EXPLORING THE IMPACT OF ADENOSINE A2B RECEPTOR ON COLORECTAL CARCINOGENESIS

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Eylül Kılıç

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We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

Serkan İsmail Göktuna (Advisor)

Pınar Önal

Bala Gür Dedeoğlu

Approved for the Graduate School of Engineering and Science:

Orhan Arıkan

Director of the Graduate School

ABSTRACT

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Eylül Kılıç

M.Sc. in Molecular Biology and Genetics

Advisor: Serkan İsmail Göktuna

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Colorectal Cancer (CRC) is one of the most prevalent cancer types, with a highly heterogeneous mutation profile, and an incidence rate ranking third and lethality ranking second worldwide. Metastasis is an important factor in CRC mortality and recurrence, having been observed in a fourth of the patients diagnosed. While the effect of purinergic signaling components has been studied in cancer progression and metastasis, with varying contributions, research on the adenosine A2B receptor (A2BR) has been overlooked in the past due to its low affinity to adenosine. The intrinsic effects of A2BR are highly context-dependent, and research on how it may affect CRC progression and metastasis is limited. In this study, we used stable A2BR overexpressing CRC cell lines as well as A2BR selective antagonists to examine the role of A2BR in CRC progression. We observed a decrease in cancer growth upon A2BR overexpression, both *in vitro* and *in vivo*. Moreover, A2BR overexpression led to a lower migratory ability and shifted the EMT marker profile of the CRC cell lines. Once we pharmacologically inhibited the A2BR activity, we observed a significant increase in cell proliferation, migration, and invasion in the CRC cell lines as well as a shift towards a more mesenchymal EMT marker profile. Furthermore, our patient survival analyses indicate a higher overall and recurrence-free survival for CRC patients with high A2BR expression. Overall, these findings indicate a tumor-suppressive role for A2BR in CRC and that pharmacological inhibition may lead to a more aggressive phenotype.

Keywords: Colorectal Cancer, Purinergic Signaling, A2B Receptor

ÖZET

ADENOZİN A2B RESEPTÖRÜNÜN KOLOREKTAL KARSİNOGENEZ ÜZERİNDEKİ ETKİSİNİN ARAŞTIRILMASI

Eylül Kılıç

Moleküler Biyoloji ve Genetik, Yüksek Lisans

Tez Danışmanı: Serkan İsmail Göktuna

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Kolorektal kanser (KRK), heterojen bir mutasyon profiline sahiptir ve kanserler arasında vaka oranı açısından en yaygın üçüncü, ölüm oranı açısından da en yaygın ikinci sırada yer almaktadır. Metastaz, KRK'ye bağlı ölüm ve nüksün önemli bir faktörüdür ve tanı konulan hastaların dörtte birinde gözlemlenmektedir. Pürinerjik sinyal sistemi bileşenlerinin kanser gelişimi ve metastazdaki etkileri daha önce araştırılmış olsa da, adenozine olan düşük afinitesi nedeniyle adenozin A2B reseptörü (A2BR) üzerine yapılan çalışmalar geçmişte büyük ölçüde ihmal edilmiştir. A2BR'nin intrinsik etkileri oldukça bağlam bağımlıdır ve KRK ilerleyişi ve metastazı üzerindeki olası etkileri yeterince araştırılmamıştır. Bu çalışmada, A2BR'nin KRK ilerleyişindeki rolünü incelemek amacıyla stabil A2BR aşırı ifade eden KRK hücre hatları ve A2BR'ye özgü antagonistler kullanılmıştır. A2BR aşırı ifadesi hem *in vitro* hem de *in vivo* ortamlarda tümör büyümesinde azalmaya neden olmuştur. Ayrıca, A2BR aşırı ifadesi hücrelerin göç yeteneğinde düşüşe yol açmış ve KRK hücre hatlarının epitel-mezenkimal geçiş (EMT) belirteç profilini değiştirmiştir. A2BR aktivitesi farmakolojik olarak inhibe edildiğinde ise hücre çoğalması, göçü ve istilası anlamlı bir artış gözlemlenmiş; ayrıca EMT belirteç profilinde daha mezenkimal bir görünüme kayma saptanmıştır. Hasta sağkalım analizlerimiz ise A2BR ifadesi yüksek olan KRK hastalarında genel ve nükssüz sağkalımın daha yüksek olduğunu göstermektedir. Genel olarak bu bulgular, A2BR'nin KRK'de tümör baskılayıcı bir rol üstlendiğini ve farmakolojik inhibisyonunun daha agresif bir fenotipe yol açabileceğini ortaya koymaktadır.

Anahtar kelimeler: Kolorektal Kanser, Pürinerjik Sinyal Sistemi, A2B Reseptörü

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Chapter 1
Introduction

1.1 Colorectal Cancer

Colorectal cancer is the uncontrolled cell growth seen in the lower end of the digestive tract where the colon and the rectum are present (Yang et al., 2024). Out of the 36 various cancer types recorded by the International Agency for Research on Cancer, colorectal cancer (CRC) is estimated to have the third-highest incidence rate along with the second-highest mortality rate among both sexes worldwide, making CRC one of the most highly prevalent cancers worldwide (Roshandel et al., 2024; Sung et al., 2021). It is also estimated that CRC incidence may increase by more than 60% by the year 2040, further emphasizing the importance of research on CRC (Ionescu et al., 2023; Roshandel et al., 2024).

1.1.1 Risk Factors of Colorectal Cancer

CRC is a multifactorial disease, and thus, several factors may increase the risk of its development. These factors can be categorized as modifiable and non-modifiable risk factors (Dekker et al., 2019; Keum & Giovannucci, 2019; Roshandel et al., 2024; Simon, 2016).

1.1.1.1 Modifiable Risk Factors

An unhealthy diet, alcohol consumption, smoking, and obesity are among the most prevalent modifiable factors. A review constructed by Veettil et al. encompassing the findings of 45 meta-analyses has shown that CRC incidence has a very significant association with dietary factors such as consumption of red meat, alcohol, dietary fiber, calcium, and yogurt. While high consumption of red (more than 100g/day) and processed meat (more than 50g/day), as well as alcohol, increases the CRC risk, the opposite was observed with the high consumption of dietary fiber, calcium, and yogurt (McNabb et al., 2020; Veettil et al., 2021; Zhao et al., 2017). Alcohol, in particular, was observed to have a dose-depending risk association with CRC where heavy drinkers who intake more than 50g of ethanol per day were at the highest risk. The analysis shows that heavy drinkers have as

high as 21% higher risk of mortality as well as 52% higher risk of incidence of CRC compared to non-drinkers (Cai et al., 2014).

Smoking in the long term, which causes inhalation of about 70 known carcinogens, is another important dose-dependent factor that has shown an increased risk of colon cancer incidence up to 39% in male smokers and 20% in female smokers (Botteri et al., 2020; Cheng et al., 2015; Gram et al., 2020; Ionescu et al., 2023). Botteri et al. have also identified specific CRC phenotypes associated with smoking which are the CpG island methylator, and microsatellite instability phenotypes (Botteri et al., 2020).

Obesity is a known risk factor for various malignancies including CRC. The effect of obesity on CRC is the topic of many studies including meta-analyses and large-scale cohort studies. These studies report that there is a significant association between obesity with a high BMI and the development of CRC (Bull et al., 2020; Roshandel et al., 2024; Soltani et al., 2019). This association can be explained by higher levels of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-a), that are present in obese individuals which promote the growth of cancer cells (Ye et al., 2020). More so, obese individuals may have higher levels of bile acid which was shown to promote CRC by damaging the intestinal epithelium, inducing oxidative stress, and causing genomic instability (T. T. Nguyen et al., 2018)

1.1.1.2 Non-modifiable Risk Factors

Knowing non-modifiable risk factors is important for individuals to take preventative measures despite their inability to alter the factor itself. Age is the most prevalent risk factor for CRC incidence as more than 90% of cases are seen in individuals aged 50 and older. There also seems to be a higher incidence risk in men compared to women, especially in high-income countries where men are almost 1.5 times more likely to develop CRC (Sung et al., 2021).

Germline mutations to the APC gene and genes involved in DNA mismatch repair are some of the common genetic risk factors for CRC development, especially in individuals diagnosed younger than 50 years of age. While these germline mutations significantly increase the likelihood of developing CRC, they only make up approximately 5-10% of

CRC cases (Valle et al., 2019; Yurgelun et al., 2017). Furthermore, certain diseases such as ulcerative colitis (UC), cystic fibrosis (CF), and diabetes are known to significantly increase CRC risk (Kim & Scherer, 2021; Scott et al., 2020; Wang et al., 2021). Patients with CF are especially at risk, up to 6 times more likely to develop CRC, because of their loss of CFTR function (Scott et al., 2020).

Exposure to abdominopelvic radiation is a prominent risk factor for subsequent CRC development in childhood cancer survivors who received radiotherapy treatment for their primary cancer (Henderson et al., 2012). While a secondary CRC diagnosis is also common in prostate cancer (PC) survivors who received radiotherapy, patient analysis has shown that this may not be related to the radiation as their risk of developing CRC is not significantly higher than PC survivors who have not received radiotherapy (Ho et al., 2020).

The composition of the intestinal microbiota is also associated with CRC incidence. The microbiome can interact with intestinal cells and influence physiological processes such as metabolism and immune response, thus, may have a role in CRC initiation and progression. Research has shown that CRC patients have a higher presence of certain species such as *Fusobacterium nucleatum* and *Bacteroides fragilis* in their microbiome (Dai et al., 2018; Wong & Yu, 2019).

1.1.2 Mechanisms Driving Colorectal Cancer

CRC is a molecularly heterogeneous disease with multiple subtypes where several genetic and epigenetic alterations accumulate to drive its progression (H. T. Nguyen & Duong, 2018). More than 90% of CRCs are adenocarcinomas deriving from the glandular colonic and rectum epithelium, from which about 65% develop sporadically and the remaining 35% of the cases are affected by heritable factors. These heritable factors mainly include having a family history of colorectal cancer or being affected by hereditary cancer syndrome (Fearon, 2011; Fleming et al., 2012; Huang & Yang, 2022). Due to the molecular heterogeneity, CRC development may follow different molecular pathways, and understanding these molecular pathways is essential for the identification of biomarkers, shaping future research, and discovering treatment methods. The three major pathways

causing genomic instability that are currently known are the chromosomal instability (CIN) pathway, the microsatellite instability (MSI) pathway, and the CpG island methylator phenotype (CIMP) pathway (Pierantoni et al., 2024; Yang et al., 2024).

1.1.2.1 The CIN Pathway

The CIN pathway affects around 85% of all sporadic CRC cases which makes it the most common pathway observed in CRC (Huang & Yang, 2022). CIN is a type of genomic instability where a large portion or the entirety of the chromosome is being duplicated or deleted. This can lead to changes in the chromosome number, also known as aneuploidy, as well as the loss and gain of function of multiple tumor suppressor genes and oncogenes that have roles in cell cycle regulation, apoptosis, and proliferation (Pino & Chung, 2010). Just as the multistep genetic model of colorectal cancer suggests, CIN arises from multiple genetic hits that are necessary for driving the development of CRC (Fearon & Vogelstein, 1990). Several mutations in major genes are associated with the CIN pathway.

The initiation of CRC development starts with the adenomatous polyposis coli (APC) loss of function mutation. APC is an important negative regulator of the canonical Wnt/β-catenin pathway and its inactivation causes an increase in the activation of Wnt. This increase promotes the proliferation and metastatic progression of the cancerous cells (Stanczak et al., 2011; Yang et al., 2024). APC loss of function is of utmost importance for CRC development as the mutation has been detected in around 90% of the patients with CRC (Coppedè et al., 2014).

The initiation is followed by the gain of function mutation of the KRAS proto-oncogene. KRAS activation can lead to various effects such as the promotion of cell growth, migration, angiogenesis, and differentiation as well as inhibition of pro-apoptotic pathways through its downstream effectors. KRAS mutations are found in around 40% of CRC and are generally associated with an increase in tumor aggressiveness (Dinu et al., 2014; Neumann et al., 2009).

Loss of heterozygosity (LOH) in chromosome 18q follows the KRAS activation. This deletion inactivates multiple tumor suppressor genes such as Cables, DCC, SMAD2, and SMAD4. Previous studies show that up to 70% of chromosome 18q LOH occurring in CRC shows loss of Cables expression which leads to an increase in cell proliferation and impaired differentiation. While DCC and SMAD2/4 genes are also located in 18q, surprisingly, mutations in these genes in CRC are much less significant at only up to 6-10% of cases (Kirley et al., 2005; Park et al., 2007; Yang et al., 2024).

The loss of function mutation to the TP53 tumor suppressor gene on chromosome 17p is the last step of the CIN pathway. TP53 encodes the p53 protein which typically acts as a transcription factor for a variety of genes; however, it can also interact with effectors such as p21 which will lead to the cell cycle arrest via inhibition of cyclin-dependent kinases. Abnormal p53 function is observed in many human cancers including CRC, with this dysfunction seen in up to 50-75% of the cases (Liebl & Hofmann, 2021). P53 is also involved in the regulation of many essential cellular responses such as apoptosis, angiogenesis, differentiation, cell cycle regulation, cell migration, and immune response (Pino & Chung, 2010; Vogelstein & Kinzler, 2004). Mutation to the TP53 gene typically is a sign of the transition from adenoma to carcinoma in CRC (Baker et al., 1989; H. T. Nguyen & Duong, 2018).

