes isolated from cervical cancer cells. Exosomes were isolated from cervical cancer cells and expression was confirmed by western blot.

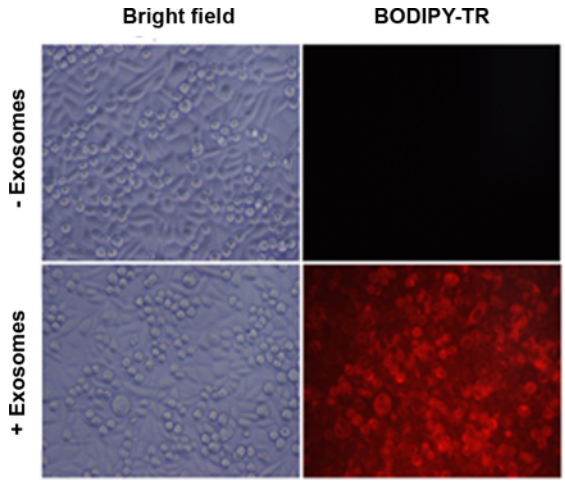


Figure 26. Invasion of cervical cancer cell derived exosomes. Labeled exosomes were plated into modified Boyden chambers to assess invasion (24 hours). Ability to penetrate the matrix was determined by Bodipy-TR ceramide signal seen in cancer cells plated below the inserts.

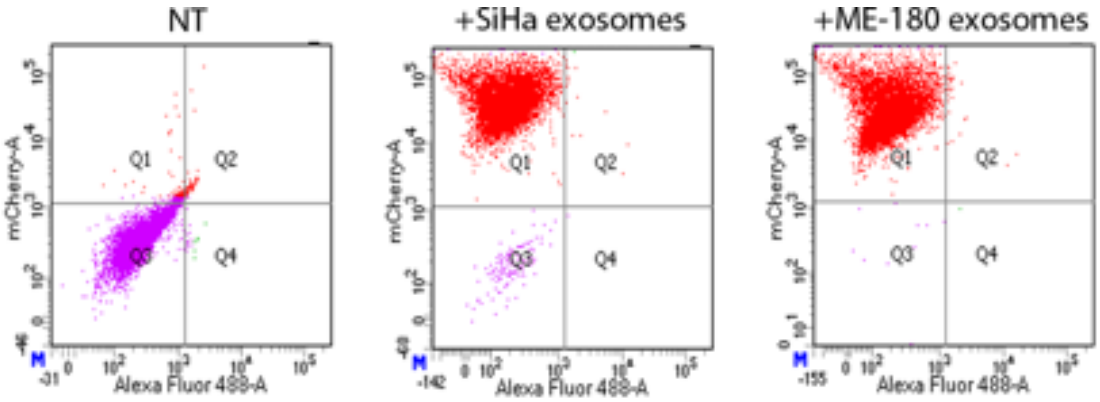


Figure 27. Uptake of cervical cancer cell exosomes by fibroblasts. Flow cytometry results are shown above, the mCherryA positive population represents the fibroblasts that have taken up Bodipy-TR ceramide labeled cervical cancer exosomes.

Since endocytosis is energy dependent process, cells put at 4°C show the uptake of exosomes is compromised and it completely abrogated this process. Moreover, to determine if exosomes were a key carrier of HPV viruses, we incubated fibroblasts with Bodipy-TR ceramide labeled exosomes from SiHa and ME-180 cervical cancer cells for 24 hours. We detected Alexa-488 conjugated HPV E7 and the Bodipy-TR ceramide label in fibroblast by flow cytometry. Our data showed that fibroblasts can uptake exosomes containing HPV E7 proteins (Figure 28).

NOF treated with cervical cancer supernatant showed enhanced migration and an increase in some pro-inflammatory genes, which are both supportive of these cells differentiating into CAFs. SiHa cells conditioned with these converted fibroblast cultured media resulted in significantly increased invasion when compared to cells treated with conditioned media from NOF (60 vs. 111, $p<0.0001$, Figure 29).

