AMERICAN UNIVERSITY OF BEIRUT

ALPHA-2C ADRENOCEPTOR PROMOTES THE MALIGNANT PHENOTYPE OF HUMAN COLON CANCER CELLS

by
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AN ABSTRACT OF THE THESIS OF

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Title: Alpha-2c Adrenoceptor Promotes the Malignant Phenotype of Human Colon Cancer Cells

Introduction: Cancer remains one of the leading causes of death worldwide, with colorectal cancer (CRC) being the third most common type. Accumulating evidence shows that stress plays a pivotal role in the malignant phenotype of CRC, by promoting hallmarks of CRC including cellular proliferation, migration, invasion, and angiogenesis. During stressful situations, the sympathetic nervous system is activated, causing an elevation in levels of catecholamines including epinephrine which elicits its effects mainly by activating adrenoceptors. Stimulation of adrenoceptors induces malignancy of CRC. In the presence of blockers for both β and α_1-ARs, epinephrine continued to promote a malignant phenotype. This indicates that receptors other than β and α_1-ARs, namely α_2-ARs, are involved. In our CRC cell line model, the expression of α_{2A}-AR and α_{2B}-AR is extremely negligible. Interestingly, the role of α_{2c}-AR in CRC malignancy remains obscure. Here, we proposed that activation of α_{2c}-AR potentiates the malignant phenotype of human CRC cells.

Methods and Results: Treatment of human colon cancer cell line, SW480, with increasing concentrations of α_2-AR specific agonist (UK 14,304; 1, 10 or 100 nM) for different time points (24, 48, or 72 hours) did not cause a significant change in proliferation. However, using monolayer scratch assay, a significant increase in migration was observed after treatment with UK 14,304 (100 nM). This was further supported by a transwell migration assay where UK 14,304 increased cell migration. Pretreatment with a specific α_{2c}-AR antagonist (JP 1302; 100 nM) abolished the UK 14,304-induced cell migration. This increase in migratory capacity was concomitant with increased Rho activation evident by translocation of Rho from the cytoplasm to the cell membrane as well as via a Rhotekin-binding activation assay. The induced Rho activation could be mediated by increased oxidative stress since treatment with UK 14,304 increased NADPH oxidase activity and ROS production. Because ROS is known to induce migration of these cells, we incubated cells with a ROS scavenger (NAC 12.5 mM) or NOX1/4 dual inhibitor (GKT 137831; 20 μM). Our results also show that NAC or GKT abolished UK 14,304-induced cell migration. Because ERK1/2 and FAK are crucial in cell migration, we looked at their phosphorylation levels. Interestingly, UK 14,204 activated both ERK1/2 and FAK. Pretreatment with PF-573228 (10 μM), a FAK-specific inhibitor, or PD98059 (20 μM), an ERK1/2 inhibitor, abolished UK-induced migration. Moreover, adhesion of SW480 colon cancer cells to TNF-alpha-activated HUVECs was promoted by UK 14,304. This UK 14,304-induced adhesion was significantly reduced when cells were pre-treated with JP 1302. Moreover,

UK 14,304 caused a dramatic increase in transendothelial migration of SW480 through HUVECs; however, UK 14,304 failed to cause this increase in the presence of JP1302, clearly indicating that α_{2c}-AR activation may promote intra- or extravasation. Because breaking the endothelial cell barrier requires matrix digestion, we measured the levels of matrix metalloproteinases (MMPs). Importantly, stimulation of α_{2c}-ARs by UK14,304 increased the production of MMP2 and MMP9, and JP 1302 abolished this UK14,304-induced production. Since vascular endothelial growth factor (VEGF) is essential for angiogenesis, we also assessed the expression of VEGF following stimulation with UK14,304. Treatment with UK 14,304 significantly increased VEGF expression.

Conclusion: Taken together, our data suggests that α_{2c}-AR potentiates the migratory and invasive capacities and hence the malignant phenotype of human colon cancer cells. Therefore, α_{2c}-AR may represent a novel target in the treatment of CRC.

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CHAPTER I

INTRODUCTION

A. Background Information

1. Epidemiology

Following cardiovascular diseases, cancer is the second leading cause of death and accounts for 13 percent of worldwide mortalities (1). Cancer is defined as the uncontrolled division of abnormal cells, which can then invade different parts of the body and spread to other organs - a process referred to as metastasis. Despite major advancements in cancer research, colorectal cancer (CRC) remains the third most common type of cancer. A recent World Health Organization (WHO) report revealed that in 2015 alone, colorectal cancer estimated 774000 lives globally (1). For the year 2018, the American Cancer Society estimated 97,220 new cases of colon cancer and 43,030 new cases of rectal cancer in the United States (2). Colorectal cancer is seen in a wide geographic area. It is most common in industrialized countries such as in Australia, Western Europe, North America, and New Zealand (3).

2. Stages of CRC

CRC usually begins as a non-cancerous polyp that grows slowly on the inner lining of the colon or rectum. The most common type of CRC is an adenomatous polyp or adenoma. Up to one-half of a given population might develop one or more adenomas during their lifetime; however, fewer than one tenth of these adenomas might progress into

cancer (4). Adenocarcinoma is cancer arising from the inner lining of the colorectum and accounts for almost 96 percent of all CRC (4). The prognosis of CRC is invariably dependent on the degree of bowel wall invasion, lymph node metastasis, as well as distant metastases (3). Three different systems are used for the staging of CRC: Astle-Coller system, Dukes system, and TNM system. American Joint Committee on Cancer (AJCC) and the Union for International Cancer Control (UICC) revealed the TNM classification, which takes into account the aforementioned mediators. Indeed, the TNM classification is by large the most important assessment in the clinic that is used to decide on the treatment options (3). Stage 0 refers to carcinoma in situ, where the tumor is all contained within the inner bowel lining. Stage I is when the tumor has grown through the inner bowel lining reaching the muscle wall of the bowel (4). Stage IIA is a tumor that has grown through the outer bowel lining but without the involvement of nearby tissue or lymph nodes. Stage IIB is a tumor that has grown to the visceral peritoneum. Stage IIC indicates a tumor that has spread through the wall of the colon or rectum and has grown to nearby tissues, yet without the involvement of lymph nodes (4). Stage IIIA is when the tumor has grown into the muscle layers of the intestine and has spread to at least 1-3 lymph nodes. Stage IIIB is a tumor that has spread to surrounding organs and into 1 to 3 lymph nodes. Stage IIIC refers to a tumor that has spread to 4 or more lymph nodes, but not to distant pats of the body (4). Stage IVA refers to cancer that has spread to a single distant part of the body, such as the lungs or liver. Stage IVB is cancer that has spread to more than one distant part of the body (4).

3. Screening

Earlier diagnosis through screening and advances in treatment modalities have improved CRC survival rate. For instance, the mortality from CRC decreased by approximately 35 percent from 1990 to 2007, and in 2012 mortality was down by 50 percent (5). The American Cancer Society recommends screening at the age of fifty for people with average risk of CRC but at an earlier age for people with increased risk such as family history, diabetes, or inflammatory bowel disease (IBD). The decision to be screened after age 75 should be made on an individual basis (4). There are several screening tests that could be used to detect colorectal cancer. These include guaiac-based fecal occult blood test, sigmoidoscopy, colonoscopy, and computed tomography (CT) colonography (4).

4. Treatment

The National Comprehensive Cancer Network (NCCN) guidelines recommend surgery as a first line of treatment. Adjuvant chemotherapy, based on the drug fluorouracil (5-FU), is indicated for stage II- high risk or stage III patients. 5-FU is a thymidylate synthase inhibitor and blocking this enzyme prevents the synthesis of pyrimidine thymidine, which is a nucleoside required for DNA replication (4). Common 5-FU toxicities include gastrointestinal effects, such as mucositis, nausea, and vomiting. Moreover, redness, swelling, and pain on the palms of the hands and/or soles of the feet (hand-foot syndrome) may occur in patients receiving continuous fluorouracil infusion (4). In patients receiving bolus fluorouracil infusions, myelosuppressive effects are more

common (4). Oxaliplatin is often part of the adjuvant chemotherapy as well (4). Oxaliplatin is an alkylating agent that forms both inter- and intra-strand cross links in DNA, which prevents DNA replication and transcription resulting in cell death. Neurotoxicity is the most frequent dose-limiting toxicity of oxaliplatin (6).

Biologically targeted therapies may also be administered either alone, or in combination with chemotherapy to relieve symptoms. The Food and Drug Administration (FDA) approved biologically targeted drugs that act on a protein called vascular endothelial growth factor (VEGF) and others that target epidermal growth factor receptor (EGFR) (4). VEGF inhibitors, such as bevacizumab, inhibit angiogenesis by binding to the pro-angiogenic factor VEGF and preventing its binding to VEGF receptor. Common side effects of VEGF inhibitors include high blood pressure, low white blood cell count, diarrhea, fatigue, headaches, and loss of appetite (4). EGFR signaling cascade plays a pivotal role in epithelial mesenchymal transition (EMT) (7). During EMT, epithelial proteins, such as E-cadherin, α-, β- and γ-catenin are downregulated, while mesenchymal proteins, including vimentin, fibronectin, are upregulated, both of which lead to increased migratory capacity and invasiveness (7). Two classes of EGFR inhibitors have been developed: monoclonal antibodies (mAbs) that target the extracellular domain of EGFR, such as cetuximab, and small molecule tyrosine kinase inhibitors that target the receptor catalytic domain of EGFR, such as gefitinib and erlotinib (7). Most common side effects of mAbs include skin problems such as papulopustular rash because EGF and EGFR are involved in stimulating epidermal and dermal regeneration. Other side effects include headaches, fever, fatigue, and diarrhea (7). Erlotinib is known to inhibit Ras signaling, a downstream signaling molecule of EGFR. It is proposed that targeting downstream

signaling molecules of EGFR may have lower cutaneous side effects (8). KRAS is an effector molecule responsible for EGFR signaling cascade. Activating mutations in KRAS induces resistance to EGFR-targeted mAbs. Thus, it is suggested that all patients with CRC are routinely tested for KRAS mutations, with EGFR-targeted mAbs being a potential therapeutic approach only for patients exhibiting wild-type KRAS (9). Moreover, studies have interestingly shown that resistance to EGFR inhibitors may be partly mediated by activating VEGF-dependent signaling cascades. Therefore, combining anti-EGFR (erlotinib or cetuximab) and anti-VEGF (bevacizumab) agents proved to be a promising therapy in preclinical and clinical studies (10).

5. Complications of CRC

Despite the significant improvements made in treating CRC, liver metastasis still remains a major challenge in prolonging survival. Around half of CRC patients sustain liver metastasis of whom fifteen percent fit the criteria to receive surgical resection (11). Patients with liver metastasis have poor prognosis and a median survival of 5 to 20 months with no treatment. The best prognosis for patients who present with local metastasis is a survival rate at 5 years of 11 percent. In fact, a 5-year survival is exceptional even in patients with limited metastatic disease. This is clearly indicative of an increasing need for newer therapeutic drugs (12).

B. Risk Factors

1. Age and Genetics

There are several risk factors associated with CRC (Fig. 1). The risk of CRC increases progressively after the age of 40 and drastically after the age of 50. Indeed, approximately 90 percent of CRC cases are diagnosed in people aged 50 and older (13). The median age at diagnosis for colon cancer is 68 in men and 72 in women. Moreover, a personal history of adenomatous polyps or a personal history of inflammatory bowel disease (IBD), whether ulcerative colitis or Crohn's disease, multiples the relative risk of colorectal cancer (13). Also, people with family history of adenomatous polyps or colorectal cancer in one or more first-degree relatives are at increased risk. In addition, an estimated 5 to 10 percent of colorectal cancer cases result from recognized hereditary conditions such as familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC), also called Lynch Syndrome (13). Lynch syndrome, accounts for 2 to 4 % of all colorectal cancers, and results from an inherited defect in either the MLH1 or MSH2 gene- genes that are involved in repairing DNA damage. In fact, the lifetime risk of CRC in people with Lynch Syndrome is as high as 80% depending on the gene affected. On the other hand, FAP results from mutations in the APC gene that a person inherits from his or her parents. FAP accounts for 1% of all CRC (13).