1.1.2.2 The MSI Pathway

Microsatellites are the short repeating DNA sequences found throughout the human genome and due to their repeating nature, they are at high risk for mutations that will, if not corrected, cause microsatellite instability (MSI) (Nojadeh et al., 2018). Under normal conditions, the DNA mismatch repair (MMR) mechanism is responsible for correcting the errors accruing during DNA replication and recombination such as insertions, deletions, and addition of incorrect bases. Thus, MMR is also responsible for correcting such mutations happening in the microsatellite regions of the DNA, meaning the MMR mechanism must be disturbed in order for the mutations to accumulate and cause MSI (Battaglin et al., 2018).

While MSI is a well-established molecular pathway in CRC, only around 15% of CRC cases arise from MSI. Lynch syndrome, also known as hereditary nonpolyposis colorectal cancer (HNPCC), is a heritable syndrome that includes germline mutations to the genes essential for the MMR mechanism including the mutL homolog 1 (MLH1) gene (Battaglin et al., 2018). While individuals identified with Lynch syndrome are at a much higher risk of developing CRC, they only make up less than 5% of the overall CRC cases, and only about a quarter of the CRC is derived from MSI (Hampel et al., 2005). The rest of the MSI-associated CRCs are sporadic in nature and generally result from the hypermethylation to the MLH1 promoter as seen in the CIMP phenotype, epigenetically inactivating MLH1 and disturbing the MMR mechanism (Shaikh et al., 2024; Sinicrope, 2010; Yang et al., 2024).

1.1.2.3 The CIMP Pathway

Epigenetic regulation controls gene expression without altering the DNA sequence and plays a critical role in maintaining genomic stability in normal tissues. DNA methylation is one such epigenetic mechanism that is well-studied in CRC and analyses show that about 17% of sporadic CRC cases are classified as CIMP tumors(Lao & Grady, 2011; Ogino, 2006).

In normal tissues, most CpG sites are methylated except for the generally unmethylated CpG islands which are parts of the genome that are about 200-500 bases in length and contain more than 50% of GC content. These CpG islands are generally located in the promoter regions of genes and their hypermethylation can silence the genes they are associated with. This gene silencing caused by CpG island hypermethylation is also a hallmark of CIMP-positive CRC (Hughes et al., 2012; Lao & Grady, 2011; Malki et al., 2020; Yang et al., 2024). CIMP-positive CRCs are also frequently associated with BRAF and KRAS mutations, as well as, microsatellite instability (Mojarad et al., 2013).

1.2 Metastasis in Colorectal Cancer

Metastasis is the ability of the cancer cells to arise in tissues and organs different than the one they originated from. In order to do this, the cancer cells must go through a series of biological changes which will give them the ability to invade tissues, survive dissemination through the body, and colonize distant organs. Metastatic cancers tend to be much more

lethal for the patients compared to harboring only primary tumors due to this dissemination. (Gerstberger et al., 2023).

Metastatic CRC (mCRC) is diagnosed in up to 25 % of patients with CRC, from which 30% of the patients are likely to experience a relapse due to metastasis after the initial treatment. Despite the efforts to cumulate novel treatment methods to resist mCRC, it remains a deadly disease where the 5-year survival rate is only about 14% (Rumpold et al., 2020). There are many factors both within the cancer cells and in the surrounding tumor microenvironment (TME) that help drive the mechanisms for the metastasis of CRC.

1.2.1 Genetic Mutations

TP53 is an essential tumor suppressor gene that harbors mutations most frequently in many human cancers. mCRC is one of these cancers where 60% of the patients have some form of p53 mutations, furthermore, the presence of mutations leading to both GOF and LOF of p53 was linked to worse survival compared to CRC patients with wild-type p53 expression (Nagao et al., 2022; Shin et al., 2023).

As mentioned, CRC may harbor LOF and/or GOF mutations for the TP53 gene which may influence the outcome of the disease. LOF mutations in TP53 promote carcinogenesis as expected by its tumor-suppressive nature via promoting cell migration, invasion, and metastasis. The missense GOF mutations, however, promote carcinogenesis by taking advantage of the transcription factor properties of p53 via the interaction of mutant p53 with the promoters of CRC stem cell markers such as LGR5, CD44, and ALDH1 (Solomon et al., 2018).

There are many studies investigating the molecular makeup of mCRC to determine which mutations are aberrant in order to better understand the disease and develop therapies accordingly. One of these studies performed mass sequencing of more than 900 different samples taken from patient tumors and found four genes that were recurrently mutated: APC, TP53, KRAS, and PIK3CA(Lee et al., 2021; Lu et al., 2023).

1.2.2 Epithelial-Mesenchymal Transition (EMT)

The epithelial-mesenchymal transition (EMT) is the reprogramming of epithelial cells to take on a mesenchymal phenotype. Under normal physiological conditions, this process occurs during embryonic development and tissue repair, however, cancer cells may also take advantage of this mechanism to develop a more aggressive characteristic. EMT helps cancer cells invade neighboring tissues, survive in circulation, and colonize distant organs, thus promoting metastasis (Lu et al., 2023; V. Mittal, 2018). A complete EMT is not necessary for cancer cells to uphold these functions, in fact, cancer cells generally favor partial or hybrid EMT for its greater adaptability. Often in mCRC, cancer cells exist in a hybrid EMT state where epithelial and mesenchymal cells cluster together for a more successful seeding into distant organs like the liver (Mizukoshi et al., 2020; Sinha et al., 2020). After this, the cells must go through mesenchymal-epithelial transition (MET) in order to reverse the EMT process and further develop in the metastasized organ (Lu et al., 2023).

The EMT process requires the collaboration of various molecular signaling pathways and transcriptional regulators (Vu & Datta, 2017). One of the most recognizable features of EMT is the changes in the regulation of epithelial and mesenchymal markers. Epithelial markers such as E-cadherin, ZO-1, and occludin show a decrease in expression whereas the mesenchymal markers such as N-cadherin, vimentin, and fibronectin show an increase in expression when EMT is initiated (Loh et al., 2019; Lu et al., 2023). Upon going through these changes, the morphology of the previously polygonal epithelial cells morphs into one resembling mesenchymal cells with an elongated spindle-like appearance (V. Mittal, 2018).

1.2.2.1 Transcription Factors

There are three core transcription factor (TF) families involved in EMT which are the Snail, Twist, and Zeb families of TFs. These TFs control the expression of epithelial and mesenchymal genes and promote metastasis.

SNAIL has many roles in the EMT of mCRC cells including the regulation of other EMT TFs such as SLUG, ZEB1, and TWIST1. SNAIL expression is directly correlated with

canonical WNT signaling and its interaction with B-catenin also promotes the WNT/b-catenin signaling, leading to a positive feedback loop that favors EMT. Some characteristics of SNAIL in terms of EMT are the suppression of the CDH1 gene that encodes E-cadherin, which is an important epithelial junction protein, and the promotion of tumorigenicity and cancer stem cell characteristics of CRC cells via upregulating the IL-8 expression (Bazzichetto et al., 2022; Lu et al., 2023; Wang et al., 2013).

TWIST1 is another important TF involved in mCRC and is typically expressed in the tumor stroma. TWIST1 also has a role in suppressing E-cadherin expression and promoting the expression of mesenchymal markers like N-cadherin and fibronectin (Vu & Datta, 2017). TWIST1 expression can be regulated by multiple factors, one of which is by its upstream regulator SNAIL as previously mentioned. Research has shown that TWIST1 expression is also related to epigenetic modifications and hypoxic conditions. Methylation of the TWIST1 and TWIST2 promoters in the stroma was observed to be an alternative method to regulate their expression and lack of this methylation caused CRC to have a higher-grade budding phenotype. Another study has shown that hypoxia-inducible factor-1α (HIF1A) also induces TWIST1 expression under a hypoxic microenvironment, promoting EMT metastasis of the cancer cells (Galván et al., 2014; Lu et al., 2023; Sun et al., 2009).

ZEB family includes ZEB1 and ZEB2 transcription factors that are associated with mesenchymal differentiation and advancement of CRC tumors. ZEB1 is a transcriptional repressor of the miR-141 and miR-200c, which are members of the microRNA-200 family. This noncoding RNA family can induce epithelial differentiation of CRC cells, upregulating E-cadherin expression and thus inhibiting the migratory and invasion ability of CRC. ZEB1 inhibits the transcription of these noncoding RNAs and thus promotes the mesenchymal differentiation of CRC cells. While ZEB1 has more of a contribution to the initiation of metastasis, ZEB2 is linked to the advancement of tumors as it mainly promotes invasion. ZEB2 upregulates the matrix metalloproteinase (MMP) synthesis which will help with the invasion of CRC cells by degrading matrix membranes (Lu et al., 2023; Parfenyev et al., 2025). Expression of ZEB2 in CRC is linked to poor prognosis and a higher risk of recurrence of the cancer (Sreekumar et al., 2018).

1.3 Purinergic Signaling

Purinergic signaling is a complex network that is centered around purine nucleosides and nucleotides, the most predominant of which are adenosine and adenosine triphosphate (ATP). These molecules bind to specific plasma membrane receptors called purinergic receptors to exert their effects and influence various important cellular processes such as apoptosis, proliferation, migration, and differentiation. The main components of purinergic signaling are extracellular purines, purinergic receptors, and nucleotide metabolizing enzymes (Burnstock & Verkhratsky, 2009; Huang et al., 2021).

Among extracellular purines, ATP is one such essential extracellular messenger that typically exists in very low concentrations, at the nanomolar range, in the extracellular space while being stored intracellularly at much higher concentrations ranging from 5 to 10 mmol/L (Burnstock, 2006; Di Virgilio & Adinolfi, 2017). This major difference in concentration allows ATP to be released rapidly in response to various stimuli such as hypoxia, inflammation, mechanical stress, and cellular damage (Burnstock, 2006; Eltzschig et al., 2012; Scheuplein et al., 2009). Furthermore, the high water solubility and quick degradation of ATP by ectonucleotidases allow ATP to be tightly regulated to initiate, propagate, and terminate the signals efficiently and minimize the risk of receptor desensitization in physiological conditions. However, in pathophysiological conditions, such as within the tumor microenvironment, extracellular ATP levels can increase significantly to reach hundreds of micromolar compared to the nanomolar levels in the healthy tissue (Di Virgilio & Adinolfi, 2017). Unlike ATP, extracellular adenosine is not released from the intracellular adenosine storage. The main source of extracellular adenosine is generated by the sequential hydrolysis of the nucleotides ATP, ADP, and AMP by ectonucleotidases (Longhi et al., 2013; Zimmermann, 2000). The extracellular adenosine interacts with the P1 receptors (P1Rs) on the plasma membrane and the signal is terminated generally via the transport of the adenosine to the intracellular space by the equilibrative nucleoside transporters (ENTs). The adenosine in the intracellular space is then degraded by adenosine deaminase (ADA) into inosine which is then degraded to hypoxantine by purine nucleoside phosphorylase (PNP) (Di Virgilio & Adinolfi, 2017; Eltzschig et al., 2012).

Purinergic receptors are categorized into two subfamilies which are referred to as P1 and P2 receptors. P1Rs are G-protein-coupled receptors (GPCRs) that bind adenosine as a ligand. P1Rs include the four subtypes of A1, A2A, A2B, and A3 receptors (Fredholm et al., 2001). While A1, A2A, and A3 receptors are able to activate under physiological adenosine levels (nM) due to their high affinity, A2B receptors (A2BRs) require a higher extracellular adenosine concentration (μM) that is generally found in pathophysiological conditions in order to activate. The expression of the adenosine receptors can differ according to the tissue type. While the A1R is mainly expressed in the nervous system, the remaining subtypes can be found in various tissues including but not limited to the nervous system, colon, spleen, and testis (Sachdeva & Gupta, 2013). One of the main functions of the adenosine receptors is to couple with different G proteins in order to control the adenylate cyclase (AC) activity and subsequently influence the cytoplasmic cAMP levels. The A1 and A3 receptors decrease cAMP by coupling with the Gi protein and inhibiting AC activity whereas A2A and A2B receptors promote the activity of AC by coupling to the Gs protein and hence increase the cAMP levels (Sachdeva & Gupta, 2013). Other effectors such as phospholipase C (PLC) and mitogen-activated protein kinase (MAPK) are also regulated by adenosine receptors (Huang et al., 2021). It is suggested that the P1Rs are able to form heteromeric complexes that function in a bidirectional manner. Navarro et al. proposed that A1-A2A receptors can form a heteromer, being able to couple with both the Gi and Gs proteins, thus, being able to regulate cAMP levels in either direction. The effect of the complex activation will switch depending on the adenosine concentration, low concentrations activate the A1 subunit while higher concentrations activate the A2A subunit which will determine the direction of the outcome (Navarro et al., 2016).