To study if intervention with siRNA for specific genes involved in exosomes release pathways will abrogate the effects we have seen, we first silenced the gene SMPD2 in cervical cancer cells. Three sequences were used to validate knock down of the SMPD2 mRNA in SiHa cells (Figure. 30). All three of the sequences show a 90% knockdown of SMPD2 mRNA. Future analysis of supernatant from SMPD2 siRNA treated cells for E7 protein compared to control is necessary. This data can provide evidence that HPV uses exosomal pathways for oncoprotein release.

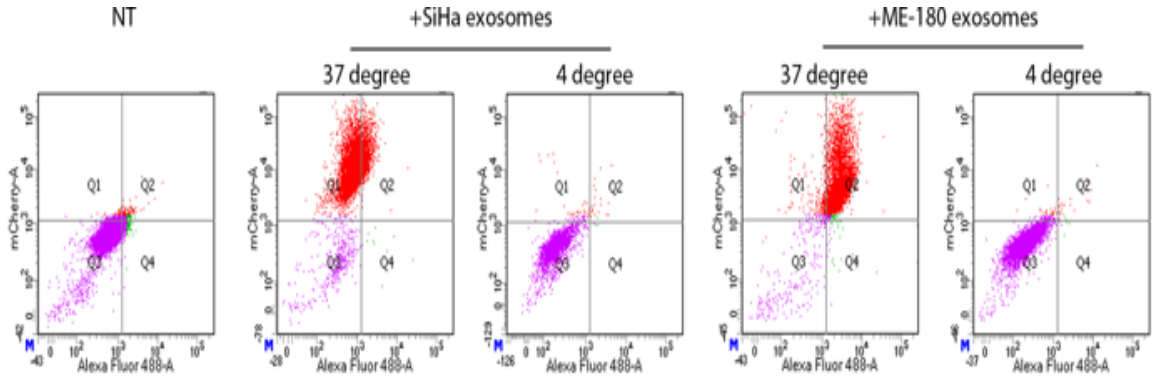


Figure 28. Detection of exosomal HPV E7 protein in fibroblasts. Flow cytometry results are shown here. The double positive population in the Q2 quadrant represents the fibroblasts that have taken up the Bodipy-TR ceramide positive exosomes and E7 protein (HPV E7 specific antibodies were conjugated to Alexa 488 fluorescent secondary antibody for detection by flow).