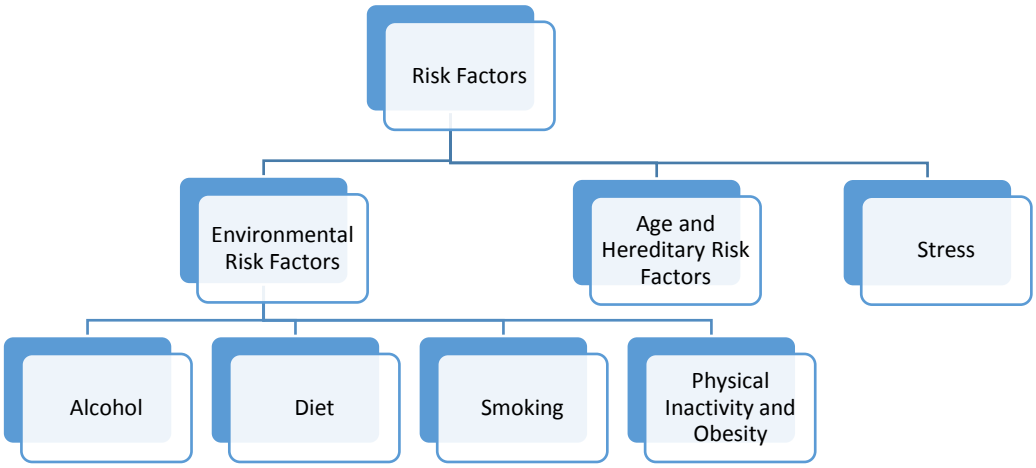


Figure 1: Colorectal cancer risk factors

2. Environment

Besides age and hereditary factors, a substantial number of lifestyle and environmental risk factors play an important role in increasing the risk of CRC (13). For instance, nutritional practices, such as high fat diets and red meat, strongly influence the risk of colorectal cancer (14). A possible reason behind this is that high dietary fat intake results in increased exposure to bile acids. At high physiologic concentrations, bile acids, mainly deoxycholic acid (DOC) and lithocholic acid (LCA), lead to the generation of reactive oxygen species and reactive nitrogen species, disruption of the cell membrane and mitochondria, induction of DNA damage, mutation and apoptosis, and ultimately cancer (15). Moreover, red meat increases the chances of developing colorectal cancer due to the high presence of heme iron. The mechanism is not clear, but heme iron has a catalytic effect on the endogenous formation of carcinogenic *N*-nitroso compounds and the formation of cytotoxic aldehydes by lipoperoxidation (14). Physical inactivity and obesity, manifested by lower metabolic efficiency and decreased insulin sensitivity, are also

associated with the increased risk of colorectal cancer (16). Cigarette smoking is known to be the leading cause of lung cancer. Remarkably, recent studies have shown that 12 percent of colorectal cancer deaths are also attributed to long-term smoking (16). Giovannucci *et al*. hypothesized that carcinogens present in cigarette smoke may initiate tumors in the colon and rectum. An induction period of 35 to 40 years may be needed to increase CRC incidence (17). Alcohol consumption also exacerbated the smoking-induced risk of CRC because DNA mutations that are caused by smoking are less efficiently repaired in the presence of alcohol (16). According to WHO, there are 2 billion people worldwide who consume alcohol on a regular basis with an average of 6.2 liters of ethanol per adult per year. A pooled analysis of 8 cohort studies in Europe and North America found that regular high alcohol intake (>45 g/day) modestly increases the risk of colorectal cancer (45 % for colon cancer and 49 % for rectal cancer) (18). Interestingly, Pederson *et al*. have shown that the risk of colorectal cancer seems to be reduced when wine is added to the alcohol intake (18).

3. Stress

Recent studies suggest that a distressed emotional state is also a potential risk factor for CRC (13). Stress is a physiologic response to any kind of threat or demand. During stressful situations, the sympathetic nervous system is activated (Fig. 2). This system acts on the adrenal medulla to stimulate the release of endogenous catecholamines. The catecholamines, epinephrine and norepinephrine, then act on adrenergic receptors that are ubiquitously expressed in different tissues.

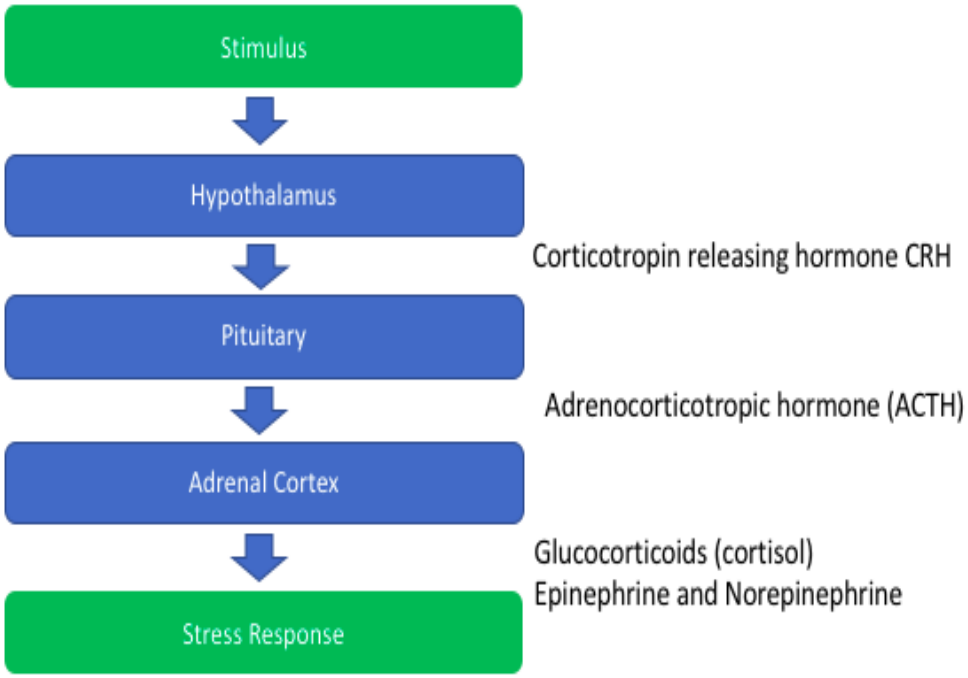


Figure 2: Stress-induced activation of the hypothalamic-pituitary-adrenal axis

Although stress is involved in physiologic reactions, it could sometimes lead to a homeostatic imbalance. Complications of chronic stress include sleep problems, depression, autoimmune diseases, heart diseases, reproductive problems, digestive problems, and cancer (19) (Fig. 3).

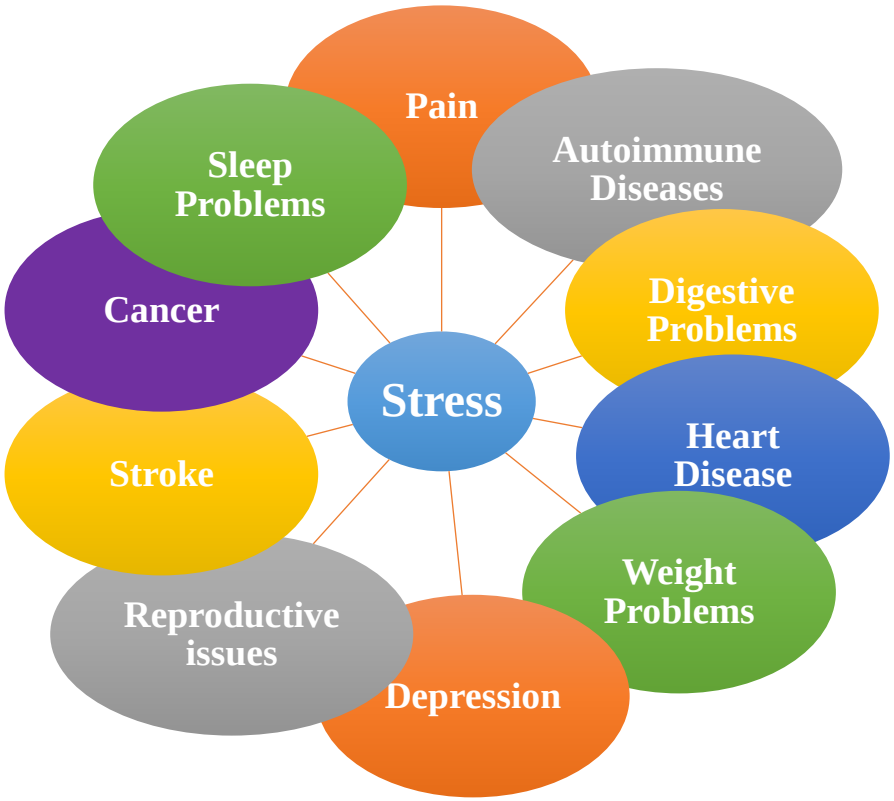


Figure 3: Complication of stress

For instance, chronic stress results in a sustained increase in cortisol and a decrease in serotonin and dopamine, both of which cause depression and sleep problems (19). Moreover, although cortisol helps in fighting inflammation, continuous increase in cortisol during chronic stress causes a dysregulation in the immune system thus precipitating inflammation (20). This unabated inflammation weakens the immune system, and autoimmune diseases, such as Celiac disease, may develop (20). Chronic stress may also result in hypertension through repeated activation of the sympathetic nervous system. Indeed, stress reduction interventions including meditation, stress management training, progressive muscle relaxation, acupuncture, and music therapy was associated with

significant blood pressure reductions (21). Studies have also shown that corticotropin-releasing hormone (CRH) inhibits hypothalamic gonadotropin-releasing hormone (GnRH) secretion, and glucocorticoids prevent pituitary luteinizing hormone and ovarian estrogen and progesterone secretion. These effects may result in female fertility problems and the "hypothalamic" amenorrhea of stress (22). Moreover, evidence has shown that mild to severe emotional stress may decrease testosterone levels and interfere with spermatogenesis in males (23).

Cancer is also a major complication of chronic stress. Indeed, stress induces a wide variety of biological responses involved in cancer progression including cellular proliferation, migration, invasion, and angiogenesis (11). Over the past 30 years, clinical and epidemiological studies have provided strong evidence supporting the link between chronic stress and cancer progression. However, there is limited evidence for the role of stress in cancer initiation. Numerous studies have revealed a possible link between stress and cancer progression, particularly breast cancer (24). Cancer patients usually live under chronic stress due to all the challenges they face in treatment and disease progression. At the molecular level, chronic stress induces the production of stress-related hormones that has been shown to cause changes in proliferation, apoptosis susceptibility, migration, and invasion potential of both normal and cancerous cells (25). Stress-related hormones increase the production of radicals, increase inflammation through the production of cytokines (26), impair the immune cell function (26), and stimulate the production of VEGF (26), insulin-like growth factor 1 (IGF-1) and other growth factors that promote the growth of the tumor (26).

The adrenoceptors, as mediators of stress, belong to a large family of G-protein coupled receptors (GPCR) (27). Once activated, they stimulate GTP-binding regulatory proteins, which in turn activates effectors including adenylyl cyclase and phospholipase C (28). There are two types of adrenergic receptors: α adrenergic receptors (αAR), which are classified into two subtypes (α_1 and α_2), and β adrenergic receptors (βAR) divided into β_1, β_2, and β_3 subtypes (29).

C. Adrenergic Receptors

1. Alpha-Adrenergic Receptors

Alpha-1 adrenoceptors are widely distributed in central and peripheral sites such as the brain, heart, blood vessels, spleen, liver, kidney, and prostate (30). Three α_1-AR subtypes have been identified: α_{1A}, α_{1B}, and α_{1D}. In the CNS, their activation initiates depolarization and neuronal excitability (27). α_1ARs are G_qPCR that activate phospholipase C (PLC) (31). PLC then catalyzes the conversion of phosphatidyl inositol 4,5-biphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). This increases intracellular calcium concentrations and stimulates smooth muscle contraction (32), mitogenesis (33), hepatic gluconeogenesis, glycogenolysis (34), apoptotic pathways, and cardiac stimulation (35). Studies have shown that α_1-ARs are also linked to other intracellular cascades including phospholipase A2, phospholipase D, MAP kinases, ROS production and NADPH oxidase. These responses are tissue and cell-type specific (34). For instance, in cardiomyocytes α_1-ARs activate MAPK pathways to induce expression of genes involved in cardiac hypertrophy. Moreover, in vascular smooth muscle cells, α_1-ARs

activate Ras/MAPK pathway which stimulates DNA synthesis and cell growth as well as proliferation (31).

Alpha-2 adrenergic receptors were initially described as presynaptic regulators that act via a negative feedback loop to control the release of norepinephrine (29). However, α_2-AR are also present on post-junctional sites. They are G_iPCR that inhibit adenylyl cyclase and mediate actions such as platelet aggregation (36), smooth muscle contraction (37), and inhibition of insulin secretion (38). Three alpha-2 adrenergic receptors have been cloned: α_{2A}, α_{2B}, and α_{2C}. α_2-ARs act both centrally and peripherally. In the CNS, α_2-ARs are involved in antihypertensive action of α_2 agonists such as clonidine. Nonselective α_2 agonist usually leads to a biphasic blood pressure response; α_{2B} -AR is responsible for the initial hypertensive effect, whereas α_{2A} -AR activation results in blood pressure decrease (29). Hence, a selective α_{2A} agonist may serve as a therapeutic target in the treatment or management of hypertension (29). Moreover, both α_{2A} and α_{2C} inhibit dopamine release in basal ganglia and serotonin secretion in the mouse hippocampal or brain cortex slices (29). α_{2A}-AR was thought to be the major inhibitory presynaptic receptor in sympathetic neurons (39). However, there is increasing evidence that α_{2C} receptor also functions as a presynaptic regulator in central and more prominently peripheral nervous tissues (39). The difference between α_{2A} and α_{2C} receptors is that α_{2A} inhibits the neurotransmitter release significantly faster and at higher action potential frequencies than α_{2C}. In the gastrointestinal tract, the inhibitory effect on the motility is mediated only by α_{2A} receptors (29).

α_{2C}-AR are predominantly expressed on cutaneous arteries and veins. They are

responsible for thermoregulation enabling the vessels to constrict during the cold. This response is exaggerated in Raynaud's phenomenon. Jeyaraj *et al*. established that when exposed to cold temperature, α_{2C} may be translocated from the intracellular pool to the cell surface. Patients with Raynaud's phenomenon experience augmented cold-induced vasoconstriction especially in the fingers and toes. Treatment with α_{2C} antagonists showed to relieve their symptoms (40). Further molecular and cellular experimentation by Bailey *et al*. showed that cold-induced activation of Rho/Rho kinase can facilitate cold-induced constriction in cutaneous arteries by stimulating the translocation of α_{2C}-ARs to the plasma membrane and also by increasing the calcium sensitivity of the contractile process (41).