The P2 receptors (P2Rs) are categorized into two subfamilies consisting of P2X and P2Y receptors. There are seven P2X receptors ($P2X_{1-7}$) which are ATP-gated homo/heterotrimeric ion channels and eight P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11-14}$) which are metabotropic GPCRs (Burnstock, 2018). Out of the homotrimeric P2XR, P2X1 and P2X3 have the highest affinity for ATP but desensitize rapidly while P2X2 and P2X5 have a lower affinity and desensitize slower, P2X4 is similar to P2X2 and P2X5 in terms of affinity but desensitize considerably slower. Lastly, P2X7 is the least sensitive to ATP and shows virtually no desensitization from prolonged activation while P2X6 cannot

form homotrimers (Schmid & Evans, 2019). Heterotrimeric P2XRs can take on various combinations such as P2X1/2 with one P2X1 and two P2X2 subunits and P2X2/3/6 with three different subunits and so on. The high variety of combinations in P2X receptors allows them to take on various functions within different tissues (Antonio et al., 2014; Coddou et al., 2011). While the P2X receptors are only activated by ATP, P2Y receptors can be activated by various nucleotides such as ATP, ADP, UTP, and UDP (Burnstock & Verkhratsky, 2009; Valera et al., 1994). P2YRs have varying sensitivity for the different nucleotides. P2Y1, for example, is more sensitive to ADP than ATP while P2Y2 is sensitive to ATP and UTP but has little affinity with ADP. More so, P2Y12 and P2Y13 have high affinity for ADP as their preferred agonist, P2Y4 for UTP, P2Y6 for UDP, P2Y11 for ATP, and P2Y14 for UDP-glucose respectfully (Abbracchio et al., 2006). Similarly with P1Rs, P2YRs couple with G proteins to regulate AC and PLC, through this coupling P2Yrs can control secondary messengers such as Ca2+, cAMP, and Inositol 1,4,5-triphosphatase (InsP3). P2Y1, P2Y2, P2Y4, and P2Y6 couple with Gq; P2Y12, P2Y13, and P2Y14 couple with Gi and P2Y11 couple with both Gq and Gs proteins (Di Virgilio et al., 2018). P2YRs take part in the regulation of many physiological functions including cell proliferation and embryonic neurodevelopment, thus, their dysfunction can influence the occurrence of various pathophysiological conditions such as neurodegeneration, and cancer (Huang et al., 2021).

Nucleotide metabolizing enzymes are the final core components of purinergic signaling and are essential for the regulation of the extracellular purine concentration which allows for a more precise control of the length and intensity of purinergic signals. While there are many enzymes in this category, the most essential ones for purinergic signaling are ectonucleotide triphosphate diphosphohydrolase-1 (CD39), ecto-5′-nucleotidase (CD73), and ADA due to their prominence in regulating ATP and adenosine driven signals (Le et al., 2019). CD39 is a plasma membrane bound enzyme that is responsible for the hydrolysis of ATP to ADP as well as ADP to AMP. CD73 is then responsible for the hydrolysis of AMP further into adenosine. This sequential activation is an essential pathway for producing extracellular adenosine in healthy tissues and is commonly exploited by cancer cells in order to elevate adenosine levels in the TME for its immunosuppressive properties (Di Virgilio & Adinolfi, 2017; Roliano et al., 2022). After the extracellular adenosine

interacts with P1Rs, it is transported into the intracellular compartment from which ADA can further metabolize a majority of it to inosine or it can be phosphorylated back into AMP by the adenosine kinase (Le et al., 2019).

Purinergic signaling allows for the immune cells in the TME to communicate with cancer cells and mediates the relationship between inflammatory responses and tumor growth. This interaction between the TME and the cancer cells plays an important role in the progression of various cancers including CRC (Di Virgilio, 2012; Roliano et al., 2022).

1.3.1 A2B Adenosine Receptor (A2BR) Signaling

As previously mentioned, A2BR is seen as the low affinity counterpart to A2AR within the adenosine receptors. Due to its low affinity, it is believed that in physiological conditions where the adenosine concentration is in the nanomolar levels, A2BR does not play a significant role compared to A2AR and that A2BR signaling shows its relevance in pathophysiological conditions, such as inflammation and cancer, where the extracellular adenosine levels are elevated to micromolar levels (Feoktistov & Biaggioni, 2011; Z.-G. Gao, Haddad, et al., 2025; Lan et al., 2018).

While the most well characterized signaling of A2BR is through the Gs protein coupling, it was observed that certain cell types such as HEK293 can mediate A2BR signaling through Gi coupling as well as fewer studies indicating coupling with Gq/11 proteins (Z.-G. Gao et al., 2018; Linden et al., 1999; Voss et al., 2022).

1.3.1.1 cAMP-PKA Signaling

A2BR controls the cAMP levels through its interaction with the Gs protein. Once A2BR is activated, it couples with the Gs protein and subsequently activates AC which converts ATP into cAMP and increases the cAMP levels. This increase leads to the activation of protein kinase A (PKA). Darashchonak et al. has found that this PKA activation through A2BR leads to the phosphorylation of cAMP response element binding protein (CREB) in trophoblast cells and induces proliferation and invasion (Darashchonak et al., 2014). Research shows that A2BR mediated cAMP-PKA signaling also has a role in promoting chloride excretion by activating CFTR (Rajagopal & Pao, 2010; Watson et al., 2016).

1.3.1.2 Calcium Signaling

A study by Gao et al. recently uncovered that A2BR activation can mediate calcium mobilization in a cell-type dependent manner. They showed that while the calcium mobilization occurs through Gq/11 protein coupling in HEK293 cell line, T24 cell line shows A2BR Gi coupling, both leading to PLCβ activation and subsequent calcium mobilization (Z. G. Gao et al., 2018; Z.-G. Gao, Gao, et al., 2025).

1.3.1.3 ERK1/2 Signaling

A2BR mediated ERK1/2 signaling is highly cell type dependent due to the ability of the A2BR to couple with different G proteins and their interactions with the downstream effectors depending on the cell type. HEK293, for example, shows a synergistic effect upon coupling of the Gi and Gs proteins which both promote ERK1/2 activation. Gi coupling does this by the PLC-PKC pathway whereas Gs does it mainly by activation of exchange protein directly activated by cyclic AMP (EPACs) through increase in the cAMP levels. Unlike in HEK293, T24 cell line shows an opposing interaction where Gi proteins activate ERK1/2 through PKC and Gs proteins suppress ERK1/2 activation through PKA and EPAC2 but not EPAC1, which also indicates that EPAC proteins show different effects on ERK1/2 activation on different cell lines (Z. G. Gao et al., 2018; Z.-G. Gao, Haddad, et al., 2025).

1.4 A2BR in Cancer

Over the years, there have been many studies regarding the role of A2BR in different types of cancer. Research shows that the expression of A2BR is higher in cancer tissues compared to the healthy tissues. In breast cancer, especially the triple negative breast cancer (TNBC) subtype, it was found that high A2BR expression led to a poor prognosis and patient survival. Similar outcomes were seen in bladder cancer, however, there seems to be contradictory findings in ovarian cancer where high A2BR expression indicates a good prognosis in early stages, suggesting a context-dependent role (Campos-Contreras et al., 2022; D. Mittal et al., 2016). Pro-proliferative and migratory abilities of cancer cells in various cancers such as melanoma, prostate cancer, and bladder cancer were reported to be amplified upon A2BR activity. These effects are linked to A2BR signaling activating

important proliferative pathways such as MAPK, cAMP-EPAC, and PI3K-Akt (Fang & Olah, 2007; Z.-G. Gao & Jacobson, 2019; Shen et al., 2018).

It has been shown that A2BR activation has a pro-metastatic role in animal models of melanoma as well as breast cancer where lung metastasis was significantly amplified upon activation. Furthermore, both genetic and pharmacological inhibition of A2BR was reported to hinder the lung metastasis induced by A2BR activation in these cancers (Cekic et al., 2012; D. Mittal et al., 2016). In CRC, Ma et al. has shown that A2BR expression is higher in the metastatic SW620 cell line compared to its non-metastatic counterpart, SW480, indicating that A2BR may have a role in metastasis in CRC (Ma et al., 2010).

A2BR can also influence cancer progression through angiogenesis and immune suppression. A2BR is highly expressed in endothelial cells and its activation can stimulate the production of vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), which are key angiogenic factors in cancer progression (Feoktistov et al., 2002; Merighi et al., 2009). A2BR activation reduces the expression of the MHC-II transactivator, essential for CD4+ T cell anti-tumor responses, which was shown to induce a more metastatic CRC phenotype (Warabi et al., 2000).

1.5 Aim of the Study

The adenosine A2B receptor has been shown to affect varying signaling pathways in different cell lines and tissues upon its activation. While the role of A2BR has been elucidated as oncogenic in certain cancer types, sufficient research has not been conducted on intrinsic effects of A2BR on colorectal cancer to come to a conclusion. Since the A2BR signaling is highly context dependent, we wanted to investigate the role of A2BR in CRC progression, with a focus on cell proliferation, tumor growth, migration, invasion, and epithelial-mesenchymal transition. We utilized stable A2BR overexpression CRC cell lines and pharmacological inhibition of A2BR by selective antagonists to characterize its effects by various *in vitro* assays and an *in vivo* xenograft model.

Chapter 2
Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Table 1. The chemicals and reagents used in the study

Product Name	Catalog Number	Company
Glycine	927-032-1000-1KG	IsoLab
Sodium Chloride	969.033.1000-1KG	IsoLab
Tween 20	39796.51	Serva
Trizma HCl	T-3253	Sigma-Aldrich
Trizma Base	T1503-1KG	Sigma-Aldrich
Dodecyl Sulfate Sodium Salt	8.22050.1000	Merck
Methanol	947.046.2500-2.5L	IsoLab
Sodium Azide	S2002-5G	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sc-2323	ChemCruz
Milk Powder	-	Pinar
Ponceau S	A1405,0025	AppliChem
β-Mercaptoethanol	A1108,0250	AppliChem
Bromophenol Blue	0449-100G	Amresco
30% Acrylamide/Bis Solution 29:1	1610156	Bio-Rad
Ammonium Persulfate (APS)	A2941,0500	AppliChem
Ethanol Absolute	920.026.2500-2.5L	IsoLab
Ethylenedinitrilotetraacetic acid disodium salt dihydrate (EDTA)	1.08421.1000	Merck
TEMED	A1148,0100	AppliChem
Glycerol 85%	927.023.1000-1L	IsoLab
Clarity Western ECL Substrate	1705060	Bio-Rad

SuperSignal West Femto Maximum Sensitivity Substrate	34095	Thermo Scientific
PageRuler Prestained Protein Ladder	26616	Thermo Scientific
cOmplete Protease inhibitor cocktail tablets	11697498001	Roche
Sodium Fluoride	1.06449.0250	Merck
Triton X-100	A1388,0500	AppliChem
Sodium Orthovanadate	S6508-50G	Sigma-Aldrich
PMSF	A0999,0025	AppliChem
EGTA	11290.01	Serva
Tetrasodium Pyrophosphate	P-8010	Sigma-Aldrich
LightCycler 480 SYBR Green I Master	04887352001	Roche
DMSO	M.6323.0100	Genaxxon Bioscience
Propidium Iodide	P4170	Sigma-Aldrich
SuperSignal West Pico PLUS	34577	Thermo Scientific

2.1.2 Kits

Table 2. The kits used in the study

Product Name	**Catalog Number**	**Company**
Pierce BCA Protein Assay Kit	23227	Thermo Scientific
E.Z.N.A.® Total RNA Kit I	R6834	Omega Bio-Tek
iScript cDNA Synthesis Kit	1708891	Bio-Rad

2.1.3 Cell Culture

Table 3. The cell culture media and solutions used in the study

Product Name	Catalog Number	Company
DMEM Low Glucose (1 g/l), with L-Glutamine, with Sodium Pyruvate	DMEM-LPA	Capricorn Scientific
DMEM High Glucose, with Sodium Pyruvate	41966029	Thermo Scientific
Mccoy´s 5A w/ LGlutamine	L0210-500	Biowest
Newborn Calf Serum	NCS-1A	Capricorn Scientific
Trypsine-EDTA 10X	X0930-100	Biowest
Puromycine	Ant-pr-1	Invivogen
PSB 603	3198	Tocris
MRS 1754	2752	Tocris

2.1.4 Buffers

Table 4. Buffers used in the study and their formula

Buffer Name	Formula
10X Phosphate-Buffered Saline (PBS)	17.02 g Na_2HPO_4, 80 g NaCl, 2 g KCl, 2 g KH_2PO_4, ddH2O up to 1 L, pH= 6.87
2X RIPA Cell Lysis Buffer	25 ml 1M Hepes, 30 ml 5M NaCl, 5 ml Triton-X100, 100 ml Glycerol, 340 ml ddH2O
1X RIPA Cell Lysis Buffer	5 ml 2X RIPA Cell Lysis Buffer, 500 µl NaF, 500 µl Na_3VO_4, 500 µl β -Glycerophosphate, 500 µl cOmplete protease inhibitor, ddH2O up to 10 ml
2X Tissue Lysis Buffer	100 mM Tris-HCl, 500 mM NaCl, 0.2 mM EGTA, 2% Triton-X, 1% NP40, 20% Glycerol, 0.5 mM Tetrasodium Pyrophosphate
1X Tissue Lysis Buffer	5 ml 2X Tissue lysis buffer, 500 µl NaF, 500 µl Na_3VO_4, 100 µl β-glycerophosphate, 10 µl DTT 1M, 200 µl PMSF 0.1M, cOmplete protease inhibitor tablet, ddH2O up to 10 ml
10X TBST	24.2 g Tris-Base, 80 g NaCl, pH=7.6, 20ml Tween, ddH2O up to 1L

10X TGS	144 g Glycine, 30.2 g Tris-Base, 10 g SDS, ddH2O up to 1L
10X Transfer Buffer	144 g Glycine, 30.2 g Tris-Base, ddH2O up to 1L
1X Transfer Buffer	100 ml 10X Transfer Buffer, 200 ml Methanol, 700 ml ddH2O
0.5% Crystal Violet	0.5 g Crystal Violet, 25 ml methanol, 75 ml ddH2O

2.1.5 Antibodies

Table 5. Antibodies used in the study

Product Name	Catalog Number	Company
A2BR	AAR-003	Alomone Labs
E-Cadherin	Sc-8426	Santa Cruz
N-Cadherin	14215	Cell Signaling
ZO-1	13663	Cell Signaling
ZEB-1	Sc-515797	Santa Cruz
Integrin-α5	4705	Cell Signaling
α-SMA	Ab5684	Abcam
Phospho-ERK	4370	Cell Signaling
β-Actin	3700	Cell Signaling
GAPDH	Sc-47724	Santa Cruz
Anti-Rabbit IgG, HRP-linked Antibody	7074	Cell Signaling
Anti-Mouse IgG, HRP-linked Antibody	7076	Cell Signaling

2.1.6 Primers

Table 6. qPCR primers used in the study

Primer Name	Sequence
A2BR-F	GCCAATTCAGTTGTCAATCCC
A2BR-R	TCTGCTTGGCAGAGAAGATAC
GAPDH-F	GCCCAATACGACCAAATCC
GAPDH-R	AGCCACATCGCTCAGACAC

2.2 Methods

2.2.1 Cell Maintenance

All cell lines used in the study were cultured in an incubator maintaining 5% CO_2 levels at 37°C. HT29 cells were grown in DMEM high glucose medium supplemented with 1% L-glutamine and 10% Newborn Calf Serum (NBCS). HCT116 cells were grown in McCoy's 5A medium supplemented with 1% L-glutamine and 10% Newborn Calf Serum (NBCS). The medium was renewed every 2-3 days and cells were passaged as necessary.