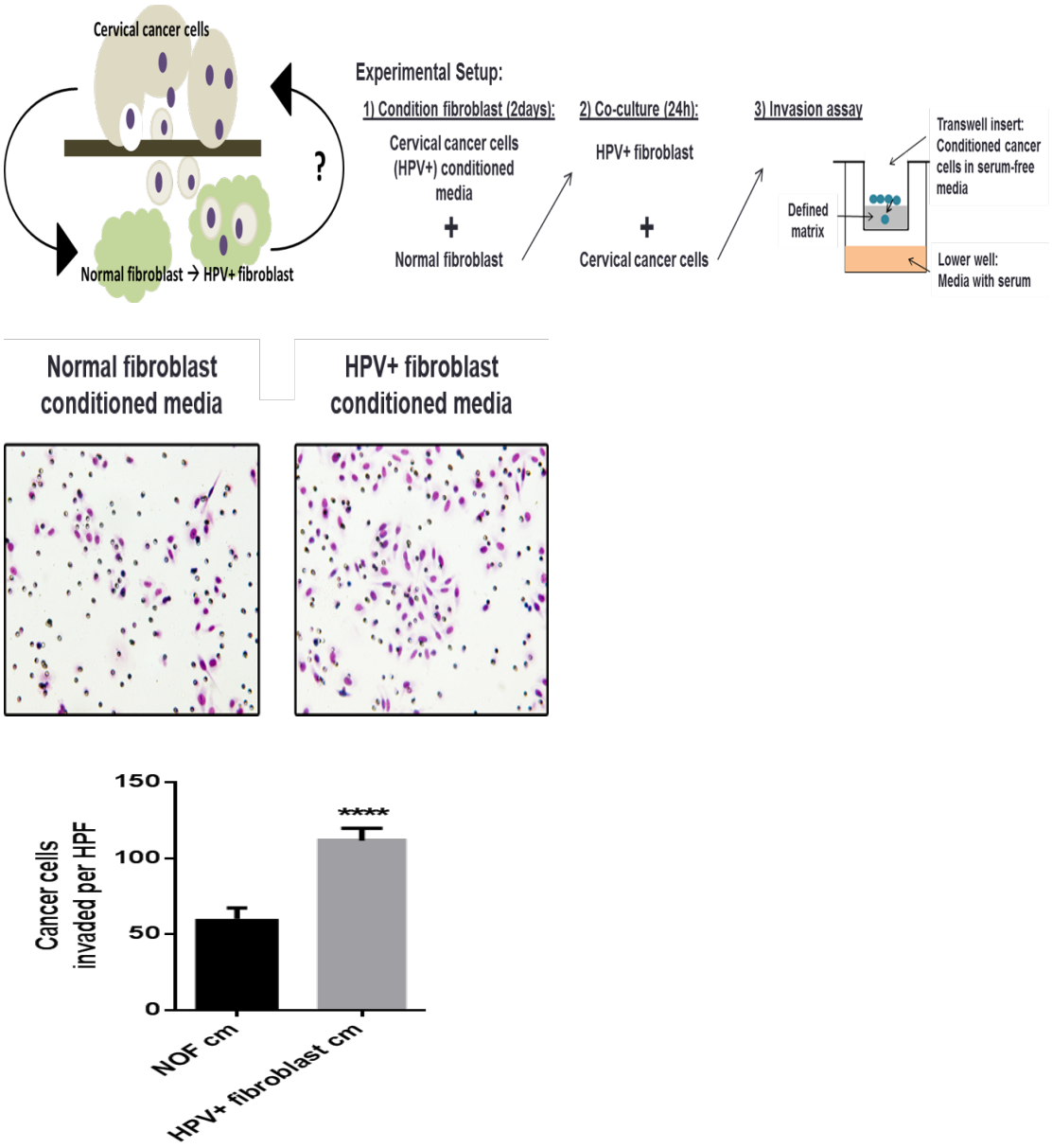


Figure 29. Invasion of cancer cells co-cultured with HPV-CAFs. Normal fibroblasts were cultured with SiHa conditioned media. HPV positive fibroblasts were co-cultured with SiHa cells for 24 hours prior to plating them into modified Boyden chambers for invasion assay (24 hours). Cell numbers per high power field (HPF, 200X) were then counted. ****indicates $p<0.0001$.

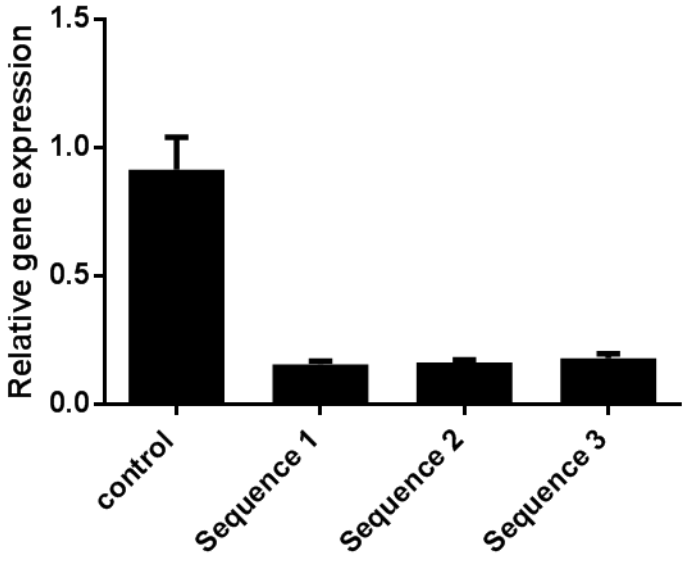


Figure 30. SMPD2 siRNA in cervical cancer cells. Three sequences were used to validate siRNA efficiency specific for the SMPD2 gene in cervical cancer cells.