2. Beta-Adrenergic Receptors

Beta-adrenergic receptors (β_1, β_2, and β_3) are G_sPCR that stimulate adenylyl cyclase and increase intracellular cAMP concentrations. Selective agonists and antagonists exist for each of the three subtypes that helped identify their physiological significance. β_1 adrenoceptors play a major role in cardiac stimulation and increase inotropy and chronotropy (42). They are also involved in renin release (43). β_2-AR are predominantly expressed in bronchial smooth muscle and their activation results in bronchodilation (44). Moreover, they are expressed on vascular smooth muscle causing vasodilation, and on cardiomyocytes resulting in increased inotropy (45). The β_3-AR are important for lipolysis in white adipose tissue and thermogenesis in the brown adipose tissue found in rodents (27). Other important physiological functions for β-adrenergic receptors include insulin and glucagon secretion from the endocrine pancreas and stimulation of glycogenolysis in

skeletal muscle and the liver (46). Myocardial infarction is characterized by increased GPCR kinases which result in desensitization of β_1-AR and β_2-AR. β_3-AR, on the other hand, lacks the GPCR kinase recognition sites and hence is not desensitized in heart failure. Having said that, β_3-AR may contribute to protective signaling pathways in the heart (47).

D. Adrenergic Receptors and Colorectal Cancer

The intertwined relation between stress and the malignant phenotype of colorectal cancer has been well documented. In particular, the adrenergic receptors, as mediators of stress, have been linked to the hallmarks of CRC such as proliferation, migration, invasion, apoptosis, and angiogenesis. As a matter of fact, the close distribution of noradrenergic fibers to the basement lamina of the colon results in the human colon being particularly sensitive to stress (48).

1. Proliferation

To begin with proliferation, studies have shown that catecholamines increase tumor cell proliferation. For instance, Lin *et al*. has shown that chronic restraint stress (CRS), induced by constraining mice horizontally in 50 ml conical centrifuge , increased the levels of norepinephrine (NE) and epinephrine (E) and stimulated the growth of CRC cell-derived tumors *in vivo*. *In vitro*, both E and NE increased the proliferation of HT-29, SW116, and LS174T CRC colon cancer cell lines in a dose dependent manner. However, when tumor cells were cultured in the presence of corticosterone, no significant differences in proliferation was observed. This implies that norepinephrine and epinephrine

are the stress hormones associated with CRC growth (49). Moreover, a non-selective α- AR antagonist (phentoalmine) and β-AR antagonist (propranolol) blocked the CRS-enhanced CRC cell growth in nude mice and CRC cell proliferation *in vitro* (49). Another study has also shown that β-AR play an important role in cell proliferation via ERK/MAPK activation pathway and β-AR blockade inhibited epinephrine induced phosphorylation of ERK1/2 and thus reduced cell proliferation (49). Moreover, epinephrine stimulated HT-29 colon cancer cell proliferation in a dose dependent manner. The activation of these receptors by epinephrine upregulated cyclooxygenase-2 (COX2) and vascular endothelial growth factor (VEGF) expression and increased matrix metalloproteinase-9 (MMP-9) activity. This resulted in the progression of CRC (50). In addition to that, β_1 and β_2-selective antagonists attenuated the stimulatory effects of epinephrine on the proliferation of HT-29 cells. Thus, epinephrine directly stimulated colon cancer cell proliferation through β-ARs which are upstream receptors of COX-2, VEGF and MMP-9 (50). This suggests that β-AR antagonist may be a potential therapeutic approach for the prevention and treatment of CRC. A protective role for non-steroidal anti-inflammatory drugs (NSAIDs), COX-2 inhibitor, has also been documented (50). Furthermore, selective blockage of β_2-AR, but not β_1-AR, resulted in downregulating survival signaling pathways such as EGFR-AKT-ERK1/2 pathway and caused G1-phase arrest (51). Contrary to these findings, Harris et. al found that β-AR stimulation decreased viability and proliferation of human SW480 colon carcinoma cells. However, α_{1B}-AR activation increased the survival of SW480, which calls for the investigation of these receptors as targets for antineoplastic agents (52). A study has shown that epinephrine may also act via a unique pathway to

induce cell proliferation and increase chemoresistance in colon cancer. miR-155, recently found to be an oncogene, promotes cell proliferation and migration and inhibits apoptosis in colon cancer cells. miR-155 is known to be upregulated by NFκB. Epinephrine treatment significantly increased NFκB activity in a dose-dependent manner and subsequently increased miR-155 expression. This contributed to an increase in proliferation and chemoresistance in colon carcinoma cells (53).

2. Migration

Adrenergic receptors have also been linked to migration. Epithelial mesenchymal transition (EMT) is a process during which epithelial cells undergo biochemical changes to acquire the characteristics of mesenchymal cells, such as increased resistance to apoptosis, increased ECM components, invasiveness, and migratory capacity (54). A recent study showed that by acting on β-AR, norepinephrine could induce EMT via decreasing epithelial marker (E-cadherin) and increasing expression of mesenchymal marker (vimentin). Two potential signaling pathways were involved: β -AR/TGF-β 1 signaling/HIF-1α /Snail and β -AR/TGF-β 1signaling/p-Smad3/Snail. These pathways indicate that TGF-β 1 signaling pathway is a significant factor of NE-induced EMT of CRC cells. Both non-selective β-AR and selective β_2-AR- antagonists were able to significantly decrease the migratory and invasive numbers of NE-treated cells; however, the numbers were still greater than in the control group indicating that β-AR antagonists only partially inhibited the migratory and invasive effect by NE (55). Epinephrine also increases migration through the activation of β–AR that increases MMP-9 activity in a COX-2 dependent

pathway (50). In line with previous studies, Masur *et al*. showed that norepinephrine augmented the migration of SW480 colon cancer cells by acting on β–AR to activate protein tyrosine kinase (PTK) (56). A nonselective β-blocker reduced the stimulatory effects of norepinephrine on migration (56). Interestingly, a β_1-blocker only marginally influenced the norepinephrine-induced locomotion. The inhibition of PTK using PP2 lead to a decrease in norepinephrine-induced locomotion from 70 % to 20 % which represents the level spontaneous locomotor activity. PP2 did not affect the spontaneous locomotor activity. However, the inhibition of PLCγ and PKCα resulted in a decrease in both spontaneous and norepinephrine induced locomotion. This suggests the use of a β_2-blocker for the preventive treatment to inhibit metastasis of colon carcinoma (56). Another study also showed that norepinephrine abolished the inhibitory effect of sunitinib, a chemotherapeutic drug, on cell migration in sunitinib treated HT-29 and CT26 colon cancer cell lines (57).

3. Invasion

Invasion is another hallmark of CRC modulated through the activation of the adrenergic system. Cell invasion is a process during which cancer cells migrate through the extracellular matrices within a tissue or infiltrate into neighboring tissues. Previous studies have shown that β adrenergic signaling modulates both invasion and tumor vascularization. Chronic stress increases VEGF, MMP9, and CD31 protein expression, all being markers of invasion and angiogenesis (11). Chronic stress also alters the primary tumor microenvironment increasing M-MDSCs infiltration, major immunosuppressive cells in the

tumor microenvironment. It also upregulated the expression of metastatic genes, ultimately resulting in increased invasion and metastasis to the liver. The use of β-blocker significantly abrogated the macrophages infiltration (11). It has been shown that APC mutations in the early stages and tumor suppressor p53 and KRAS mutations in the later stages both influence CRC progression and drive the malignant epithelial transformation. KRAS status acts as a biomarker determining the sensitization to β_2-AR blockage, similar to anti-EGFR therapy. β_2-AR blockade, and not β_1-AR blockade, strongly inhibits not only growth and viability but also invasion and migration in CRC cells that express wild-type KRAS (58).

4. Apoptosis

A hallmark of cancer is the ability of malignant cells to evade apoptosis. It has been shown that catecholamines-induced activation of β_1 and β_2-adrenergic receptors accelerate the progression of cancers including CRC. Chin *et al.* investigated the underlying signaling mechanism of the inhibition of β_1 and β_2-AR in the treatment of CRC. They established that β_2-AR blockage induced G1 phase arrest through the upregulation of p21 and p27 expression and downregulation of cyclin D1 and CDK4 expression (51). Moreover, histone H3 (Ser10) phosphorylation is involved in cell cycle progression while histone H2A.X (Ser139) phosphorylation is indicative of DNA damage. β_2-AR blockage induced H2A.X (Ser139) phosphorylation and decreased histone H3 (Ser10) phosphorylation, thus, promoting DNA damage and apoptosis through the intrinsic caspase-9 and extrinsic caspase-8 apoptotic pathways (51). Furthermore, β_2-AR blockage induced apoptosis by upregulating the expression pro-apoptotic Bcl-2 members (Bak, Bax

and Puma) and downregulating the expression of pro-survival Bcl-2 members (Mcl- 1 and Bcl-xL) (51). Thus β_2-AR blockage might be a potential therapeutic approach to halt the progression of CRC. Another study mentioned above showed that epinephrine upregulated miR-155 by increasing NFκB which ultimately results in a decrease in cisplatin-induced apoptosis in HT-29 cells (53).

5. Angiogenesis

As angiogenesis, also referred to as vascularization, is essential for tumor growth and metastasis, targeting tumor-associated angiogenesis is a promising therapeutic approach to limit cancer progression. Sunitinib is an inhibitor of several receptor tyrosine kinases including the receptors for VEGF and platelet-derived growth factor (PDGF), FMS-like tyrosinekinase-3 (FLT3), stem cell factor receptor (KIT). It exerts its chemotherapeutic action though anti-angiogenic effects. Liu *et al.* found that chronic restraint stress decreased the efficacy of sunitinib by increasing the expression of VEGF and interleukin-8 which stimulate angiogenesis. Norepinephrine induced a similar effect that was abolished by the β-antagonist propranolol (57). Thus, under psychological stress which is commonly seen in cancer patients, β-antagonist (propranolol) may improve anti-angiogenic activity of sunitinib (57). Moreover, through VEGF and CD31 staining, it has been shown that chronic stress was sufficient to cause a statistically significant increase in angiogenesis (11). Based on previous studies, which showed that the sympathetic nervous system regulates tumor vascularization and angiogenesis, Zhao *et al*. showed that a beta blocker abolished the stress-induced vascularization (11).

E. Hypothesis

Despite major advances in cancer research and treatment, CRC remains one of the leading causes of deaths worldwide (1). The hallmarks of CRC, including proliferation, migration, invasion, angiogenesis, and apoptosis have all been linked to stress and the adrenergic system. Recent studies have shown that β-ARs play a major role in accelerating the progression of CRC by modulating the aforementioned hallmarks. Therefore, β-ARs may serve as a potential target for the treatment of CRC and help in delaying the progression of CRC. It has been shown that advanced colon cancer patients on ACEIs/ARBs with β-blockers had a slower tumor progression, decreased hospitalizations, and a decreased mortality (59). Moreover, stage IV colorectal cancer patients on β-blockers had a significantly longer overall and cancer-related survival (60). Lately, there has been a debate on whether α-blockers might mimic the therapeutic role of β-blockers in CRC. Indeed, recent studies underpin an important role of α-ARs in the progression of cancer (61,62). The use of α_2-adrenoceptor antagonist, yohimbine, inhibited the proliferation of pancreatic cell lines and induced their apoptosis, suggesting that α_2-adrenoceptor antagonist can be used as an anticancer drug for apoptosis of pancreatic cells (61). Moreover, rauwolscine, an α_2-adrenoceptor antagonist, was shown to be effective in inhibiting breast cancer proliferation and may represent a possible novel adjuvant treatment for breast cancer (62). In CRC studies, in the presence of blockers for both β and α_1-ARs, epinephrine continued to promote a malignant phenotype. This indicates that receptors other than β and α_1-ARs, namely α_2-ARs, are involved. In our CRC cell line model, the expression of α_{2A}-AR and α_{2B}-AR is extremely negligible (52). Interestingly, the role of α_{2c}-AR in CRC

malignancy remains obscure. Here, we proposed that activation of α_{2c}-AR potentiates the malignant phenotype of human CRC cells.

CHAPTER II

MATERIALS AND METHODS

A. Cell lines and culture

SW480 human colon cancer cells, classified as Dukes' type B colorectal adenocarcinoma, were maintained in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, D6046) supplemented with 10% fetal bovine serum, and 1% penicillin/streptomycin. Cells were incubated at 37°C in 5% CO_2 and 95% air. The culture medium was replaced every 48 hours. Once the cells reached 90 - 95% confluency, cells were split at a ratio of 1 to 4.