2.2.2 A2BR-Selective Antagonist Treatment

The concentrations used for the antagonists were decided from previous research conducted. The antagonists were added into the medium of the cells upon seeding and renewed with every medium change. The control groups were treated with equal volumes of DMSO to the antagonists.

2.2.3 Cell Lysis

Cells were lysed to acquire protein samples of the various cell lines, with or without treatment. The medium was discarded, and cells were washed twice with 1X PBS to remove the remaining medium and dead cells. Additional 1X PBS was added to the plate and the cells were collected with the help of a scraper. After the cells were collected, they

were centrifuged at max speed for 10 minutes at +4°C and the supernatant was removed. The cells were resuspended in an appropriate amount of RIPA Cell Lysis Buffer depending on the cell number. The samples were incubated on ice for 30 minutes and centrifuged at max speed for 30 minutes at +4°C. The supernatant was then collected to be used in subsequent experiments.

2.2.4 Tissue Lysis

1X Tissue Lysis Buffer was added onto the tissue samples and the sample was homogenized in the buffer using micro pestles. The homogenized sample was then incubated on ice for 15 minutes followed by 30 minutes of max speed centrifugation at +4°C. The supernatant containing tissue lysate was collected to use in the following experiments.

2.2.5 Clonogenic Assay

The cells were seeded in the middle two wells of a 6-well plate. Around 500 cells per well were seeded to observe single colonies and the remaining wells were filled with ddH_2O. The plate was incubated for approximately 2 weeks with frequent medium changes as needed. Once the colonies reached an adequate size without touching one another, the experiment was terminated by fixing and staining the cells. The cells were first washed with 1X PBS followed by the fixation of the cells by adding ice-cold Methanol and incubating the plate at -20°C for 10 minutes. Afterward, the methanol was discarded and the surface of the wells was covered with 0.5% Crystal Violet and incubated for 30 minutes at room temperature. The crystal violet was then removed, and the wells were washed multiple times with dH_2O until no stain remained. After the plates were left to dry overnight, the colony count was analyzed using the OpenCPU software.

2.2.6 Wound Healing Assay

For this assay, the cells were seeded into a 24-well plate to have ~95% confluency after being incubated overnight. The following day, the cells were scratched with a 200ul pipette tip to create a uniform “wound” in order to observe the closure. To get rid of the now unadhered cells, the wells were thoroughly washed with 1X PBS and the medium was replaced with one containing only 1 % serum. The images of the scratch were taken at 0H

and at 24h intervals until the gap closed adequately. The area of the scratched surface at 0H and at the time of termination were quantified in ImageJ using the Wound Healing Size Tool plugin and was used to determine the percentage of the gap closed.

2.2.7 Cell Line-Derived Xenograft

6-8 weeks old nude athymic mice were used for the xenograft model. The mice used in the study were provided by the Bilkent University MBG department animal facility with the permission of the local ethics committee. The cells were grown to accommodate injection of 7 million cells on both flanks of the six mice for the two experimental groups. The cells were resuspended in 1X PBS and injected subcutaneously into both flanks while the mice were under anesthesia. The tumors were measured with a caliper approximately once every two days to determine tumor volume using the Length x $Width^2$ formula. Once the tumor volumes exceeded $1500mm^3$, the mice were sacrificed, and tumor samples were collected.

2.2.8 RNA Isolation

The cells were collected in 1X PBS as previously explained. Cell lysis and RNA isolation were performed with and according to the E.Z.N.A.® Total RNA Kit I manufacturer instructions. The RNA samples were then stored at -80°C.

2.2.9 cDNA Synthesis

The cDNA synthesis was performed using the iScript cDNA Synthesis Kit. 1000ng of RNA template was used and the protocol of the manufacturer was followed accordingly. This protocol required 4µl of 5x iScript Reaction Mix, 1µl of iScript Reverse Transcriptase, and nuclease-free water along with the RNA template for a 20µl reaction. The reaction mix was then incubated in a thermal cycler as follows: 5 min at 25°C for priming, 20 min at 46°C for reverse transcription, and 1 min at 95°C for inactivation of the reverse transcriptase. The cDNA samples were stored at -20°C.

2.2.10 qPCR Analysis

For the qPCR experiments, cDNAs were used after being diluted 1:4. To make a 10µl reaction mix; 1µl of cDNA, 5µl of LightCycler® 480 SYBR Green I Master, 1µl of 10µM

Primer mix, and 3µl of PCR-Grade water were combined. The reaction was set as provided by the Roche-LightCycler® 480 SYBR Green I Master protocol.

2.2.11 Protein Quantification

The Pierce BCA Protein Assay Kit was used to determine protein concentrations in the samples. The following concentrations of BSA was serially diluted with ddH2O to form the standard curve : 0µg/mL, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL, 70 µg/mL, 100 µg/mL. The protein samples to be quantified are diluted 1:100 with ddH2O to ensure the concentration falls within the standard curve. Both the standards and the protein samples are loaded in triplicates to a 96-well plate. BCA working solution is prepared as instructed by the manufacturer and added 1:1 on the standard and protein samples. The 96-well plate is incubated in 60°C for 50 minutes. After the active reagent changes color, the 96-well plate is cooled to room temperature. The absorbance readings at 562 nm were measured using the Synergy HT Microplate Reader.

2.2.12 Western Blot

After quantification, protein samples were boiled and ran in a SDS-PAGE. A wet transfer system was used to transfer the proteins into a PVDF membrane. Afterwards, the membrane was stained with Ponceau S to check any problems regarding the transfer process. Blocking was done with 10% skimmed milk solution made with TBST for approximately 30 minutes. Membranes were incubated with primary antibodies at +4ºC overnight and then incubated with secondary antibodies either overnight at +4ºC or for 1-2 hours in room temperature. Between every step starting from the Ponceau S staining, the membranes were washed 3 X 10 minutes with TBST. The Western Blots were revealed mainly using SuperSignal West Pico PLUS or Clarity Western ECL Substrate using the Amersham Imager 600.

2.2.13 Transwell Migration/Invasion Assay

For Transwell migration assay with A2BR overexpressed HCT116 cells. The cells were starved overnight and seeded into the inner chamber membrane with medium containing 0% serum. Below the membrane, medium containing 20% serum was added. After the cells

migrated, they were fixed with methanol and 4%PFA and then subsequently stained with Crystal Violet. For the Transwell assays with antagonist treated HCT116 cells, the cells were treated with the antagonist for 2 days before seeding. For the Invasion assay, the membrane was covered with 2% Matrigel to simulate invasion. The area of the cells that migrated/invaded was measured using the ImageJ Software.

2.2.14 Prognostic Analyses

For the prognostic analyses, the ADORA2B probeset "205891_at", which exhibited the highest mean expression among ADORA2B probesets in both GSE39582 and GSE17536 datasets, was chosen. To choose optimal cut-off values for the probability of prognosis, log-rank multiple cut-off (LRMC) graphs were plotted, and the significant points were chosen according to the expression thresholds on the x axis and the p-values shown on y axis, poor and good prognosis indicated by the red and blue colors. The 25th percentile, median and 75th percentile values were shown through the vertical lines, whereas the p-value indicator at p=0.05 has been shown as the horizontal line. IBM SPSS software was used for the cox regression analysis and the Kaplan Meier plots were made using the GraphPad Prism software.

Chapter 3
Results

3.1 Protein and mRNA expressions of A2BR in various cell lines

In order to analyze the CRC cell lines that were available to us in terms of A2BR expression, we measured the A2BR mRNA and protein expressions in the 7 CRC cell lines (HT29, HCT116, SW480, SW620, SW48, RKO, DLD1) and a healthy colon cell line (CDD-18Co). We observed that, in terms of mRNA expression, the healthy colon cell line had the lowest relative A2BR expression compared to the CRC cell lines (Figure 1A). However, in terms of protein expression, CDD-18Co expressed A2BR similarly to RKO, which had the highest mRNA expression, as well as showing relatively higher protein expression than most CRC cell lines (HCT116, SW480, SW620, SW48, and DLD1) (Figure 1B). HT29 has the lowest mRNA expression out of the CRC cell lines, however, its protein expression seems to be the highest after normalizing the quantified A2BR with the GAPDH loading control (Figure 1A-B). It is possible that this is due to differences in GAPDH expressions between cell lines with different characteristics as GAPDH expression in HT29 cells seem much lower compared to the other CRC cell lines (Figure 1B). Interestingly, the A2BR expression of SW480 cell line is higher, both at the protein and mRNA levels, compared to its metastatic counterpart, SW620 (Figure 1A-B). This may hint that A2BR expression may have a suppressive effect on metastasis. The discrepancy between mRNA and protein expressions should be further investigated.

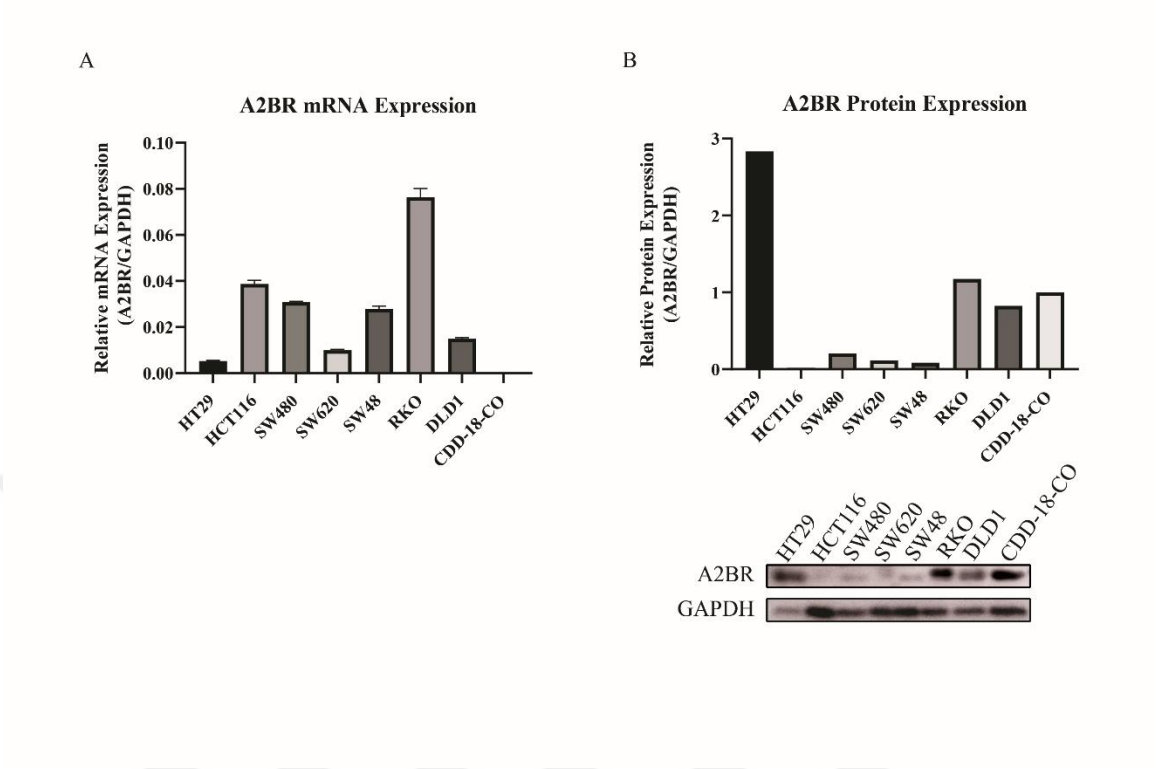

Figure 1: A2BR Expression in CRC cell lines and CDD-18-CO, a healthy colon cell line. The A2BR expression was measured in 7 different CRC cell lines and 1 healthy colon cell line by **A)** qPCR analysis for mRNA levels and **B)** Western Blotting for protein levels.