Discussion

Biological significance of stress in cervical cancer progression

While chronic stress has been previously implicated in the progression of many cancers, the biological significance in cervical cancer had not been explored. In our detailed investigation of how chronic stress contributes to cervical cancer progression, we first show daily restraint stress led to increases in tumor weight and number of nodules in an orthotopic murine model of cervical cancer. The use of a non-specific beta-blocker, propranolol, completely abrogated this effect. This finding is suggestive that there is a link between sustained adrenergic signaling and cervical cancer progression. We provide evidence that HPV oncogene transcription and translation increases after catecholamine exposure. This is the first time HPV has directly been shown to respond to adrenergic signaling. The relationship between catecholamines and increased HPV is important, as HPV is the primary cause of cervical cancer and is capable of manipulating numerous pathways in cancer cells [66]. The significance of HPV oncogenes in a chronic stress model is most dramatically illustrated by the complete abrogation of tumor growth and number of tumor nodules when E6/E7 siRNA was used.

In the current study, HPV protein was elevated in tumors and serum from restraint stress animals when compared to control. This data shows catecholamines can increase the secretion of HPV in addition to enhancing its transcriptional activity in cancer cells. Although HPV has been detected outside of the cell [67], the mechanism by which the virus is maintained extracellularly is

not understood. We demonstrate, for the first time, cervical cancer derived exosomes contain HPV oncoproteins. Perhaps the mechanism the virus uses to maintain latent and persistent infection in an individual is through packaging itself in cancer cell derived exosomes. Further we show that the HPV containing exosomes directly interact with fibroblasts, suggesting a novel mechanism HPV uses for infecting non-epithelial cell populations in the tumor microenvironment. Our study provides new directions for defining precise mechanisms by which HPV plays a role in cervical cancer tumor progression.

Mechanisms

To our knowledge, this is the first time neuroendocrine modulation of HPV *via* SNS has been demonstrated. In this study, we prove NE-induced increases in E6/E7 are required for cervical cancer cell invasion. Additionally, cervical cancer cells expressing E6/E7 show an increased resistance to anoikis when treated with NE. Together these results demonstrate the consequences of catecholamine-mediated increases in HPV oncogenes.

The unique and heterogeneous expression of β-receptors in these cervical cancer cell lines makes delineating one overarching mechanism and pathway driving increases in HPV oncogenes intricate. To study this mechanism, siRNA targeting specific ADRβs will be most definitive in identifying the signaling pathway driving these increases. More evidence is needed to support both ADRβ-dependent and HPV-dependent processes and their interplay. We did not address this issue specifically, however a model with HPV negative cells that

have a functional response to adrenergic stimulus could address the catecholamine-dependent effects of chronic stress on tumor progression. When stable expression of HPV is added into this specific model and compared, we hypothesize the stress effect will be amplified.

While many studies have focused on the effect of HPV in tumor cells and how the virus function plays a role in oncogenesis [68], we provide a role of the virus in conditioning the microenvironment and how it may support progression of cervical cancer. The exosomes from cervical cancer cells were shown to contain the oncogene E7, supporting a route by which cancer cells are directly modulating their environment *via* oncogene transfer. After determining the ability of exosomes to penetrate a basement membrane like setting, we wanted to examine the capability of these exosomes to be uptake by cells found in the microenvironment such as; immune cells, macrophages, endothelial cells, or fibroblast. The data presented here shows fibroblasts can uptake cervical cancer cell derived-exosomes. Another avenue we can explore to determine the significance of the exosomes is their role *in vivo* in tumor progression by using CD63-GFP labeled cancer cells. Analysis of these tumors and the surrounding tissue can help us understand the extent to which the exosomes are released *in vivo* and where they are primarily taken up or located. Furthermore, we can explore the effect of the exosomes on the other cell types in the microenvironment that we did not elucidate on here.

Several other experiments are necessary to dissect specifically what the exosomes alone are contributing to the fibroblast. Experiments addressing the

cervical cancer cell derived exosomes are critical so we can know more about their content, release, and uptake. The exosomal release mechanisms are of primary interest because interfering with these pathways can abolish the communication and delivery of these oncogenes from the tumor in the first place. To block exosomal release, inhibitors or siRNA for Rab family members (Rab11, Rab27, Rab35), SMPD2 shRNA, or inhibitors of the endosomal sorting complexes required for transport (ESCRT) machinery can be used [55]. Experiments using these types of inhibitors will definitively support if the exosomes are the component in the cervical cancer cultured media that are responsible for altering the fibroblast by transferring HPV oncogenes to them. Furthermore, the inhibitors that show most efficacies *in vitro* can be formulated for application in an *in vivo* setting. Studies addressing exosomal release and developing ways to disrupt these pathways could provide beneficial therapeutic targets, which could be used in adjuvant to current treatment options.