B. Immunofluorescence staining of alpha-2c adrenoceptor.

SW480 cells were seeded on cover slips in 12-well plates at a density of 2×10^5 cells per well in complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 70 % confluency. Cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 to 20 min at RT. Cells were then washed three times with PBS, blocked with 3% normal goat serum (NGS) in PBS for 1 hour at RT and incubated with the primary antibodies: α_{2C}-AR (Abcam, ab46536) and Caveolin (Abcam, ab17052) in 1% NGS/PBS overnight at 4°C. Following incubation, cells were washed three times with PBS and placed for 2 hours at RT in the presence of fluorescein-conjugated secondary antibody Alexa Fluor (Abcam, ab150080) diluted at 1:500 in PBS. After washing three times with PBS, sample

cells were mounted with DAPI (Abcam). Cover slip was placed on a slide and mounted with antifade. The slide was examined under laser scanning confocal microscope.

C. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide Assay

SW480 cells were seeded in 96-well plates at a density of 7×10^3 cells per well in 200 µl complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 60% confluency. The medium was removed, and cells were serum-starved for 24 h prior to treatment. Cells incubated in culture medium alone served as a control for cell viability. Cells were treated with different concentrations of α_2-AR specific agonist (UK 14,304; 1, 10, or 100 nM) for different time points (24, 48, and 72 h) in serum-starved medium. Following treatment with UK 14,304 (Abcam, ab120773), 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) solution (5 mg/ml) was added to each well (final MTT concentration of 0.5 mg/ml) for 1 hour. The plates were then incubated under 95% atmosphere air and 5% CO_2 at 37°C for 1 hour. Wells were then aspirated, and 200 µL of DMSO were added to each well and gently mixed for 20 minutes to dissolve the formazan crystals. Treatments were performed in triplicates, and optical densities were read on an Elisa Multiscan EX Reader (Thermo) at a wavelength of 550 nm. Results are expressed as a percentage of control, the viability of which is assumed to be 100%.

D. Monolayer Scratch Assay

SW480 cells were seeded in 12-well plates at a density of 3×10^5 cells per well in 1 ml complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 90-95 %

confluency. The medium was removed, and cells were serum-starved for 24 h prior to treatment. A uniform wound was scratched on the cell monolayer using a 10 µL sterile pipette tip. The remaining cells were then washed twice with PBS and cultured in a serum-starved medium containing 0.5% FBS. Cells incubated in culture medium alone served as a control. Cells were treated with 7 different conditions: α_2-AR specific agonist (UK 14,304; 100 nM), α_{2C}-AR specific antagonist, JP 1302 (Abcam, ab141129; 100nM), pretreatment with JP 1302 for 30 minutes followed by UK 14,304, NOX 1/4 enzyme inhibitor (GKT 137831; 20 µM), pretreatment with GKT 137831 for 1 hour followed by UK 14,304, ROS Scavenger (N-acetyl cysteine NAC; 12.5 mM), pretreatment with NAC for 1 hour followed by UK 14,304. The wound healing was monitored at 0, 2, 4, 6, 8, 12, and 24 hours. Micrographs were taken, in marked areas. The distance was measured in µm. The quantification of the evolution of the healing was analyzed with ZEN lite from Zeiss Microscope software.

E. Transwell Migration Assay

SW480 cells were seeded in the upper chamber of a 24-multiwell insert system (8 um pore size, PET membrane) at a density of 4×10^4 cells per insert in 200 µl serum-starved DMEM. 700 µl of complete DMEM containing 10 % FBS was added to the lower chamber to act as a chemoattractant. Cells incubated in culture medium alone served as a control for cell migration. Cells were pretreated with JP 1302 (100nM) for 30 minutes followed by UK 14,304 (100nM). SW480 cells were placed at 37°C in a 5% CO_2 humidified incubator and allowed to migrate for 24 hours. After incubation, the media in inserts was aspirated and

wells were washed twice with PBS. Cells were then fixed with 4% formaldehyde in PBS for 10 minutes. The fixation solution was aspirated, and cells were washed twice with PBS. Methanol (100%) was then added for 20 minutes. Methanol was aspirated, and cells were washed twice with PBS. Later, cells were stained with 1% crystal violet for 5 minutes. Cells were washed twice with PBS. Non-migrated cells were removed with sterile cotton-tipped applicator. The membrane was cut with a blade and mounted with antifading agent. The slide was observed using a fluorescent microscope.

F. Adhesion assay

Cell culture plates were coated with collagen and Human Umbilical Vein Endothelial Cells (HUVECs) were then seeded and allowed to grow to reach a confluent monolayer. TNF-α (25 ng/ml) was added for 6 hours to stimulate HUVECs prior to the addition of SW480 cells. SW480 cells were transfected with *Renilla* luciferase and resuspended in DMEM containing 1% BSA. Cell suspension was added to HUVECs in the absence or presence of UK 14,304. After 1 hour, wells were washed three times with PBS to remove unattached cells. Adherent cells were then lysed with a luciferase lysis buffer (Promega, Madison, WI, USA) and light units measured according to the manufacturer's protocol.

G. Transendothelial migration

Transwell filters were coated with collagen and allowed to dry overnight. HUVECs were then seeded at a density of 2×10^5 cells per well onto the rehydrated membrane and allowed to grow to reach a confluent monolayer. In order to stimulate HUVECs, TNF-α (25 ng/ml) was added for 6 hours. SW480 cells were then loaded on top

and incubated overnight in the absence or presence of UK 14,304. Non-migrated cells were removed with sterile cotton swab, whereas SW480 cells on the bottom were stained and quantified.

H. NADPH Oxidase Activity

SW480 cells were seeded in 6-well plates at a density of 3×10^5 cells per well in 2 ml complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 60% confluency. The complete DMEM was removed and the cells were serum-starved for 12 h prior to treatment. Cells incubated in culture medium alone served as a control. The cells were treated with α_2-AR specific agonist (UK 14,304; 100 nM; 10 min), α_{2C}-AR specific antagonist (JP 1302; 100 nM), or pretreatment with JP 1302 for 30 minutes followed by UK 14,304 for 10 min. The cells were then collected by adding 50 µl lysis buffer (20 m*M* K_2HPO_4 [pH 7.0], 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 0.5 µg/ml leupeptin). The lysates were rotated for 1 hour at 4 degrees Celsius. Protein concentrations were determined using Lowry protein Assay. To start the assay, 20 µg of homogenates was added to 50 m*M* phosphate buffer (pH 7.0) containing 1 m*M* EGTA, 150 m*M* sucrose, 5 µ*M* lucigenin, and 100 µ*M* NADPH. Photon emission expressed as relative light units was measured every 20 or 30 s for 4 minutes in a luminometer. A buffer blank (<5% of the cell signal) was subtracted from each reading.

I. Dihydroethidium staining (DHE)

SW480 cells were seeded on a cover slip in 12-well plates at a density of 1.5 x 10^5 cells per well in 1 ml complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 40 % confluency. The complete DMEM was removed and the cells were serum-starved for 12 h prior to treatment. Cells incubated in culture medium alone served as a control. The cells were treated with α_2-AR specific agonist (UK 14,304; 100 nM; 10 min), α_{2C}-AR specific antagonist (JP 1302; 100 nM), or pretreatment with JP 1302 for 30 minutes followed by UK 14,304 for 10 min. The media was then aspirated, and cells were washed twice with cold PBS. DHE stain (5 µM) was added to the wells (1ml/well) in the dark. Cells were incubated for 1 hour at 37°C in a 5% CO_2 humidified incubator. The stain was then removed, and wells were washed once with cold PBS. The cover slips were observed using Microscope Zeiss Axio.

J. Determination of Rho translocation by Western blot analysis

SW480 cells were seeded in 6-well plates at a density of 3×10^5 cells per well in 2 ml complete DMEM and placed at 37°C in a 5% CO_2 humidified incubator until 70% confluency. After removing the medium, cells were serum-starved for 24 h prior to treatment. Cells incubated in culture medium alone served as a control. Cells were treated with α_2-AR specific agonist (UK 14,304; 100 nM) for 10, 30, and 60 min in serum-starved medium. The cells were then homogenized in cold homogenization buffer (mM): 100 Tris-HCl (pH 7.4), 1 EGTA, 1 EDTA, 1 PMSF, and 1 Na3VO4. The homogenates were centrifuged at 100,000 g and 4 °C for 20 min. The supernatant (cytosolic fraction) was

collected, and the pellet (membrane fraction) was re-suspended in homogenization buffer containing 1% Triton X-100. Protein concentrations were determined using Lowry protein Assay. Protein levels of Rho were determined by Western blot using monoclonal Rho antibody (Abcam, ab187027).

K. Rhotekin-binding activation assay

SW480 cells were grown to 80% confluence and then made quiescent in 0.5% FBS-DMEM. Cells were treated with UK 14,304 (100 nM) for 10, 30, and 60 minutes. Using Rhotekin Rho binding domain, RhoA activation was measured by G-LISA according to manufacturer recommended protocols (Cytoskeleton). Data were expressed as fold-change in RhoA activity (means ± SE).

L. Western blotting

Cells were lysed using 10 mM Tris pH 6.8, 2% SDS. Gels were loaded with 25-50 μg of protein lysates on an SDS-PAGE. After transferring the proteins to a nitrocellulose membrane for 1 hour, the membrane was blocked for 1 hour in 5% skim milk, then was washed 3 times with 1xTTBS and incubated overnight with the primary antibody at 4°C. Primary antibodies of ERK 1/2, FAK, RhoA were purchased from Abcam. After incubation with the secondary antibodies for 1 hour, the protein bands were developed with the chemiluminescent reagents (Bio-Rad). The blot images were taken by using the Chemidoc MP Imaging system.

M. Measurement of matrix metalloproteinases by ELISA

SW480 cells were grown until they reached near confluence. Cells were incubated in serum-free media and pretreated with increasing concentrations of UK 14,304 (0, 3, 10, 30, and 100 nM) for 24 and 48 hours. The conditioned media were collected and subjected to an ELISA for MMP-2 and MMP-9 as per the manufacturer's protocol (R&D Systems, USA). In separate experiments, cells were also pretreated with PD98059 or JP1302 followed by UK 14,304. The conditioned media were collected and subjected to an ELISA for MMP-2 and MMP-9 as per the manufacturer's protocol (R&D Systems, USA). Experiments were conducted in triplicates, and data are presented as percent of control (vehicle).

N. Quantitative Immunoassay for Human Vascular Endothelial Growth Factor

SW480 cells were seeded in 24-well plates overnight in serum-containing culture media and then, the media was replaced by serum-free media. Cells were treated with increasing concentrations of UK 14,304 (0, 3, 10, 30, and 100 nM) and the conditioned media was collected at 24 and 48 h. VEGF level was then measured using a VEGF enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. The proteins present in the conditioned media were concentrated using the Amicon Ultra-0.5 protein purification and concentration column (Millipore) and protein concentration was assayed using the BCA protein assay kit (Thermo Scientific). Levels of VEGF were normalized to the total protein level in each sample. The assays were performed in triplicates and three independent experiments were performed. Data are presented as percent of control ± SEM.

O. Statistical Analysis.

Statistical analysis was performed by a student's t-test for either paired or unpaired observations using GraphPad Prism version 5.0 and InStat3 Software (GraphPad software, Inc. San Diego, CA). Data was presented as mean ± SEM, where n is equal to the number of times an experiment is repeated (n will be ≥3). When more than two means are used for comparison, ANOVA was used: either one-way ANOVA (with Dunnett's post hoc test) or two-way ANOVA (with Tukey-Kramer's post hoc test).

CHAPTER III

RESULTS

A. Receptor Expression

1. α_{2C}-AR is expressed on the plasma membrane of SW480 colon cancer cells

The localization of α_{2C}-AR was examined using immunofluorescence staining. The caveolin primary antibody allowed the detection of the plasma membrane. Caveolins are a family of integral membrane proteins that are principal components of caveolae membranes and involved in receptor-independent endocytosis. Immunofluorescence revealed that α_{2C}-ARs are expressed with caveolin on the plasma membrane (Fig. 4).

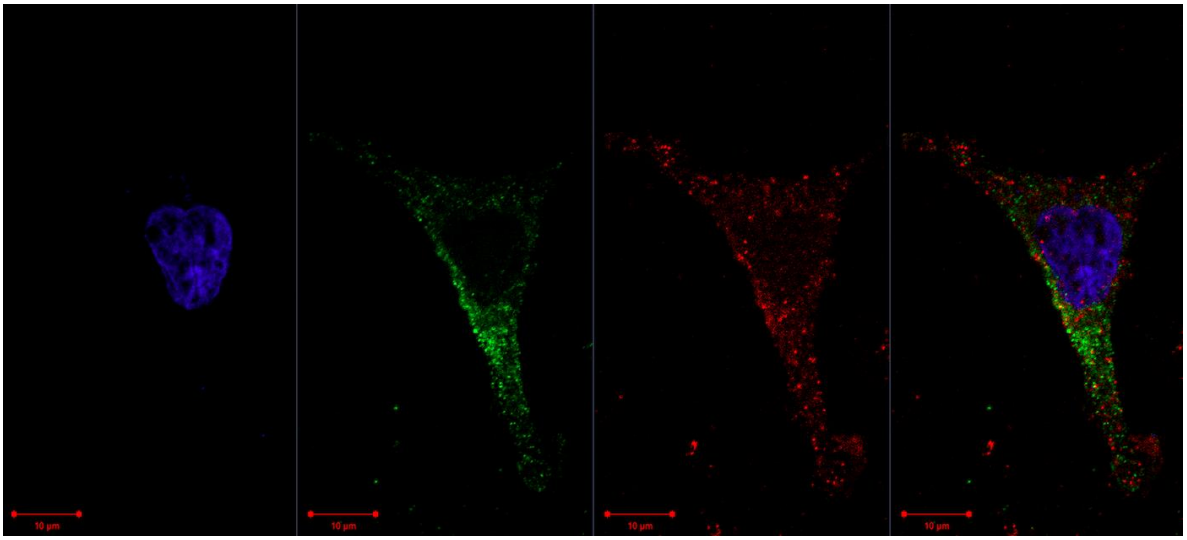


Figure 4: Localization of α2C-AR in SW480 human colon cancer cells. Immunofluorescence staining showed that α_{2C}-AR is located at the plasma membrane of SW480. The green color refers to caveolins. Caveolin allows the detection of the plasma

membrane. The red color indicates α_{2C}-AR which is expressed with caveolin at the membrane.