3.2 A2BR Expression Negatively Affects Cell Proliferation of CRC Cell Lines

We used the HT29 and HCT116 stable A2BR overexpression cell lines to determine what kind of an effect A2BR expression has on these cell lines. These cell lines were transfected with an empty pBABE vector or one containing the ADORA2B gene and are referred to as pbCT and pbA2BR cells. The A2BR overexpression in HT29 pbA2BR and HCT116 pbA2BR cell lines was confirmed by measuring the mRNA levels. Both cell lines showed a significant increase in the A2BR mRNA levels compared to the pbCT cells (Figure 2A, B).

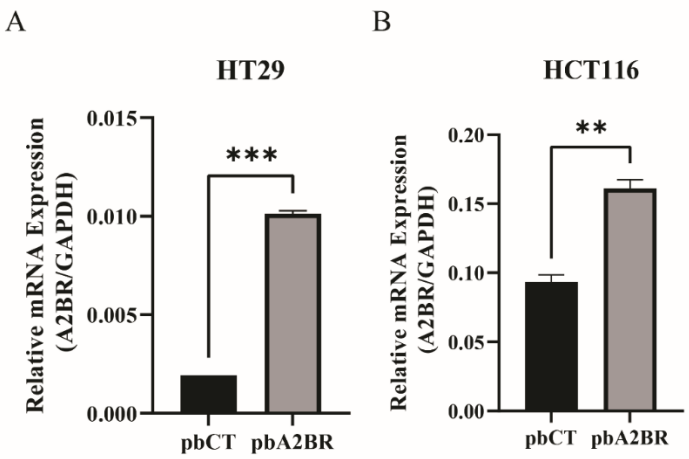

Figure 2: A2BR mRNA expression in stable overexpression cell lines. RNA samples are isolated from **A)** HT29 pbCT and pbA2BR **B)** HCT116 pbCT and pbA2BR stable overexpression cells after puromycin selection. A2BR mRNA levels are quantified by qPCR analysis and normalized according to GAPDH expression.

Next, to examine if A2BR expression had an effect on the proliferation of our overexpression cell lines, we performed flow cytometry using the Propidium Iodide (PI) stain. For both cell lines, we prepared single cell suspensions in PBS after they were grown overnight. We then fixed the cells using ethanol and treated them with RNase before subsequently staining them with PI overnight. We used the NovoCyte 3000 Flow Cytometer and its complementary NovoExpress software to analyze the cells. The analyzed cells were gated in scatter plots to remove cell debris and doublet cells, and then we used the cell cycle analysis feature of the NovoExpress software to automatically plot and analyze the cell cycle distribution in the histogram plots using the PE channel. The percentage of the pbCT and pbA2BR cells in the S-phase was compared in HT29 (Figure 3A) and HCT116 (Figure 3B) cell lines. We observed that there was approximately a 2.5% decrease in the S-phase population of the HT29 pbA2BR cells (Figure 3A) and more than 4.5% decrease in the S-phase population of HCT116 pbA2BR cells (Figure 3B). By this significant decrease in the S-phase population, we can conclude that A2BR expression has a negative effect on cell proliferation in both cell lines.

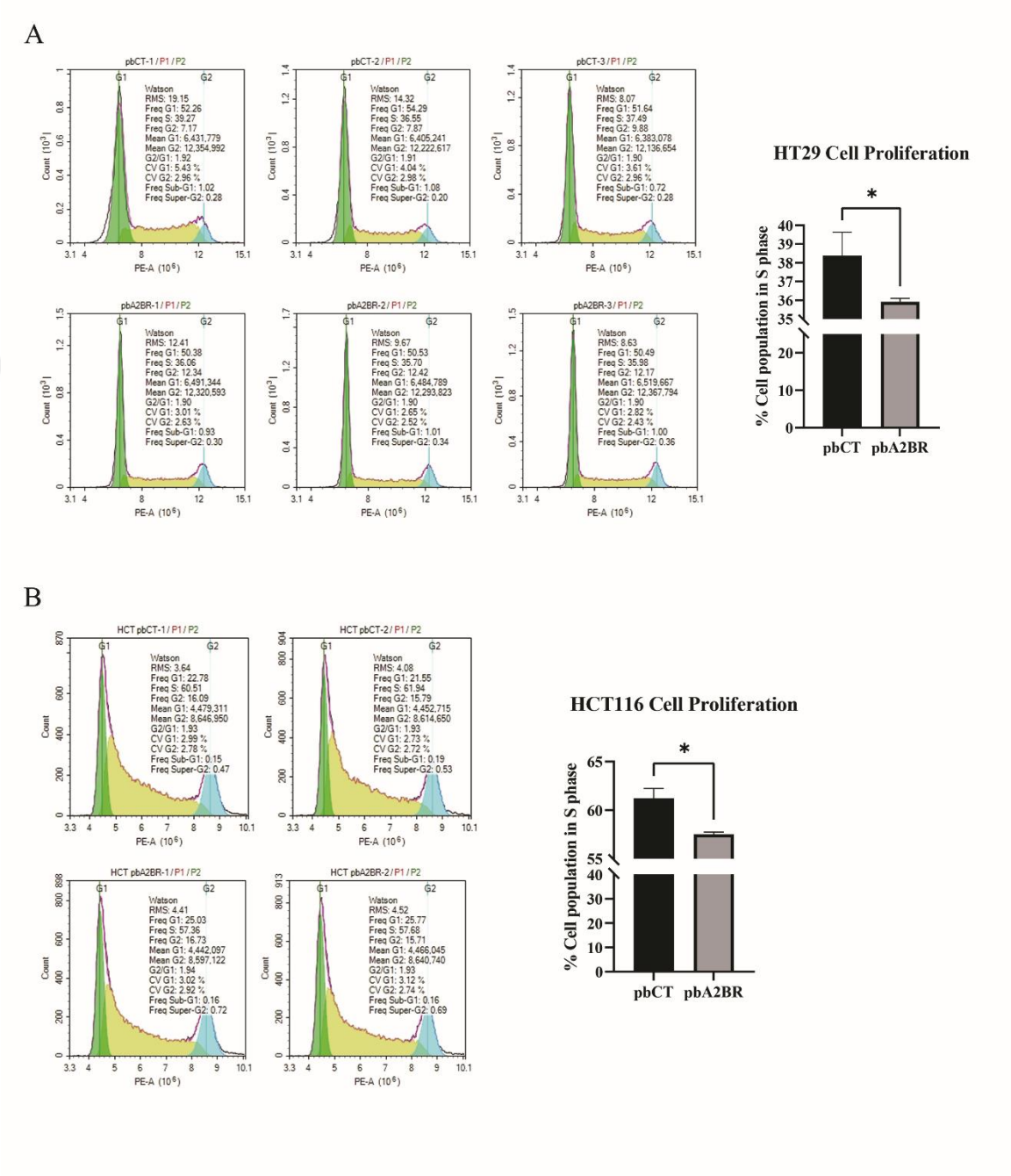

Figure 3: A2BR expression negatively correlates with cell proliferation. The percentage of cells in the S-phase of the cell cycle has significantly decreased in the **A)** HT29 pbA2BR cell line as well as the **B)** HCT116 pbA2BR cell line according to the flow cytometric cell cycle analysis.

To assess the proliferative capacity of the cells even further, we performed clonogenic assay which would inform us if the A2BR has an effect on the ability of the single cancer cells to form colonies. HT29 pbA2BR cell line was used for this assessment due to its inherently higher adherence to the plate coating compared to the HCT116 cell line which would decrease the chances of the colonies detaching during medium changes. First, the cells were collected in a single cell suspension in the growth medium. The cells were counted and adequately diluted to make sure the cell number would not exceed the capacity of the 12-well plate. After seeding, we followed the formation and growth of the colonies until they reached the desired size and changed the medium as needed. After fixing and staining the colonies, the wells were photographed and the colony numbers were counted using the OpenCPU software. Upon analyzing the results, we showed that overexpression of A2BR in the HT29 cell line significantly decreased the ability of the single cells to form colonies (Figure 4B). Furthermore, the colonies that formed in the experimental group were visibly smaller than those in the control group (Figure 4A). Overall, we concluded that the increased expression of A2BR in CRC cell lines is detrimental to cell growth *in vitro*.

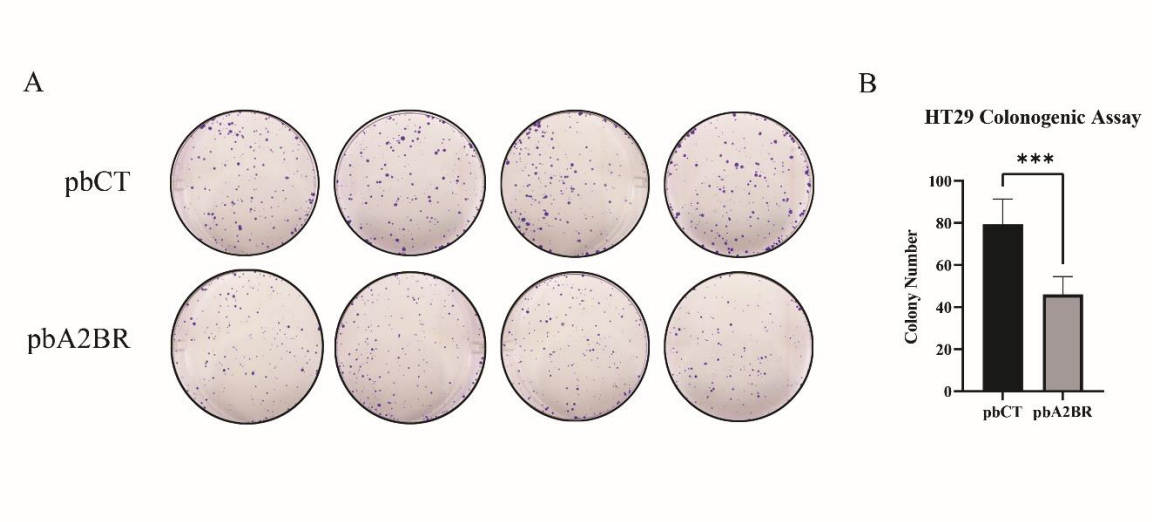

Figure 4: A2BR expression hinders the ability of the CRC cells to form colonies. The ability of the HT29 pbA2BR cells to form colonies was assessed by performing clonogenic assay. **A)** The colonies were stained with crystal violet upon reaching an appropriate size and photographed. **B)** Colony number was quantified by the OpenCFU software.

3.3 High A2BR Expression Hinders Tumor Growth in Cell Line-Derived Xenograft Model

After establishing that high A2BR expression showed a negative correlation in cell growth *in vitro*, we wanted to examine if we would have comparable results in terms of tumor growth *in vivo*. We cultured HCT116 pbCT and pbA2BR cells in large amounts to subcutaneously inject 7 million cells resuspended in PBS into each flank of the nude mice. We measured the width and height of each tumor for approximately 5 weeks to follow the tumor growth in terms of volume (Figure 5A). After tumors in the control group exceeded 1500mm^3 in volume, we sacrificed the mice and dissected the tumors (Figure 5B). The weights of the tumors were also measured after dissection (Figure 5C). Tumor tissue was collected from each mouse to check A2BR protein expression in the tumors (Figure 5D). The tumors in the A2BR overexpressed group displayed both a higher volume and weight compared to the control group. Thus, we can conclude that increase in A2BR expression leads to a decrease in tumor growth *in vivo*.