The ability of cancer cells to modulate fibroblast, and become CAFs, has been recently established for breast, ovarian, and prostate cancers [64]. We are currently showing the fibroblast conditioned by cervical cancer cells have increased migration, proliferation, and increased pro-inflammatory gene expression. Furthermore we support a paracrine feedback on the cancer cells from the reprogrammed fibroblasts, which caused an increase in cancer cell invasion. It is possible that exosomes released from the conditioned *vs.* normal fibroblast are different in content and concentration which can directly be affecting the cancer cells. The communication between the cancer cells and

fibroblasts is demonstrated here as a potential explanation for priming the tumor microenvironment. The mechanism underlying the communication from the CAFs to cervical cancer cells which leads to increased invasion needs to be dissected. This study provides a mechanism by which cell-to-cell communication is promoting cervical cancer progression *via* a positive feedback loop.

To determine if HPV is required in the CAF-like transformation of normal fibroblast, the role of HPV in the fibroblast needs to be examined. Based on our evidence, we show a novel mechanism for viral packaging and infection *via* exosomes to the fibroblasts. The specific interactions E6 and E7 proteins have in the fibroblast are unidentified to this point and a mass spectrometry analysis of this would be of interest. To address the transforming capabilities of HPV oncogenes in the fibroblasts, a stable expression of HPV E6/E7 in fibroblasts is necessary.

Clinical implications

We demonstrate a reliable animal model for studying the progression of cervical cancer. Our orthotopic model of cervical cancer closely resembles the tumor growth and spread that is seen in the clinic. This model has excellent efficiency and gives researchers the ability to accurately predict clinical outcomes.

Our study has important clinical significance. We show the significant reductions in tumor burden with the use of propranolol in cervical cancer. This is an extremely important foundation to support using β-blockers in clinic in

combination with other therapies for cervical cancer, to prolong survival and help make current therapies more effective. ADRβ2 expression was related to overall survival in cervical cancer patients, which complements why the use of beta-blockers in this population could be beneficial.

Detection of HPV in patients has recently been used as a diagnostic marker simultaneously with abnormal cervical cytology, to increase the amount of screening a patient is advised in the hopes of preventing lesions or detecting lesions in very early and treatable stages [69]. The relationship between HPV expression and progression of the disease in patients, such as tumor stage and grade or overall survival, should be further studied in patients. Also, ISH for HPV DNA in tumor tissues will further tell us more about the interplay between the microenvironment and developed tumors. We predict the patients with high-HPV in the blood will additionally have more invasive tumors, more metastasis, and poorer overall survival. This data together could be further correlated with depression or stress assessments to support a clinical relationship between psychosocial distress and cervical cancer progression.

Exosomes are currently of interest in the nanotech field for therapeutic potential. Investigation of whether or not E6/E7 silencing will minimize the progression through less transfer of oncogenes is pending. Further examination by use of high-throughput platforms such as RPPA or gene array of the cervical cancer cell derived exosomes will provide more insight into the biogenesis and content of these vesicles. This disease has a very long time until it presents with

large and bulky tumor and if we can prevent the virus from potentiating its effects in progression we eliminate a hurdle current therapies do not address.

Conclusions

Evidence presented offers a new insight into not only stress-related pathways but also viral oncology and overall tumor biology. We addressed the three hypotheses of this project. In the current study, we demonstrate a previously unrecognized role of sustained adrenergic signaling in cervical cancer tumor progression. We show chronic stress increases HPV oncogene expression *in vivo* and *in vitro*. Catecholamine-driven increases in HPV promote tumor progression through increased cancer cell invasion and resistance to anoikis *in vitro*. Further review of HPV led us to the finding that the virus is secreted and has an emerging role in manipulating the microenvironment. We offer a complex but reliable model for explaining the influence of viral transfer through exosomes to the microenvironment. More importantly, the stromal changes are shown to play a biologically significant role in promoting cervical cancer cell invasion. Collectively, these data are translatable findings that need to be further examined and pursued to uncover the mechanisms behind our research.

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Vita

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