B. Proliferation

1. The effect of α_2-AR stimulation on proliferation of SW480 cells

The effect of UK 14,304 (α_2-AR specific agonist) on SW480 proliferation was studied using MTT assay. Treatment of SW480 human colon cancer cell lines with increasing concentrations of UK 14,304 (10, 30 or 100 nM) for different time points (24, 48, or 72 hours) did not cause a significant change in proliferation (Fig. 5). (p>0.05)

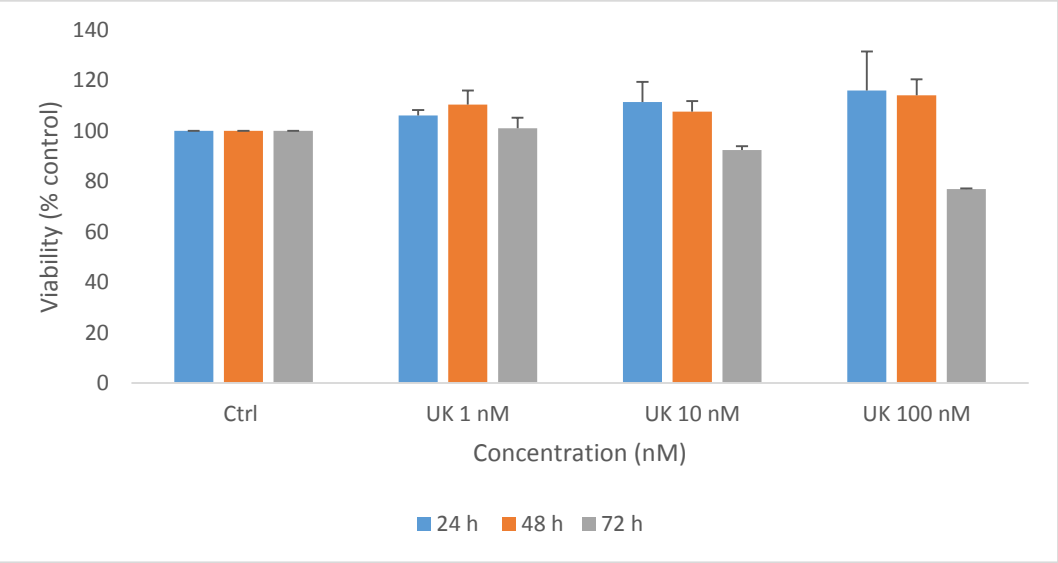


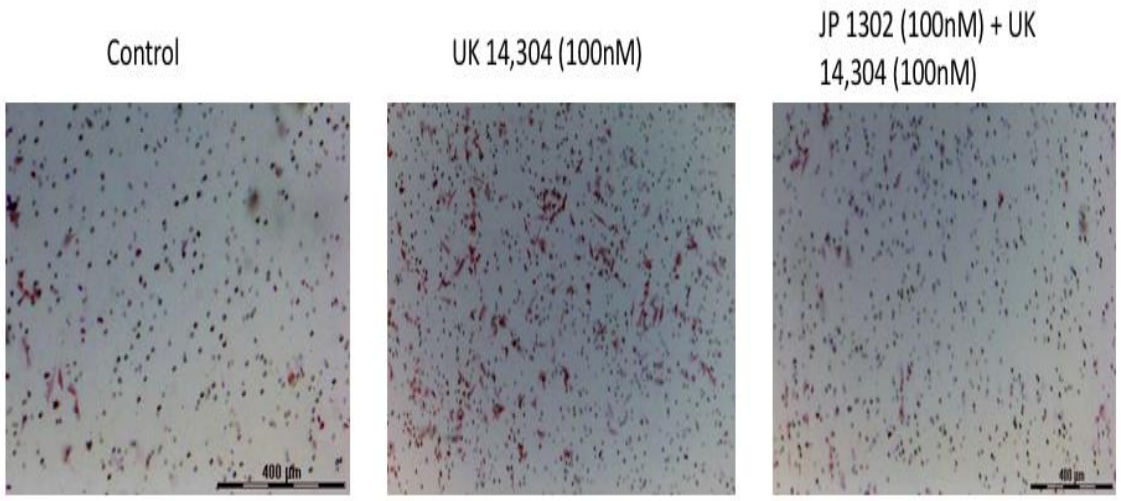
Figure 5: No significant differences in the proliferation of SW480 cells. Increasing concentrations of UK 14,304 (1, 10, and 100 nM) had no effect on proliferation at 24, 48, and 72 hours. Viability values are calculated as % of the corresponding vehicle control value and represented as mean ± SEM of three replicates.

C. Migration

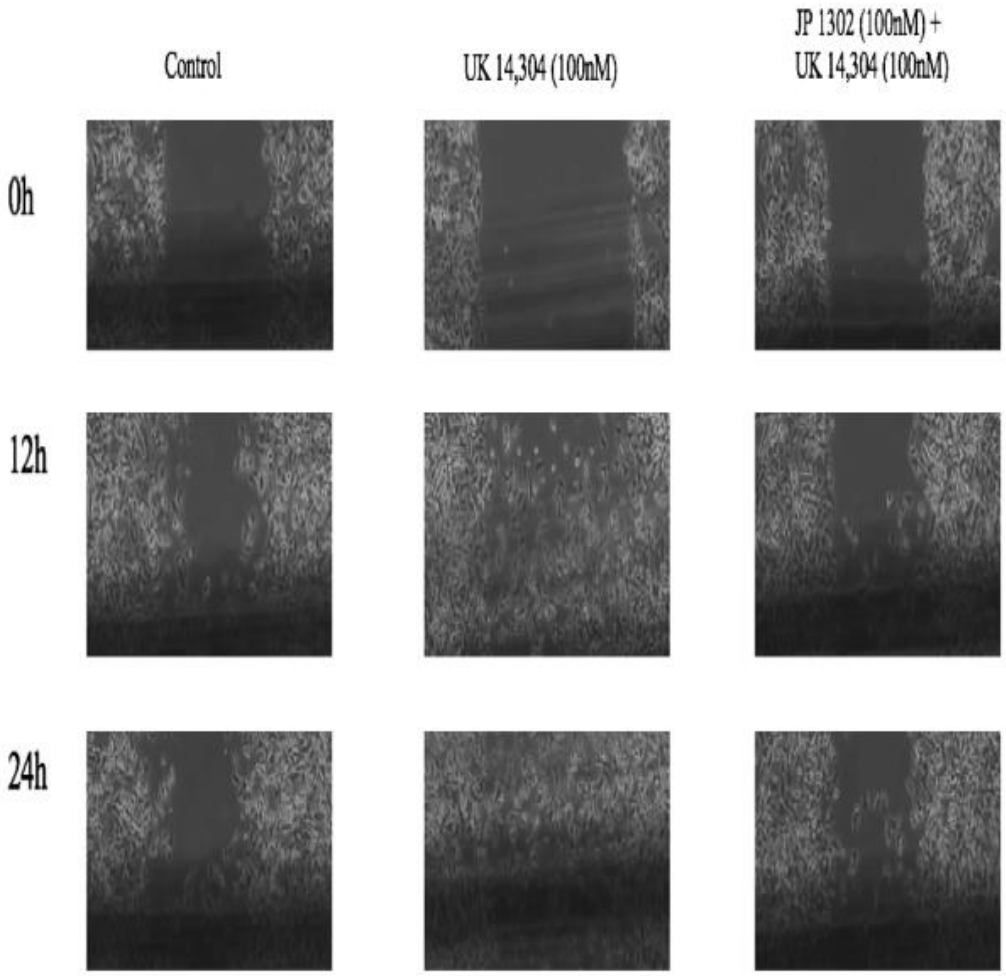
1. α_{2C}-AR stimulation induces migration of SW480 cells

The effect of α_{2C}-AR stimulation on SW480 migration was assessed using both scratch and transwell migration assay (Fig. 6). UK 14,304 (100 nM) significantly increased SW480 migration from upper to lower chamber (Fig. 6A). Similarly, UK 14,304 (100 nM) promoted wound healing significantly at 2, 4, 6, 8,12 and 24 hours (Fig. 6B and 6C) ($p < 0.01$). Pretreatment with a specific α_{2c}-AR antagonist (JP 1302; 100 nM) abolished the UK 14,304-induced cell migration both in transwell (Fig 6A) and scratch assays (Fig. 6B and 6C).

A.



B.


Control
UK 14,304 (100nM)
JP 1302 (100nM) +
UK 14,304 (100nM)
0h
12h
24h

C.

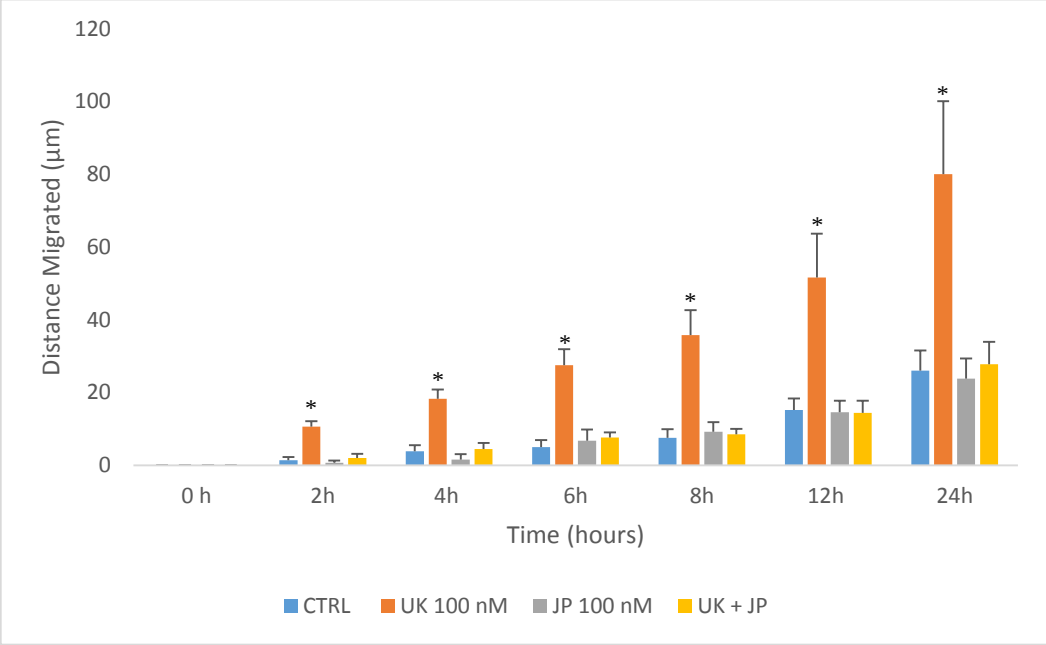


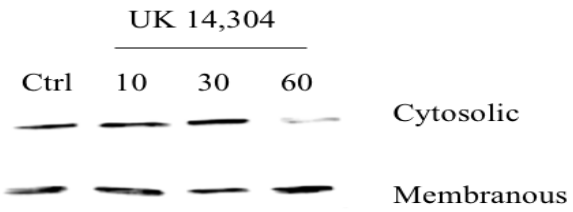
Figure 6: UK 14,304 (100nM) increases SW480 migration via the activation of α2c-AR. *(A) Representative photomicrographs of the effect of 100 nM of UK 14,304 on SW480 migrating to the lower chamber in transwell migration assay. (B) Representative photomicrographs of the effect of 100 nM of UK 14,304 on SW480 scratch wound healing at 12 and 24 hours postscratch. (C) Mean ± SEM of distance migrated in µm. Values are and represented as mean ± SEM of four replicates. * denotes $p < 0.05$.*

2. α_{2C}-AR stimulation induces RhoA translocation to the plasma membrane

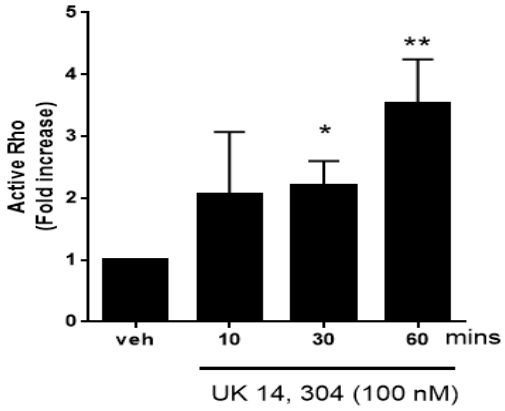
There is overwhelming evidence that Rho GTPases play a central role in cell migration (63). To assess whether α_{2C}-AR stimulates RhoA translocation to the membrane, SW480 cells were treated with UK 14,304 (100 nM) for 10, 30, and 60 minutes. RhoA

translocation was determined by western blot analyses of membrane and cytosolic proteins. Results showed that α_{2C}-AR stimulation induces RhoA translocation to the plasma membrane at 30 and 60 minutes significantly (Fig. 7A, 7B). This Rho activity was also evident by Rhotekin binding G-LISA activity assay (Fig. 7C). Moreover, this UK-induced Rho activity was inhibited by JP 1302 and PD98059 (20 μM), an ERK1/2 inhibitor, indicating that $alpha_{2c}$ acts through ERK 1/2 to activate Rho (Fig. 7C).