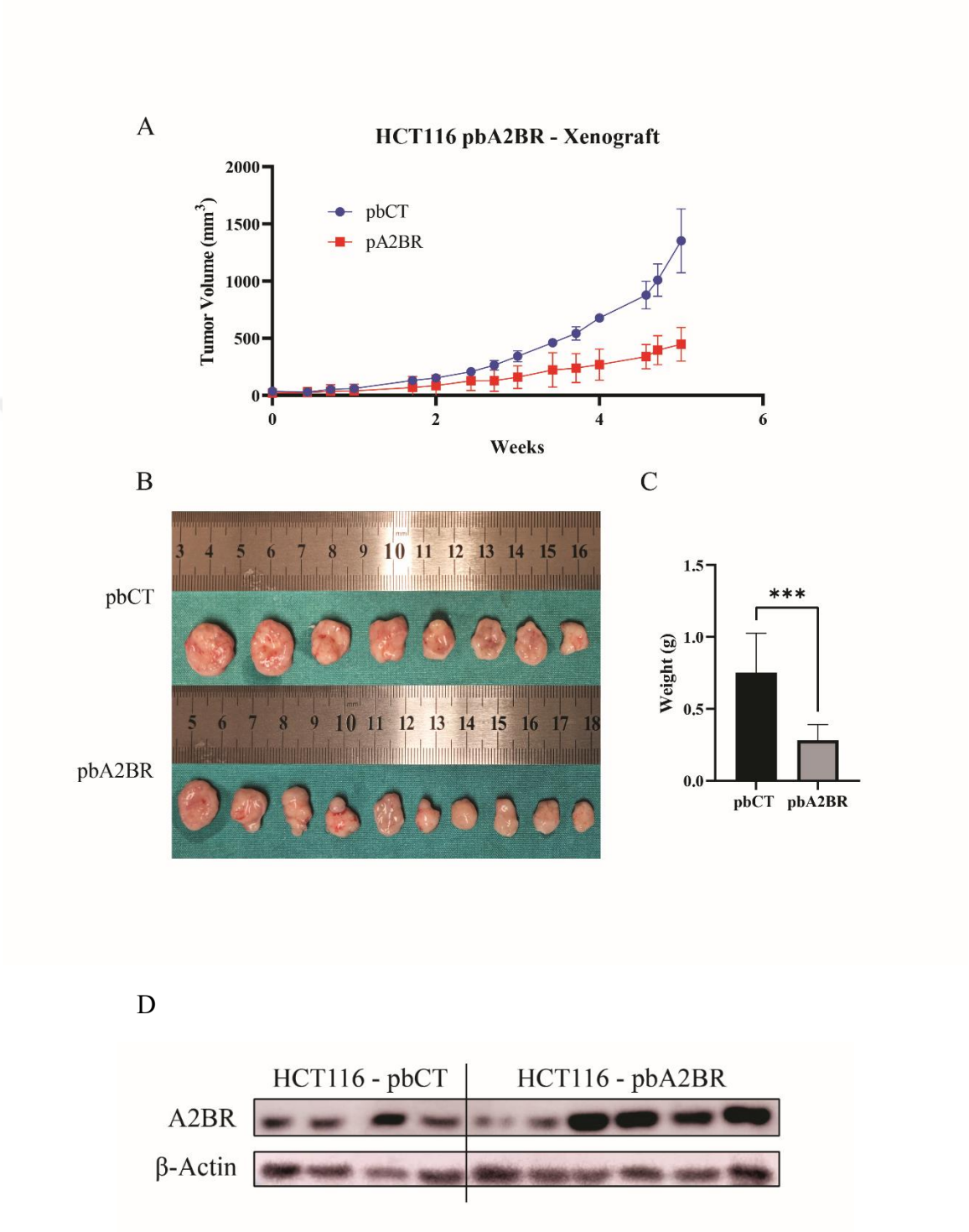

Figure 5: High expression of A2BR reduces tumor growth in cell line-derived xenografts. A) The tumor volume was calculated using the Length x Width^2 formula and the mice were sacrificed after tumor volume of one of the groups approached 1500 mm^3. **B)** The tumors were dissected and **C)** weighed to determine tumor size differences

between the groups. **D)** A2BR expression of pbA2BR tumors was confirmed by western blotting.

3.4 The Effect of Increased A2BR Expression on the Migratory Capability of CRC Cells

We performed wound healing assay on the HT29 pbA2BR cell line in order to determine if an increase in the A2BR expression affects the migration ability of the cells. Once the cells we seeded reached ~95% confluency, we scratched the cell surface with a pipette tip to induce the wound. The wound was then photographed when it was first induced (0H) and after the cells migrated adequately but have not entirely closed the wound (~96H) (Figure 6A). The wound area was quantified using the ImageJ software with the wound healing size tool plugin and the percentage of migrated cells were calculated using the area of the initial and final wound size (Figure 6B). The wound healing assay showed us that A2BR overexpressed HT29 cells had a lower migratory ability compared to the control group.

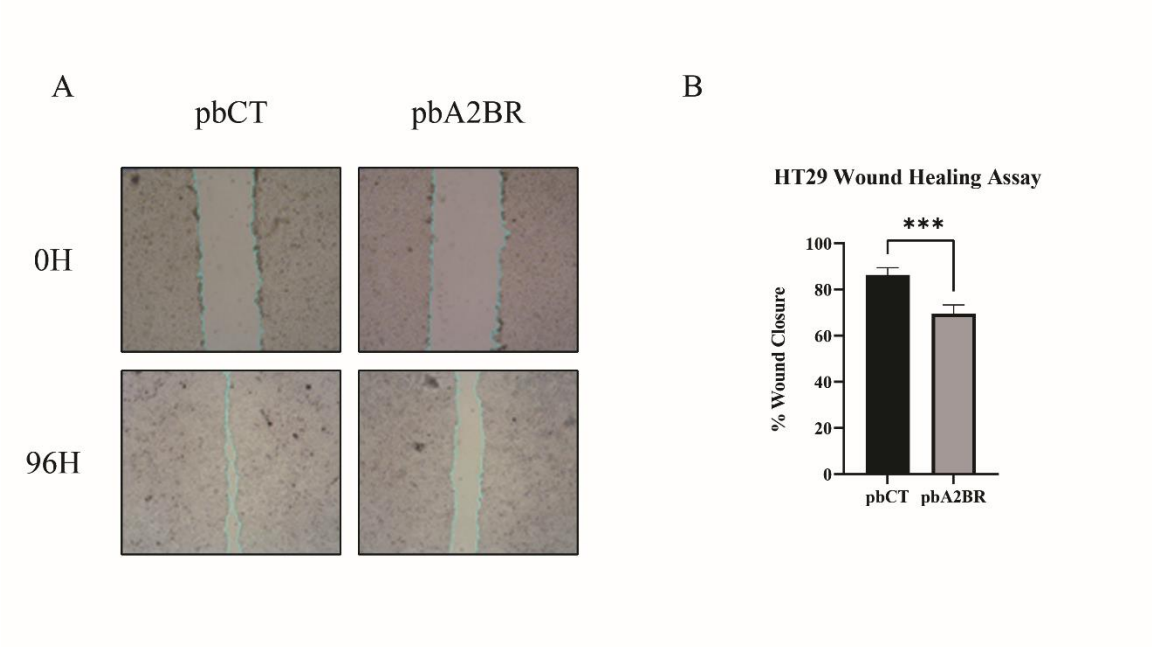

Figure 6: HT29 pbA2BR cells migrate significantly less than the HT29 pbCT cells. The scratches created for the wound healing assay **A)** were photographed at initial formation (0H) and after approximately 96H of migration of the HT29 cells. **B)** The

percentage of the wound closure via cell migration was quantified using ImageJ software by comparing the area of the initial wound and the area of the wound after closure by cell migration.

To measure the migratory ability of the HCT116 A2BR overexpression cell line, we performed a Transwell migration assay as the HCT116 cells inherently detach easily from the plate coating, not allowing for an accurate wound healing assay. The cells were seeded on the membrane of the Transwell chamber with serum-free medium. We placed 20% serum containing medium around the Transwell chamber to lure the cells into migrating to the other side of the membrane. After overnight incubation, the cells on the membrane were fixed with 4% PFA and stained with crystal violet. We carefully removed the cells that were still in the inner chamber and took pictures of the migrated cells in the outer surface of the membrane (Figure 7A). While there seems to be a tendency of decreased migration in the HCT116 pbA2BR cells, the quantified results were not significant enough to confirm any change in the migratory ability of HCT116 cells upon A2BR overexpression (Figure 7B). Overall, we can conclude that increased A2BR expression decreased the migratory ability of pbA2BR cell line but did not affect the migratory ability of the HCT116 pbA2BR cell line.

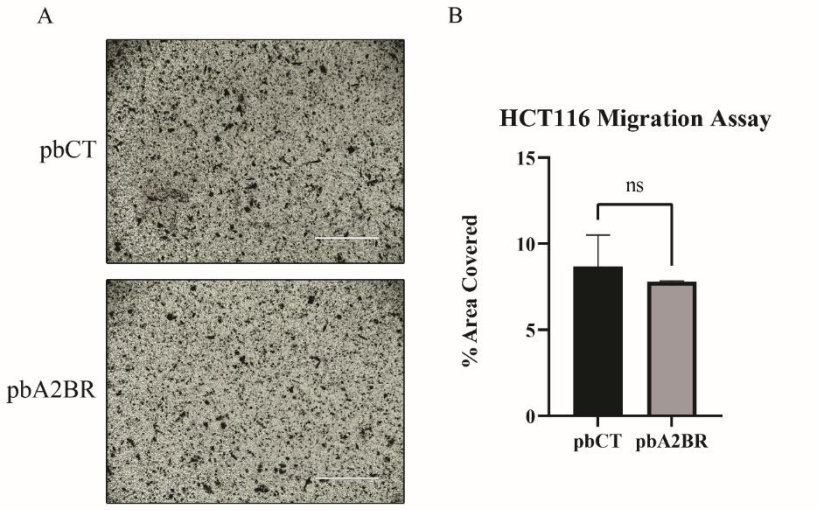

Figure 7: Increase in A2BR expression does not significantly affect the migration of HCT116 cells. A) Crystal violet stained Transwell membranes were photographed on 5 random points to assess HCT116 cell migration for the pbCT and pbA2BR cells. The

photographs were taken using the 10X objective lens and **B)** quantified by measuring the area of the migrated cells using the ImageJ software.

3.5 EMT Marker Expression Profiles Are Altered in CRC Cell Lines Upon Stable A2BR Overexpression

Upon observing A2BR induced differences in migration, we wanted to see if induced A2BR overexpression had an effect on the EMT process. After growing the stably A2BR overexpressed HT29 and HCT116 cells, we lysed them using the RIPA cell lysis buffer, performed protein quantification using the Pierce BCA Protein Assay Kit and proceeded with Western Blotting after boiling the proteins to compare protein expressions of EMT markers.

In HT29 pbA2BR cells, we observed a decrease in the N-Cadherin and a-Smooth Muscle Actin and an increase in Integrin-a5 and ZEB1 expression of mesenchymal markers compared to the control. Surprisingly, E-cadherin expression remains unaffected despite a slight increase in the ZEB1 transcription factor (Figure 8A). HCT116 cells show a more conventional EMT change upon A2BR overexpression. We observed an increase in the expression of E-cadherin and ZO-1 epithelial markers and a decrease in the expression of the a-Smooth Muscle Actin mesenchymal marker. Integrin-a5 expression has also been increased in HCT116 pbA2BR cells compared to the control group, whereas, ZEB-1 expression seems unaffected (Figure 8B).

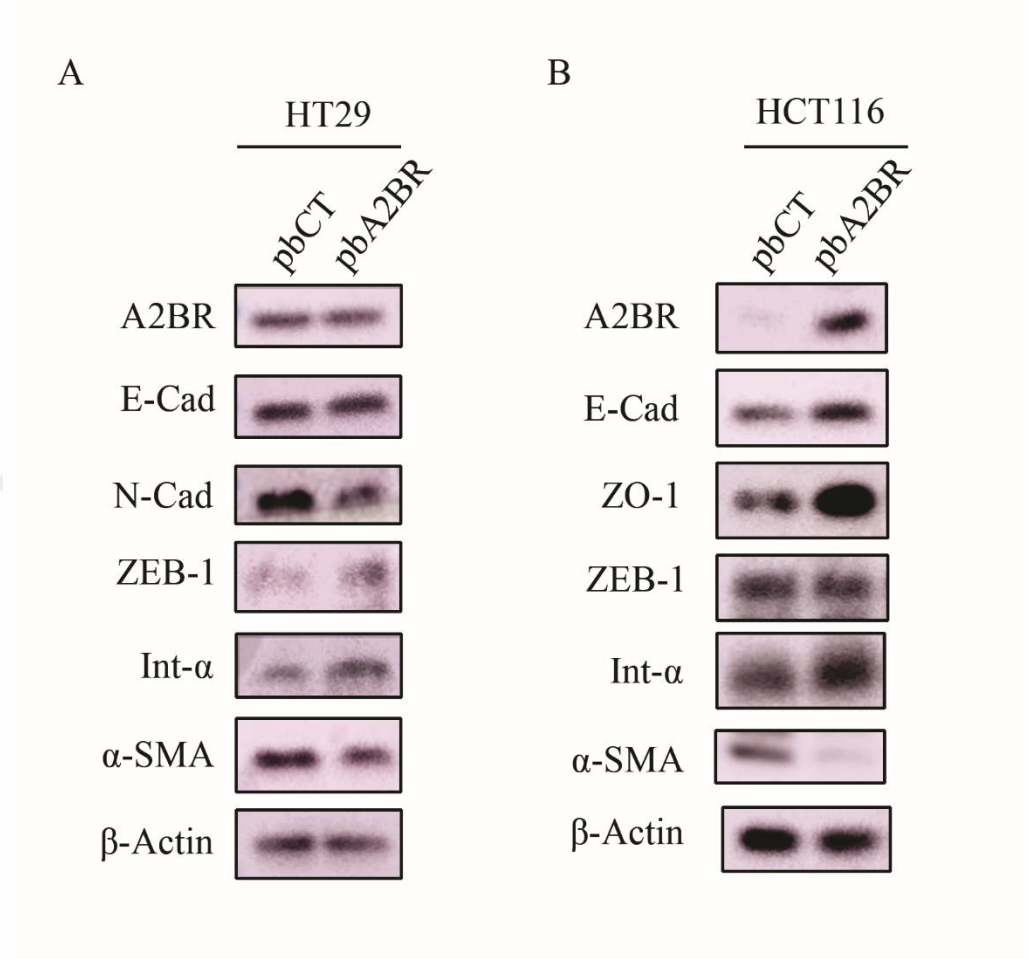

Figure 8: A2BR overexpression cell lines display an altered EMT marker profile. Protein expressions of EMT markers were analyzed to observe changes in the EMT marker profile of **A)** HT29 pbA2BR and **B)** HCT116 pbA2BR cells.

3.6 Pharmacological Inhibition of A2BR by A2BR-Selective Antagonists Increase the Proliferative Capacity of CRC cells.

After observing the effects of A2BR overexpression on HT29 and HCT116 cell lines, we wanted to inhibit A2BR activity without changing its expression levels to determine if the pharmacological inhibition of A2BR induces the expected opposing effects without manipulating the expression levels. We used the MRS1754 and PSB603 A2BR-selective antagonists to selectively inhibit the A2BR activity in these cells.

In order to measure the proliferative capacity of the HT29 cells after pharmacological A2BR inhibition, we performed clonogenic assay. The clonogenic assay was seeded as previously explained, however, 15uM of MRS1754 and 10uM of PSB603 and equal volumes of DMSO were added into the medium containing the single cell suspension before seeding. We changed the medium every 3 days to renew medium containing the antagonists and DMSO. Once the colonies reached the appropriate size, we fixed the colonies and stained them with crystal violet. Afterwards, we photographed the wells containing the colonies and quantified them using the OpenCFU software. After analysis, we observed that pharmacological inhibition of A2BR, both by MRS1754 and PSB603 treatment significantly increases the number of colonies formed from the single cell suspension, indicating an increase in the cell proliferation in the absence of A2BR activity.

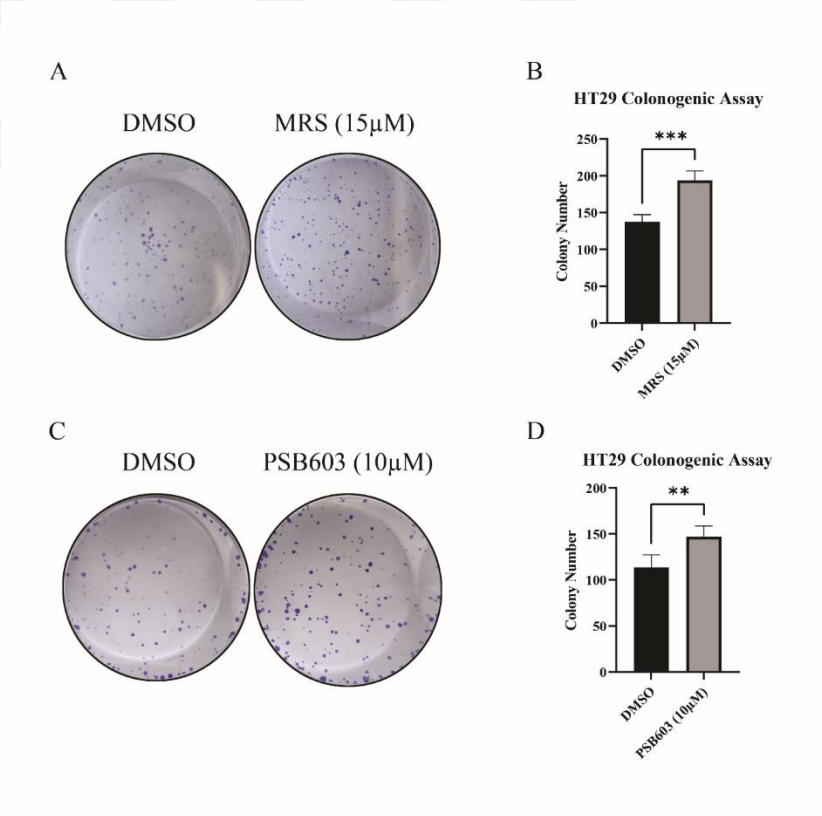

Figure 9: Treatment of HT29 cell line with A2BR-selective antagonists promote the ability of the single cells to form colonies. A) Colonies treated with DMSO and MRS1754 were stained with crystal violet after reaching an appropriate size and photographed, **B)** their colony numbers were quantified by the OpenCFU software. **C)** Colonies treated with

DMSO and PSB603 were stained with crystal violet after reaching an appropriate size and photographed, **D)** their colony numbers were quantified by the OpenCFU software.

3.7 Pharmacological Inhibition of A2BR by A2BR-Selective Antagonists Increase the Migratory Ability of CRC cells.

We performed the Transwell Migration assay to measure the migratory ability of the HCT116 cells upon pharmacological inhibition of A2BR. Before the cells were seeded in the Transwell membrane, they were treated for 2 days with DMSO, 20uM MRS1754, and 10uM PSB603 respectively. The Transwell seeding and setup was performed as previously explained. We fixed the cells with 4%PFA after 16H and stained them with crystal violet. We carefully removed the cells that were still in the inner chamber and took pictures of the migrated cells in the outer surface of the membrane (Figure 10A-C). We have observed that, upon inhibition of the A2BR activity in HCT116 cells through either antagonist, migration of the cells was significantly increased, both to the naked eye and by quantification of the area percentage of the membrane covered by the cells (Figure 10B-D).

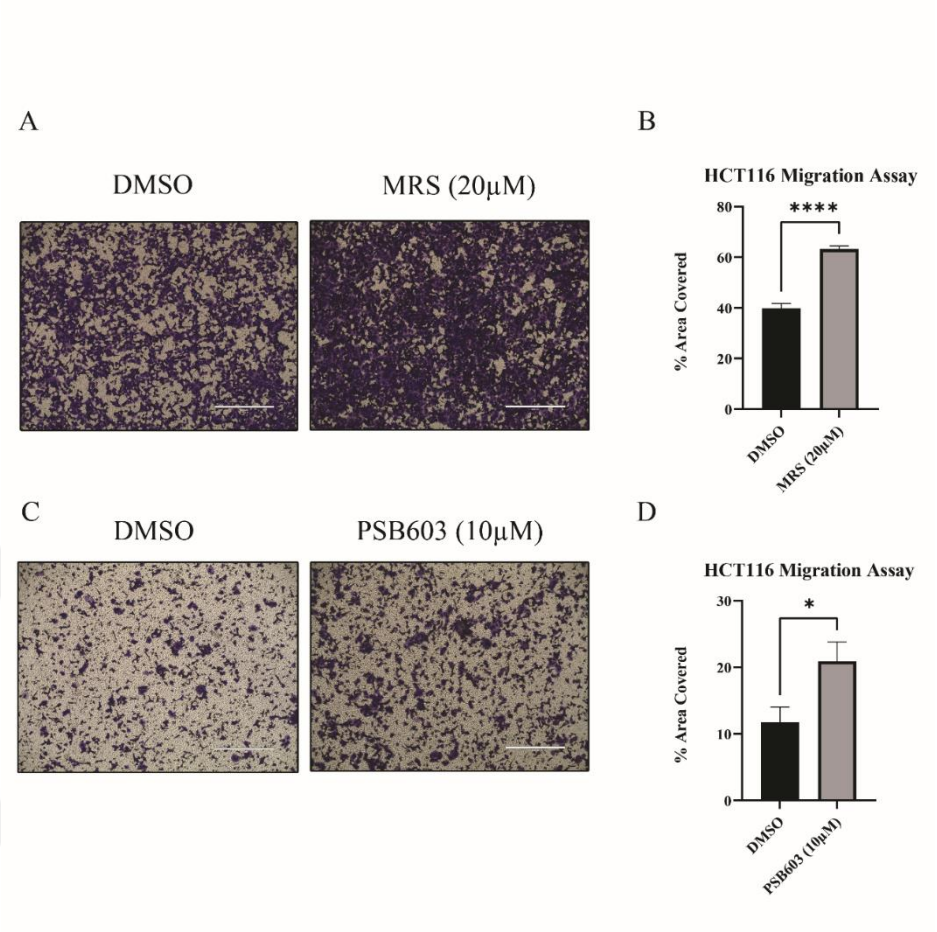

Figure 10: Treatment of HCT116 cell line with A2BR-selective antagonists promote the migratory ability of the cells. Crystal violet stained Transwell membranes were photographed on 5 random points to assess HCT116 cell migration for the **A)** 20uM MRS1754 treated and **C)** 10 µM PSB603 treated cells. The photographs were taken using the 10X objective lens and **B-D)** quantified by measuring the area of the migrated cells using the ImageJ software.

3.8 Treatment with MRS1754 A2BR-Selective Antagonist Induces Invasion in CRC

Due to the aberrant increase in the migration ability of HCT116 cells upon MRS1754 treatment, we wanted to check if similar effects would be observed in terms of invasiveness. We performed a Transwell invasion assay to test this. The cells were once again treated for 2 days with DMSO and 20 μM MRS1754. Before the cells were seeded into the membrane, the membrane was covered in a layer of 2% Matrigel to stimulate invasion. The following day, we fixed the cells with 4% PFA and stained them with crystal violet. The cells in the inner side of the membrane were cleaned without disturbing the cells on the outer side. We photographed the cells on the outer membrane (Figure 11A) and quantified the area of the invaded cells using the ImageJ software (Figure11B). We saw that, upon MRS1754 treatment, the HCT116 cells were significantly more invasive compared to the DMSO treated HCT116 cells.

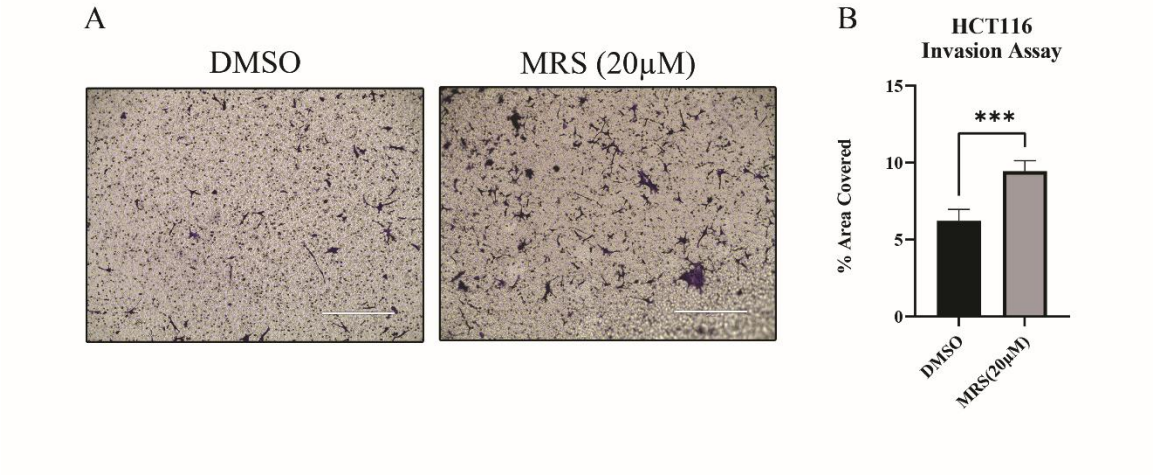

Figure 11: Invasiveness of HCT116 cells increase upon MRS1754 treatment. Crystal violet stained Transwell membranes were photographed on 5 random points to assess HCT116 cell invasion in the **A)** DMSO and 20 μM MRS1754 treated cells. The photographs were taken using the 10X objective lens and **B)** quantified by measuring the area of the migrated cells using the ImageJ software.

3.9 Treatment with A2BR-Selective Antagonists Lead to a Change in the EMT Marker Expression Profile of HCT116 Cells

We wanted to check if the EMT marker profiles of the HCT116 cells were changed upon the A2BR inhibition, as both migration and invasion assays indicated a more aggressive phenotype upon antagonist treatment. We treated the HCT116 cells with DMSO, 20 μM of MRS1754, and 10 μM of PSB603 for 2 days. Samples were prepared for western blotting as explained previously.

The results show that EMT marker protein expressions were changed when the cells were treated with the A2BR selective antagonists (Figure 12). In both of the antagonist treated samples, we saw an increase in the integrin-α, α-smooth muscle actin (α-SMA), and N-Cadherin mesenchymal markers and a decrease in the ZO-1 epithelial marker. E-Cadherin protein expression has shown different profiles in MRS1754 and PSB603 treated cells, increasing after MRS1754 treatment and decreasing upon PSB603 treatment. ERK phosphorylation, which may increase due to increase in cancer cell proliferation, was enhanced in MRS1754 treated cells and remained unchanged upon PSB603 treatment.

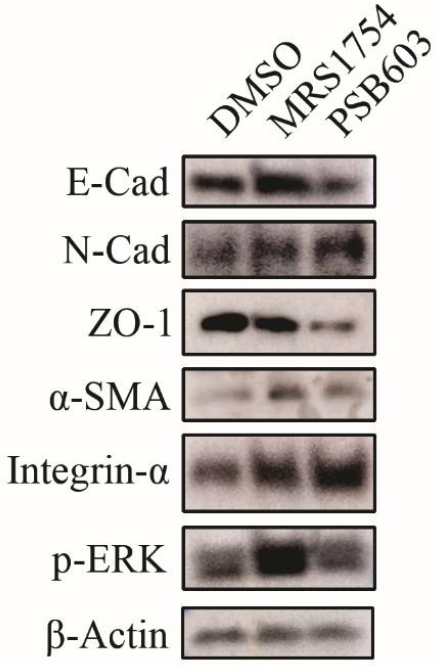

Figure 12: Upon treatment with A2BR-selective antagonists, HCT116 cells show a more metastatic EMT profile. HCT116 cells treated with DMSO, 20 μM MRS1754, and 10μM PSB603 for 2 days were lysed and protein expressions of certain EMT markers were identified by western blotting.

3.10 A2BR Expression is Directly Correlated with a Better Prognosis According to the Survival Analysis of CRC Patient Data.