A.



B.



C.

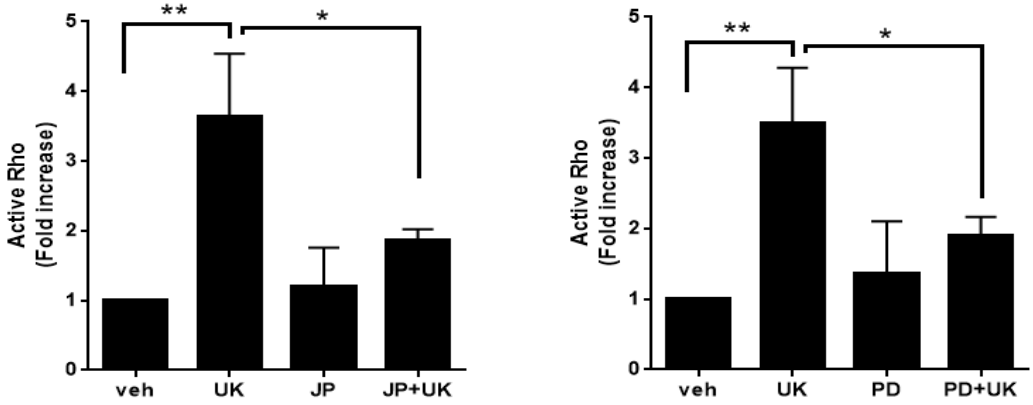


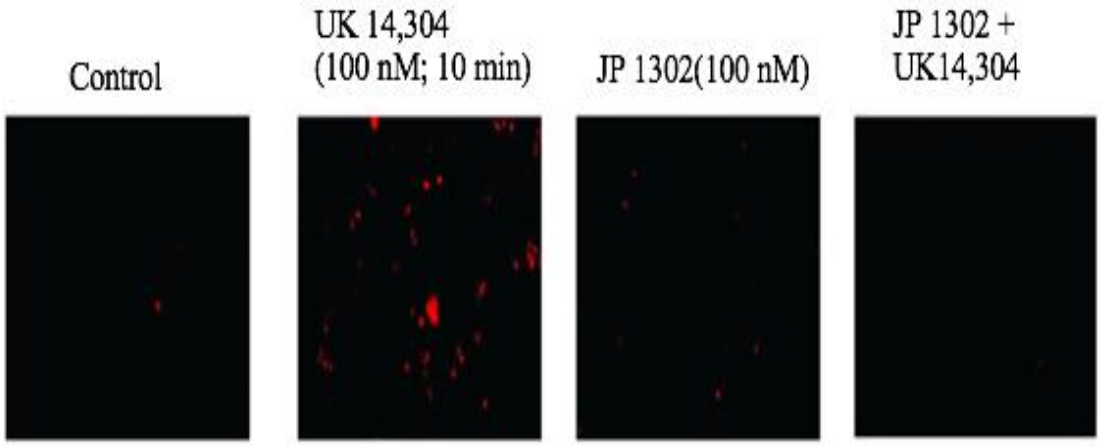
*Figure 7 : SW480 cells were treated with UK 14,304 (100 nM) for 10, 30, and 60 minutes. Cytosolic and membranous protein lysate were then subjected to Western blotting. (A) Western blot of cytosolic and membrane RhoA. (B) Histograms showing the quantitation of membranous RhoA/cytosolic Rho A. Values are the mean ± SEM of three replicates. (C) Cells were treated with UK 14,304 and subjected to G-LISA for active Rho. * denotes $p<0.05$ and ** <0.01*

3. α_{2C}-AR stimulation increases ROS production and NADPH oxidases activity

Several reports indicate that cell migration or RhoA activation could be mediated by an increase in oxidative stress. To assess whether α_{2c}-AR stimulation increases ROS production, DHE staining was performed. Cells were treated with UK 14,304 (100 nM) for 10 minutes followed by DHE staining. UK 14,304 significantly increased ROS production (Fig. 8A). Pretreatment with JP 1302 (100 nM) abolished the UK 14,304 – induced ROS production indicating that α_{2c}-AR is responsible for the increase in oxidative stress (Fig.

8A). Treatment with UK 14,304 (100 nM) also increased NADPH oxidases activity (NOX1 and NOX4, which are important mediators of ROS production. Pretreatment with JP 1302 (100 nM) significantly decreased NADPH oxidases activity implicating a role for α_{2c}-AR in NOX 1/4 activation (Fig. 8B).

A.



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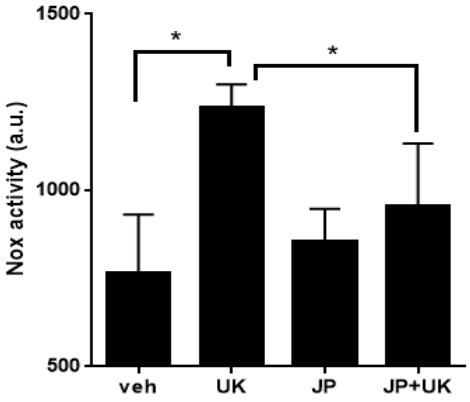


*Figure 8: α2c-AR stimulation increases ROS production NADPH oxidases activity. SW480 cells were treated with UK 14,304 (100 nM) for 10 min and DHE staining (Fig. 8A) and NADPH oxidase activity (Fig. 8B) assays were performed. (A) UK 14,304 increases ROS production and this was abolished by pretreatment with JP1302. (B) UK 14,304 increases significantly NADPH oxidases activity and this was also abolished by JP 1302. Values are represented as the mean % change from the control ± SEM of three replicates. * denotes p < 0.05.*

4. α_{2C}-AR induced oxidative stress induces migration

To assess whether the α_{2c}-AR-induced oxidative stress increases SW480 migration, a scratch assay was performed. SW480 cells were treated with α_2-AR specific agonist (UK 14,304; 100 nM) in the absence or presence of JP 1302 (100 nM), GKT 137831 (20 µM), a NOX 1/4 enzyme inhibitor (;), or, ROS Scavenger (N-acetyl cysteine NAC; 12.5 mM). Results show that UK 14,304 significantly increases SW480 migration. Pretreatment with JP 1302, NAC, or GKT 137831 abolished this effect. This indicates that α_{2C}-AR induced oxidative stress increases migration (Fig. 9).

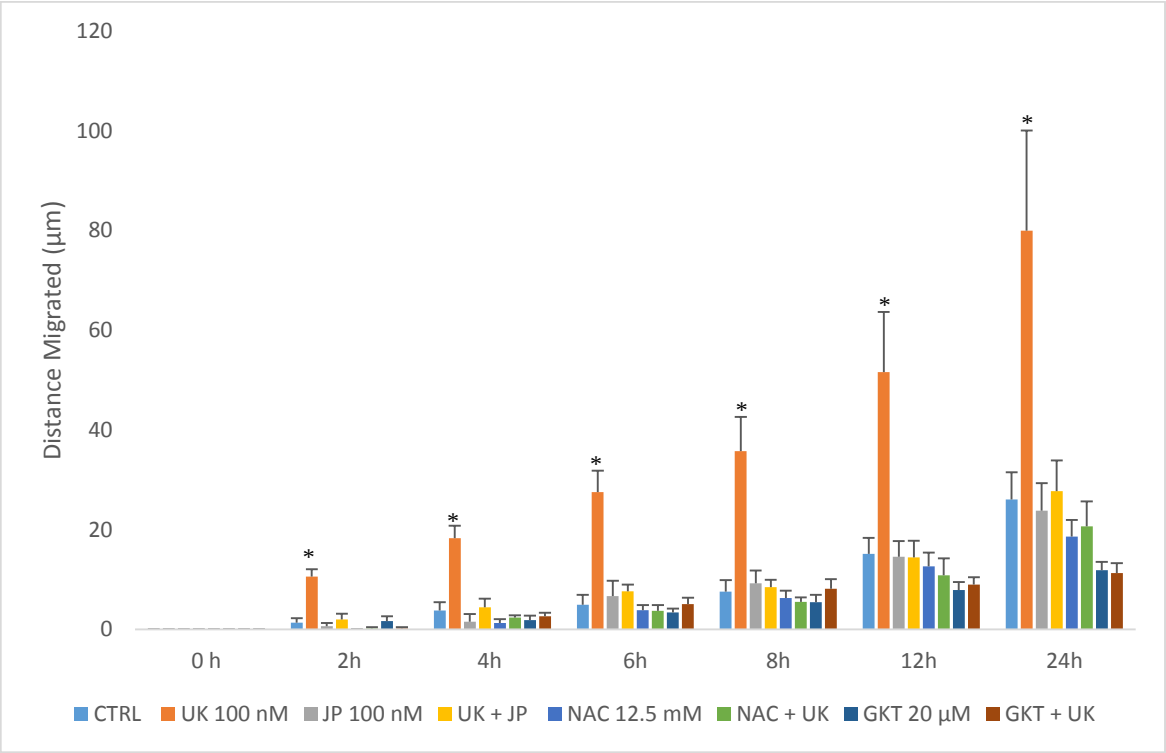


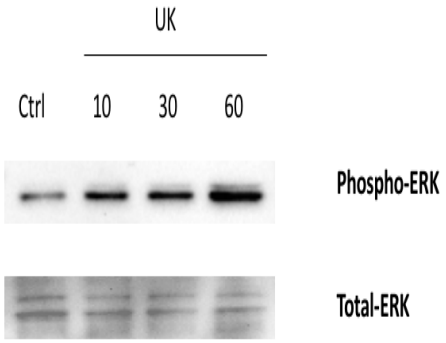
*Figure 9: α2c-AR -induced oxidative stress increases migration of SW480 colon cancer cells. Scratch assay of SW480 cells that were pretreated with JP1302, NAC, and GKT 137831 followed by UK 14,304 was performed. Results show that α_{2c}-AR increases migration through increased oxidative stress. Values are represented as mean ± SEM of four replicates. All treatments are significantly different ($p < 0.05$) from UK 14,304 alone. * denotes $p < 0.05$.*

5. α_{2C}-AR stimulation increases ERK 1/2 and FAK activity

Because ERK1/2 and FAK are crucial in cell migration, we sought to determine the ERK1/2 and FAK phosphorylation levels. SW480 cells were treated with UK 14,304 (100 nM) for 10, 30, and 60 minutes. ERK 1/2 and FAK phosphorylation was assessed using western blot. UK 14,304 (100 nM) significantly increased the phosphorylation of

ERK 1/2 (Fig. 10A, 10B) at 60 minutes (p<0.05). It is worth mentioning that our preliminary data show that in the presence of JP 1302, UK 14,304 fails to cause an appreciable increase in ERK 1/2. This suggests that α_{2c}-AR is the mediator of UK-induced ERK 1/2 phosphorylation. Similarly, UK 14,304 increased FAK phosphorylation (Fig. 10C) which was abolished by JP 1302 (Fig. 10D). Moreover, pretreatment with PF-573228 (10 µM), a FAK-specific inhibitor, or PD98059 (20 µM), an ERK1/2 inhibitor, abolished UK-induced migration.

A.



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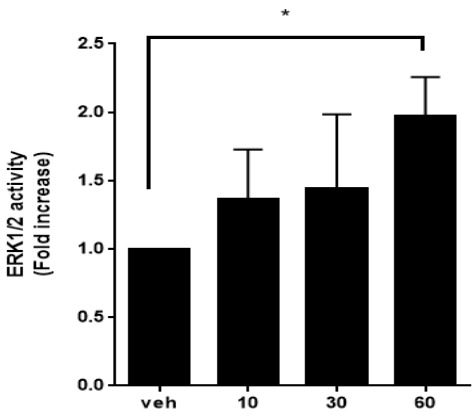

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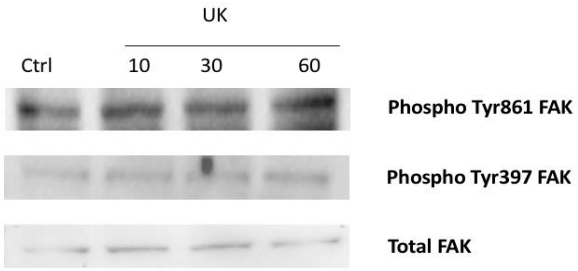

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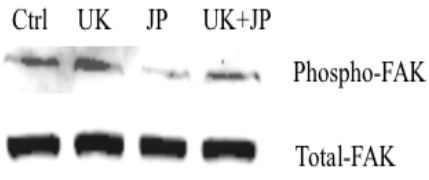

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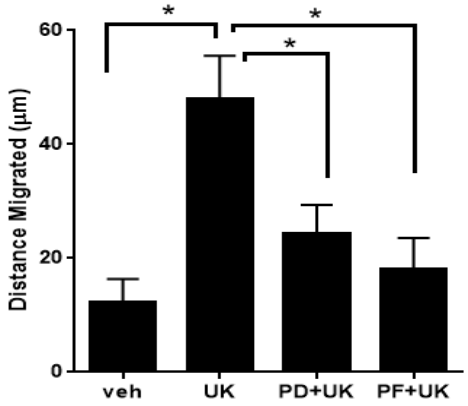

Figure 10: α2c-AR stimulation increases ERK 1/2 and FAK activity which are crucial for migration. (A) Western Blot for ERK 1/2. (B) Histogram showing quantitation of phosphorylated ERK 1/2 / total ERK 1/2. (C,D) Western Blot for FAK. (E) Pretreatment with PF-573228 or PD98059 abolished UK-induced migration. Values are represented as the mean of fold change ± SEM of three independent experiments.

D. Adhesion, Transendothelial Migration, and Invasion

1. α_{2C}-AR stimulation promotes adhesion and transendothelial migration

UK 14,304 (3, 10, 30 and 100 nM) promoted the adhesion of colon cancer cells to TNF-alpha-activated HUVECs. At 100 nM, UK14,304 caused a 325 ± 73% increase in adhesion (p<0.05) (Fig. 11A). After adhering to endothelial cells, cancer cells tend to modify the integrity of the endothelial barrier especially during intra or extravasation. Indeed, UK 14,304-caused a dramatic increase in transendothelial migration of SW480

through HUVECs. This increase was abolished by JP 1302, clearly indicating that α_{2c}-AR activation may promote transendothelial migration (Fig. 11B).

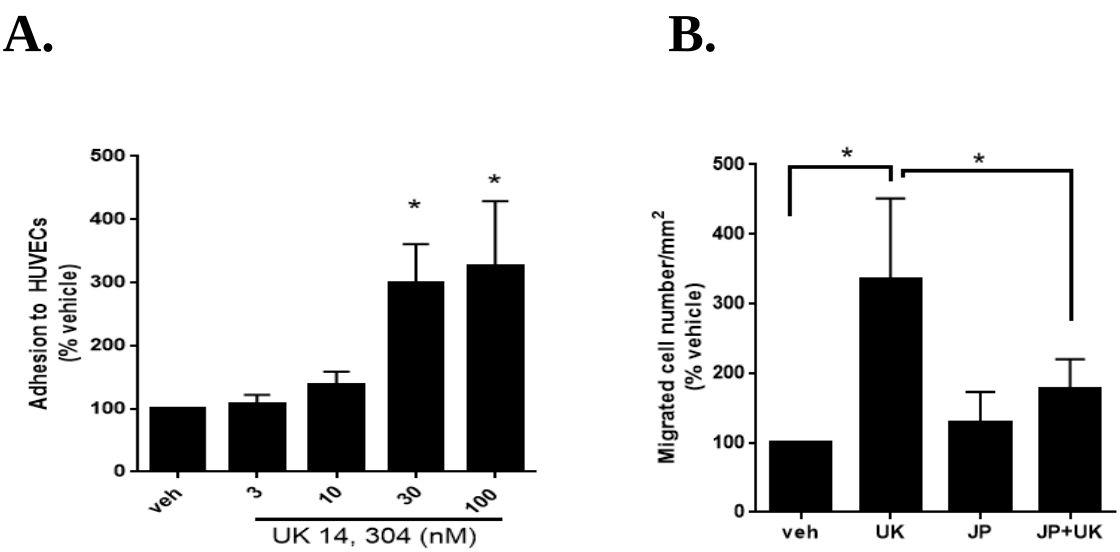

Figure 11: α2c-AR stimulation increases adhesion and transendothelial migration. (A) Cells were loaded on top of TNF-alpha-activated HUVECs and adhesion was measured. (B) Cells that penetrated through an endothelial cell layer were measured.

2. α_{2C}-AR stimulation increases MMP-2 and MMP-9 expression

Because breaking the endothelial cell barrier requires matrix digestion, we measured the levels of matrix metalloproteinases (MMPs). SW480 cells were incubated in serum-free media and pretreated with increasing concentrations of UK 14,304 (0, 3, 10, 30, and 100 nM) for 24 and 48 hours. The conditioned media were collected and subjected to an ELISA for MMP-2 and MMP-9. Results show that UK 14,304 significantly increases MMP-9 (Fig. 12A) and MMP-2 (Fig. 12B) in a concentration and time-dependent manner.

For instance, UK 14,304 (10, 30 and 100 nM) significantly increase MMP-9 and MMP-2 expressions at 24 and 48 hours. Pretreatment with ERK1/2 inhibitor (PD98059) (Fig. 12C) or JP 1302 (Fig. 12D) abolished the increase in MMP-2 indicating that α_{2C}-AR and ERK1/2 are significant mediators of the increased production of MMPs (Fig 11c).

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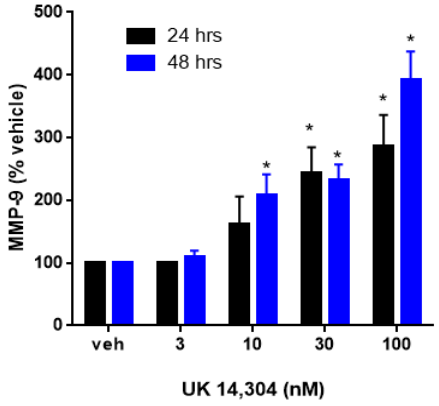

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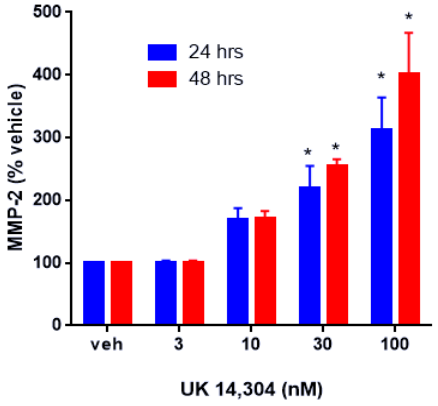

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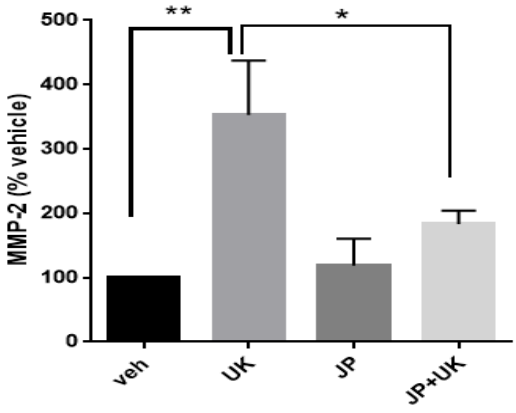

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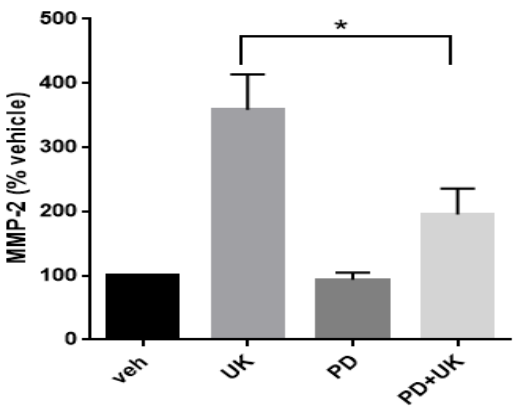

*Figure 12 : α2C-AR stimulation increases MMP-2 and MMP-9 expressions. UK 14,304 (10, 30, and 100 nM) increases MMP-9 and MMP-2 expressions significantly at 24 and 48 hours. (A) Histogram showing the quantitation of MMP-9 expression. (B) Histogram showing the quantitation of MMP-2 expression. (C) Histogram showing the quantitation of MMP-2 expression at 24 hours after pretreatment with JP1302. (D) Histogram showing the quantitation of MMP-2 expression at 24 hours after pretreatment with PD98095. Data represented are mean ± SEM of % MMP level in the corresponding vehicle control treatment (n = 3 replicates). * denotes p<0.05 and ** <0.01*

E. Angiogenesis

1. α_{2C}-AR stimulation increases VEGF expression

Since vascular endothelial growth factor (VEGF) is essential for angiogenesis, we assessed the expression of VEGF following stimulation with UK14,304. SW480 cells were treated with increasing concentrations of UK 14,304 (0, 3, 10, 30, and 100 nM) and the conditioned media was collected at 24 and 48h. VEGF level was then measured using a VEGF enzyme-linked immunosorbent assay kit. UK 14,304 (100 nM) significantly increase VEGF expression at 24 hours, while UK 14,304 (30 and 100 nM) significantly increase VEGF expression at 48 hours (Fig. 13A) (p<0.01). Pretreatment with JP1302 abolished the increase in VEGF (Fig. 13B).

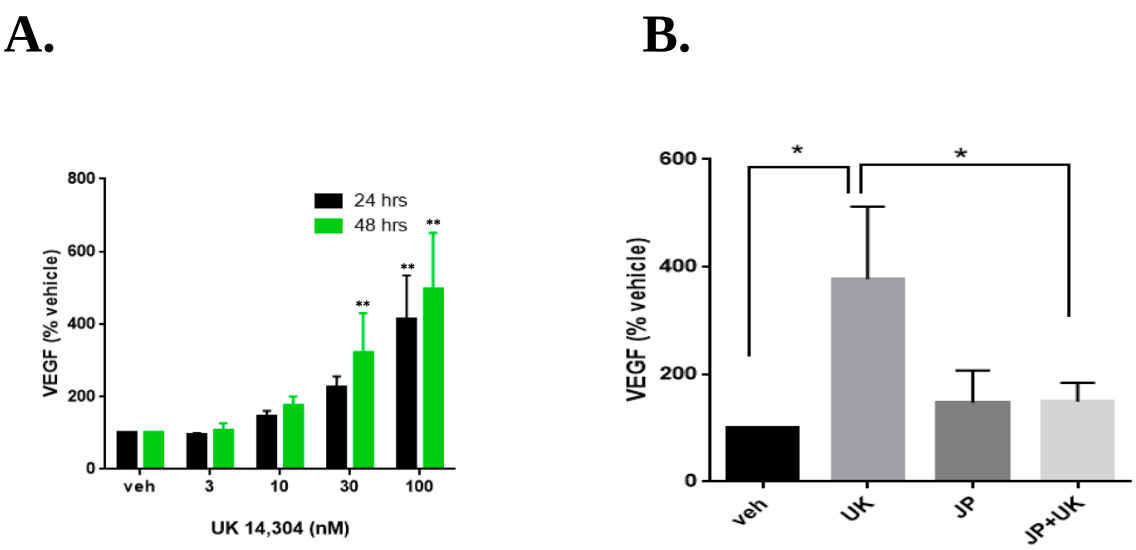

Figure 13: α2C-AR stimulation increases MMP-2 and MMP-9 expressions. (A) Histogram showing the quantitation of VEGF expression. (B) Histogram showing the quantitation of VEGF expression at 24 hours after JP 1302 pretreatment. Data represented are mean ± SEM of % MMP level in the corresponding vehicle control treatment (n = 3 replicates).

CHAPTER IV

DISCUSSION

Increasing evidence indicates that stress induces a wide variety of biological responses involved in cancer progression such as cellular proliferation, migration, invasion, and angiogenesis (11). Studies have shown that adrenoceptors, as mediators of stress, play a central role in promoting the malignant phenotype of CRC (49-53, 55-57). Interestingly, the role of α_{2c}-AR in CRC malignancy remains obscure. This is the first study depicting the role of α_{2C}-AR in enhancing the malignant phenotype in colorectal cancer. Our findings demonstrate that stimulation of α_{2c}-AR increases migration, adhesion, transendothelial migration, as well as invasion and angiogenesis markers, all of which are hallmarks of CRC malignancy.

Immunofluorescence staining revealed that α_{2C}-ARs are expressed on the plasma membrane of SW480 human colon cancer cells. This is in discordance with vascular smooth muscle cells where α_{2C}-ARs are generally retained in the perinuclear *trans*-Golgi compartment (64). During stressful conditions, or in response to cold temperature, α_{2C}-ARs are mobilized to the cell surface, which leads to their functional activation (40, 64). Interestingly, when expressed on the cell surface, α_{2C}-ARs are resistant to phosphorylation by G-protein-coupled receptor kinases (GRKs), resulting in reduced interaction with β-arrestin (65). Ultimately, this leads to decreased desensitization and internalization compared to other α_2-ARs (65). In our study, treatment of SW480 cells with UK 14,304 for 60 minutes did not decrease the expression of α_{2C}-ARs on the plasma membrane compared

to the control. Although the agonist, UK 14,304, was kept for 60 continuous minutes, the surface expression was not affected. This further validates the fact that α_{2C}-ARs are resistant to desensitization.

Activation of α_2-ARs did not cause a significant increase in proliferation of SW480 cells. This is in conflict with the literature in which α_2-AR agonists induced cancer cell proliferation. For instance, α_2-adrenoceptor agonists, clonidine and dexmedetomidine, significantly enhanced proliferation of the mouse mammary tumor cell line MC4-L5 (66). Moreover, rauwolscine, an α_2–AR antagonist, inhibited breast cancer proliferation (62). It has also been shown that the use of α_2-AR antagonist, yohimbine, inhibited the proliferation of pancreatic cell lines by deregulating signal transduction pathways, potentially involving the MAPK, and induced their apoptosis (61). This suggests that α_2-adrenoceptor antagonist can be used as an anticancer drug for apoptosis of pancreatic cells (61). Contrary to our findings, Schaak *et al*. showed that UK 14,304 enhances proliferation of Caco2-3B when placed in medium containing trace amounts of growth factors (0.5% FCS) (67). However, no significant difference in cellular proliferation was observed in either the absence or presence of high concentrations of FCS (67). This suggests that α_2-AR agonist may be inefficient in quiescent cells. As such, it acts on slowly growing cells, thus behaving as a co-mitogen on Caco2-3B cells (67). However, a growing body of evidence now suggests that decreased proliferation may be requisite for increased migration. As discussed below, activation of α_2-AR caused a significant and robust increase in migration that was notable as early as 2 hours. Further studies are needed to better delineate the interplay between proliferation and migration. It would be critical to determine if increased

proliferation may prevent such increased migration, or at least that decreased proliferation may actually promote migration of certain cancer cells (68).

Cancer cell migration is a crucial step in metastasis. Our results demonstrated that activation of α_{2C}-AR increases migration of colon cancer cells. Previous studies support the notion that the stimulation of adrenoceptors results in increased migratory capacity of colon cancer cells (50, 55, 56). The role of α_{2C}-ARs in CRC migration, however, remains poorly investigated. To the best of our knowledge, this is the first study showing that stimulation of α_{2C}-ARs increases migration.

This increase in migratory capacity was concomitant with increased Rho activation, which was evident by the translocation of Rho from cytoplasm to the membrane, as well as via a Rhotekin-binding activation assay. Rho GTPases act as molecular switches, cycling between the GTP-bound active form and GDP-bound inactive form. The Rho subfamily consists of RhoA, RhoB, and RhoC proteins (69). It has been shown that RhoA and RhoC expressions are upregulated in human tumors, whereas RhoB expression is often dowregulated (69). There is overwhelming evidence that RhoA is involved in virtually all stages of cancer progression, including cancer cell proliferation, migration, invasion, evasion from apoptosis, and metastases (69). Cancer cells invade tissues via single cell migratory mechanisms, which include mesenchymal and amoeboid migration, or by collective cell migration. In mesenchymal migration, the cells are elongated, and ECM degradation is essential for movement. In amoeboid migration, cells are rounded, and movement is driven by actomyosin-based cortical contraction (69). It has been established that RhoA is important for both mesenchymal and amoeboid migration (69). Moreover, RhoA can regulate the release of MMPs, which affects matrix remodeling and tumor

invasion (69). Some reports also suggest that RhoA, RhoC, and the downstream target ROCK are required for cancer cell extravasation (69). Increasing evidence demonstrates that Rho proteins modulate actin polymerization, depolymerization, and activity of actin-associated myosins, which are all essential for cell migration (70). A recently published study shows that blocking the expression of RhoA in LoVo colon cancer cells transfected with pshRNA-RhoA decreased the migratory and invasive capabilities (71). Additionally, it was shown that RhoA is linked to the invasion of blood vessels and lymph nodes in colorectal cancer samples (72). It is noteworthy that patients who expressed higher levels of RhoA proteins had a markedly poorer 5-year survival rate after surgery (72). This indicates that RhoA may be considered as a marker of poor prognosis in CRC and may serve as a potential target for CRC treatment (72).

The induced Rho activation could be mediated by increased oxidative stress since α_{2C}-AR stimulation increased NADPH oxidases activity and ROS production. A recently published study supports the notion that ROS in turn activates Rho/ROCK pathway (73). Reactive oxygen species are byproducts of aerobic metabolism. ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^·$), which contribute to both physiological and pathological conditions (74). Importantly, Hurd *et al*. established the role of ROS in activating signaling pathways involved in cell migration and invasion (74). Redox signaling is initiated by the generation of ROS via NADPH oxidases in response to a stimulus, such as growth factors including tumor necrosis factor-α (TNF-α), angiopoietin-1, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and extracellular matrix components. ROS then induces protein changes that are necessary for cellular migration. Once the stimulus is no longer present, ROS is

degraded, and homeostatic balance is restored (74, 75). Important targets of ROS during cell migration include modulators of receptor-tyrosine kinases (RTKs) such as protein kinase C (PKC) and protein tyrosine phosphatases (PTPs). Oxidation activates PKC, whereas it inactivates PTP. This allows RTK-induced activation of MAPK signaling pathway, which promotes tumor cell migration (76).

NADPH oxidases are a major source of ROS production. NADPH oxidase family consists of 7 members: Nox1 to Nox5 and the dual oxidases Duox1 and Duox2 (77). The major product of Nox4 and Duox is hydrogen peroxide (H_2O_2); yet, some studies report that Nox4 also generates superoxide anion. Nox1-Nox3 and Nox5 produce superoxide anion (O_2^-) (77). Accumulating evidence suggests that Nox proteins are overexpressed in a variety of cancer cells. Nox4 was shown to be highly expressed in gastric cancer (78) and lung cancer (79), whereas Nox1 was upregulated in pancreatic cancer (80) and colon cancer (80). Moreover, Nox5 appeared to be overexpressed in malignant melanomas (80). A recently published study established the role of Nox1 in colon cancer growth (81). Inhibition of Nox1 expression decreased ROS production and produced a G1 block in cell cycle progression. This block was associated with a decrease in phosphorylation of MAPK signaling pathway enzymes, including ERK1/2, and a decrease in cyclin D1 (81). Moreover, Nox1 inhibition lead to a decrease in blood vessel growth, VEGF expression, and migration of HT-29 colon cancer cells (81). This suggests that Nox1 may serve as a potential target in CRC treatment (81). Remarkably, Nox1 was also revealed to be overexpressed in colon cancer with activating mutations in K-Ras (82). Indeed, 80 percent of tumors with K-Ras mutations displayed a twofold or greater increase in Nox1 mRNA compared to normal tissues (77). It has also been demonstrated that NADPH oxidases play

a critical role in allowing increased glycolysis in cancer cells. Cancer cells typically switch from oxidative phosphorylation to glycolysis for generation of energy. NADPH oxidases generate NAD+ substrate which is essential for the glycolytic pathway (83). Blocking NADPH oxidases would halt the glycolytic pathway resulting in suppression of tumor growth (83). Our results show that reducing oxidative stress, either through a ROS scavenger or a NOX1/4 dual enzyme inhibitor, leads to a significant decrease in migration of SW480 cells.

This study established that α_{2C}-AR stimulation increases the phosphorylation of ERK1/2 and FAK, both of which are important for cell migration. Furthermore, we report here that inhibiting ERK1/2 or FAK significantly abolished α_{2C}-AR-induced migration. This is in line with several lines of evidence which established ERK1/2 phosphorylation is important for progression of colorectal cancer (84). Indeed, blocking ERK1/2 pathway decreases proliferation, metastasis, and angiogenesis of cancer cells (85). Moreover, RAS-RAF-MEK-ERK signaling pathway was shown to be over-activated in cancers with KRAS, NRAS, and BRAF mutations (86). FAK is a protein tyrosine kinase that is overexpressed in advanced cancers, including kidney, brain, lung, ovarian, cervical, pancreatic, colon, breast, and skin cancer (87). FAK expression was proved to be modulated via ERK1/2 signaling pathway (88). FAK activation promotes cancer progression and metastasis (87). Indeed, FAK inhibitors induces tumor cell apoptosis, reduced metastasis, and inhibited angiogenesis (87). In addition, inhibition of tyrosine phosphorylation at the Y397 site of FAK decreases colon cancer cell viability (89).

Invasion is also an important hallmark in cancer progression. Here, we established that α_{2C}-AR stimulation increased the production of matrix metalloproteinases, key

mediators of extracellular matrix (ECM) degradation. In addition, inhibiting JP1302 abolished the observed MMP-2 increase, clearly implicating the α_{2C}-AR subtype. It has been shown that MMPs play a critical role in tumor progression, including angiogenesis, invasion, and metastasis (90). Many CRC studies revealed a strong association between MMP-2 and MMP-9 levels and worse CRC prognosis (90). For instance, MMP-9 expression was higher in CRC patients having lymph node metastasis (90). Intriguingly, a study postulated that serum MMPs may be used as markers for CRC invasion with a greater sensitivity than carcinoembryonic antigen and CA19-9, two biomarkers currently used in clinical practice (91). Moreover, it has been established that upregulation of MMP-2 and MMP-9 may be mediated through the activation of MAPKK, such as ERK1/2 (92). This is in line with our findings where PD98095, a MAPKK inhibitor, decreases α_{2C}-AR- induced MMP-2 expression.

Angiogenesis is an essential step in cancer progression. Our findings demonstrate that α_{2C}-AR stimulation increases VEGF expression. Previous studies have shown that VEGF is highly expressed in approximately 50 % of CRC patients with minimal or no expression in normal colonic mucosa (93). Moreover, VEGF expression was higher in late CRC stages compared to early stages (93). Thus, VEGF may be considered a valuable prognostic factor in CRC (93). Another study revealed that Nox1 played a critical role in Ras-induced upregulation of VEGF expression and angiogenesis (94).

This study dissected the signaling pathway of α_{2C}-AR-induced CRC malignancy. Stimulation α_{2C}-AR activates ERK 1/2, FAK, and NOX, increases VEGF, MMP-2, and MMP-9 expression, and induces RhoA translocation to the membrane (Fig. 14). These

proteins together promote CRC malignancy by increasing migration, invasion, and angiogenesis.

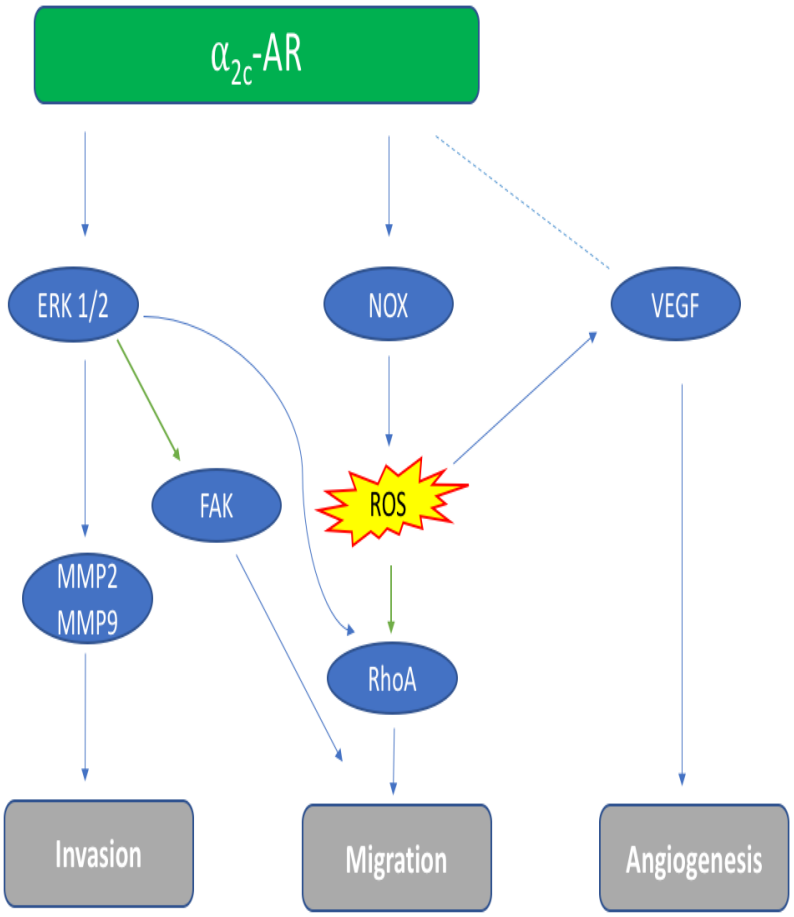

Figure 14: The proposed signaling pathway of α_{2C}-AR-induced CRC malignancy

CONCLUSION

This is the first study to reveal the importance of α_{2C}-AR in enhancing the malignant phenotype in colorectal cancer. Our findings show that stimulation of α_{2C}-AR increased migration, invasion, adhesion, transendothelial migration, and angiogenesis, thus potentiating the malignant phenotype of human colon cancer cells. Therefore, α_{2c}-AR may represent a novel target in the treatment of CRC.

LIMITATIONS AND FUTURE PERSPECTIVES

In this study, we only used SW480 human colon cancer cell line. Therefore, our ongoing and future studies will focus on the role of α_{2C}-ARs in promoting CRC malignancy in a battery of cell lines including ones that represent advanced stage colorectal cancer cell lines such as SW620, HT-29, LoVo, and HCT-116. However, it will be imperative that we first determine if α_{2C}-ARs are expressed in these cell lines.

We only used a pharmacological agonist of α_{2C}-AR. Future studies will also dissect the role of the natural agonist, epinephrine, in modulating the migratory capacity of these cells.

Another limitation is the fact that we did not study the role of α_{2C}-ARs in *in vivo* tumorigenesis. Therefore, an *in vivo* model will be established to mimic the biological process of CRC progression. Cells stably transfected with α_{2C}-ARs will be utilized in an orthotopic animal model of CRC. This would allow the prediction of clinical outcomes of α_{2C}-AR antagonists in CRC malignancy.

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