In order to examine the clinical relevance of our findings, we wished to identify if A2BR expression had a significant effect on CRC patient prognosis. The analyses were performed by Dr. Seçil Demirkol Canlı, who collaborated with us in this project, and by using the "205891_at" A2BR probeset which exhibited the highest mean expression among A2BR probesets in both GSE39582 and GSE17536 datasets.

The first panels show Log-Rank Multiple Cut-off (LRMC) analyses for recurrence-free (Figure 13A) and overall survival (Figure 13B), where each point represents a probability of good (represented by the blue points) or bad prognosis (represented by the red points) . The blue points above the horizontal threshold, where p=0.05, signify probabilities where high A2BR expression is linked to significantly improved survival. The second panels show the Kaplan-Meier curves that were made using the optimal cut-off values represented by the vertical lines in the LRMC graphs. For recurrence-free survival (Figure 13A) the most meaningful cut-off value of 8.126242 was used. For overall survival (Figure 13B) the most meaningful cut-off value of 8.334324 was used. This data represents a total of 519 patients divided to low and high expression groups, the number of patients in each group for recurrence-free survival are as follows: n=344 (Low Expression) and n=175 (High Expression). For overall survival the number of patients in each group are as follows: n=415 (Low Expression) and n=104 (High Expression) The plots show that patients with high A2BR expression have significantly better recurrence-free (Figure 13A) and overall survival (Figure 13B) compared to the patients with lower A2BR expression. These results indicate that high A2BR expression is correlated with a better prognosis in CRC patients.

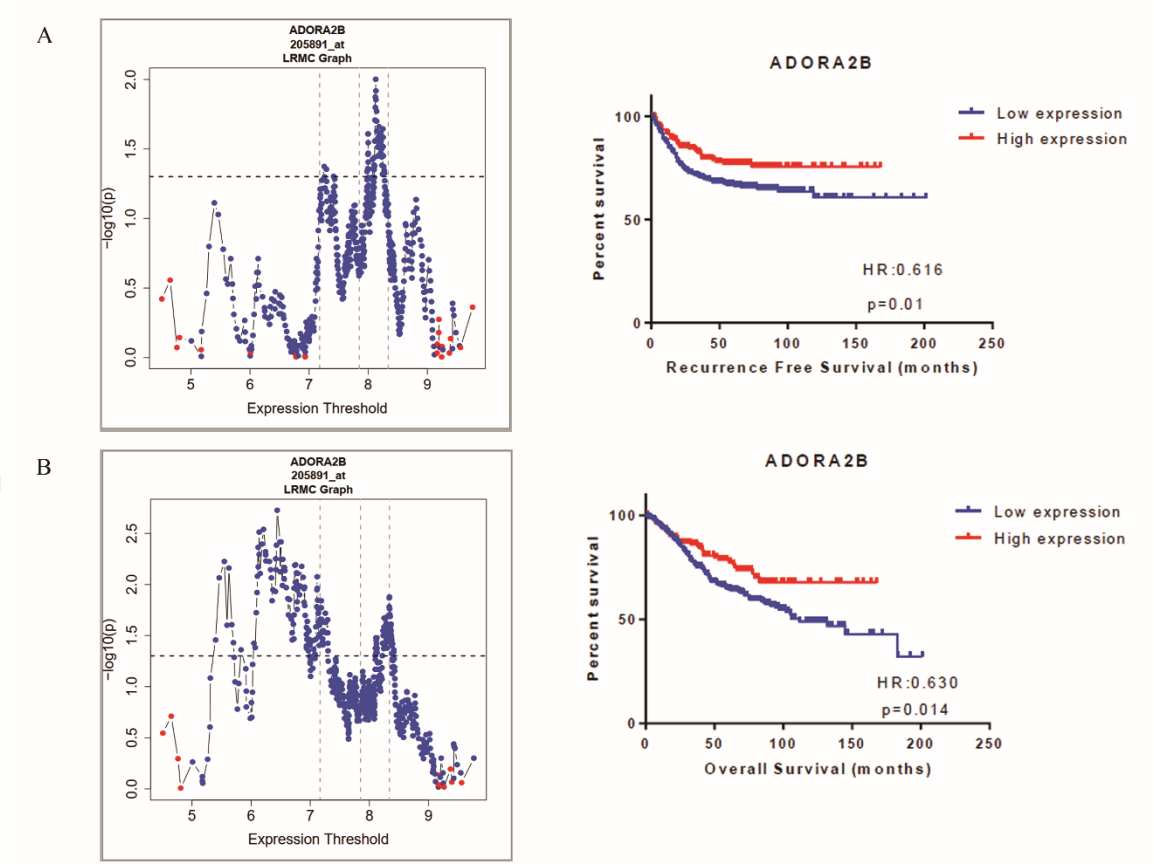

Figure 13: High A2BR expression in CRC indicates a better prognosis. This data represents a total of 519 patient data points divided into low and high expression groups. The LRMC Graphs and the Kaplan-Meier patient survival analyses show **A)** recurrence-free survival and **B)** overall survival prognosis in patients with high or low A2BR expression in CRC.

Chapter 4
Discussion

Colorectal cancer is one of the most prevalent malignancies, having the third highest incidence rate and second highest mortality rate among the 36 recorded cancer types worldwide (Sung et al., 2021). More so, this incidence rate is estimated to increase to even higher numbers in the following years, with an estimate of 60% increase by the year 2040 (Ionescu et al., 2023; Roshandel et al., 2024). A fourth of the patients diagnosed with CRC experience metastasis with a 30% relapse rate, it is important to note that metastatic CRC also possesses a very low 5-year survival rate of about 14% in patients (Rumpold et al., 2020). For these reasons, research on factors driving CRC progression and metastasis is of utmost importance as this information would be essential in development of therapies to halt the cancer progression.

Extracellular adenosine and its interaction with the A2B adenosine receptor have been reported as a factor that promotes tumor progression and metastasis. However, much of the research on CRC focuses on the immunosuppressive effects of A2BR (Hajizadeh et al., 2020). The effect of A2BR on cancer progression intrinsically has been studied in multiple cancer types, such as triple-negative breast cancer, oral cancer, and prostate cancer, and signified that A2BR expression in these cancers had a cancer promoting role (Kasama et al., 2015; D. Mittal et al., 2016; Wei et al., 2013). However, this role may differ in cancers originating from different tissues, A2BR expression in ovarian cancer has shown contradictory results where A2BR reduced cell migration and induced a more epithelial phenotype (Campos-Contreras et al., 2022). There has not been adequate research in terms of how A2BR expression may affect CRC progression in a purely intrinsic manner, and since A2BR signaling is vastly tissue and context dependent on its effects, we aimed to explore the effects of A2BR expression in CRC progression.

We showed that A2BR expression in the CRC cell lines were much higher than the healthy colon cell line at the mRNA level, which is consistent with studies showing enhanced A2BR expression in CRC patient tissues compared to healthy ones (Ma et al., 2010). While previous research has shown that A2BR mRNA expression is higher in the metastatic

SW620 cell line compared to its non-metastatic counterpart SW480 (Sepúlveda et al., 2016), our results contradict this finding and indicate a lower A2BR mRNA expression in the metastatic cell line. Surprisingly, in terms of protein levels, we observed a much different A2BR profile where the healthy colon cell line shows higher A2BR levels than most of the CRC cell lines. This discrepancy should be further investigated.

Since A2BR signaling can be context dependent, we performed our experiments in two different cell lines to see if the results would be reproducible. We chose HT29 and HCT116 cell lines which have contrasting characteristics. These cell lines differ in terms of their origins, aggressiveness, as well as molecular backgrounds despite both being CRC cell lines. HT29 cell line originates from the primary tumor of a 44-year old female whereas HCT116 cell line originates from the primary tumor of a 48-year old male. HCT116 cells display a more aggressive phenotype with very limited capacity to differentiate compared to HT29 cell line. Molecularly, HCT116 has mutations in KRAS and PIK3CA genes and shows high microsatellite instability, whereas, HT29 cells have mutations in the TP53 and BRAF genes with a microsatellite stable phenotype (Berg et al., 2017; *HCT116 Cell Line - A Comprehensive Guide to Colorectal Cancer Research*, n.d.; *HT-29*, n.d.). Using these cell lines allowed us to explore if the effects of A2BR changes upon CRC cells with different molecular subtypes as well as aggressiveness and also allowed for the implementation of different experimental methods.

The findings in this study are in support of a larger project on purinergic signaling in CRC. Previously, using stable A2BR knockout cell lines generated with CRISPR/Cas9, we have shown an increase in proliferative ability and anchorage-independent growth, as well as, improved migration and a shift towards a more mesenchymal EMT profile in HT29 and HCT116 cell lines (Uygur, 2021). Later, we have also observed that stable knockout of A2BR significantly increased tumor growth in vivo by using a cell line derived xenograft model (data not shown). To follow up on this, we used stable A2BR overexpression cell lines to observe if the changes brought upon by A2BR knockout could be reversed upon overexpression. We also pharmacologically inhibited A2BR activity in HT29 and HCT116 cell lines with A2BR-selective antagonists to observe the effects of A2BR without manipulating its expression.

MRS1754 and PSB603 antagonists were used to inhibit A2BR activity. Both of these antagonists bind selectively and competitively to the A2B receptor with high affinity, not allowing for endogenous agonists to activate the receptor (Goulding et al., 2018; Ji et al., 2001). Unlike MRS1754, PSB603 has also been suggested to allosterically bind to A2BR and inhibit its activation dose-dependently despite stimulation with agonists (Goulding et al., 2018). Furthermore, while both antagonists are A2BR selective, MRS1754 has an approximately 255-fold selectivity over other adenosine receptors while PSB603 has a selectivity of over 17000-fold. (*MRS1754 | A2BR Antagonist | Probechem Biochemicals*, n.d.; *PSB 603 | Adenosine A2B Receptors*, n.d.).

In both cell lines, stable overexpression of A2BR reduced the number of cells entering the S-phase, indicating a decrease in the proliferation of the cells. This could be due to the elevated cAMP levels upon coupling of A2BR with Gs protein. Increase in cAMP levels were shown to decrease cyclin A expression in HepG2 liver cancer cell line, a similar mechanism may be taking place which could disrupt the entry into S-phase in the A2BR overexpressed CRC cells (Massimi et al., 2017). It is also possible that these cell lines regulate the cell cycle progression through different mechanisms upon A2BR overexpression. We have observed that while there is an increase in the population of cells in the G1 phase for HCT116 cell line, this is not the case for HT29 cell line. This could indicate that A2BR overexpression is leading to G1 arrest in HCT116 cells but not HT29 cells.

The colony forming ability was also significantly reduced in the A2BR overexpressed HT29 cells and significantly increased in HT29 cells treated with the A2BR selective antagonists (MRS1754 and PSB603). These results suggest that A2BR has an anti-proliferative role in HT29 and HC116 cells *in vitro,* however, a more direct proliferation assay should be done to confirm these findings. In our cell line derived xenograft model using HCT116 stable A2BR overexpression cells, we observed a significant decrease in both the volume and weight of the tumors compared to the control group. Furthermore, the tumors in the experimental group with low A2BR expression are correlated with higher than average tumor weights, further emphasizing the role of A2BR in tumor growth. Similar findings were observed in previously performed cell line derived xenograft models

using HT29 A2BR overexpression cell lines (data not shown). This indicates that A2BR expression also inhibits tumor growth *in vivo*.

Our data also indicates that A2BR may have an anti-metastatic role in CRC. HT29 cells overexpressing A2BR significantly reduced migration, whereas HCT116 cells with A2BR overexpression only showed a non-significant trend in terms of migration reduction. Our inability to observe a significant migration reduction in HCT116 cells could be because this cell line already exhibits a more aggressive characteristic (Rajput et al., 2008) compared to HT29 cells and simply overexpressing A2BR without further stimulation may not have been adequate to suppress the migratory ability of a more aggressive cell line.

Due to the higher aggressiveness and carcinogenic potential of the HCT116 cell line, we have decided to focus our migration and invasion assays with antagonists to the HCT116 cell line. Upon inhibition of A2BR activity with antagonists, we were able to observe how A2BR affects HCT116 motility. This time, our results showed a significant increase in both the migratory and invasive abilities of the HCT116 cell line. Furthermore, we observed a more mesenchymal EMT marker profile upon treatment with MRS1754 and PSB603 in HCT116 cells. While E-cadherin expression seems to have increased upon MRS1754 treatment, this could be due to partial EMT which gives a survival advantage during metastasis (Sinha et al., 2020). It should also be mentioned that there seems to be a slight inconsistency in the loading control of the antagonist treated samples. This is likely because of the use of β-Actin, which is a cytoskeletal protein involved in cell motility, as a loading control where the morphology of the cells is altered in the experimental group. A non-structural housekeeping protein such as GAPDH would be a more appropriate loading control, however, it could not be used due to its unavailability at the time.

The EMT marker profile in A2BR overexpressed HCT116 cells showed an opposite profile, compared to the pharmacological inhibition, as expected. A2BR overexpressing HCT116 cells showed a shift towards a more epithelial profile, however, changes in the A2BR overexpressing HT29 cell line were not as clear. Although we saw an increase in ZEB1 expression, E-cadherin levels were unchanged and N-cadherin expression was decreased. Furthermore, an increase in Integrin-5α and a decrease in α-SMA expressions, both being mesenchymal markers, were observed. It seems as though EMT is trying to be

initiated in A2BR overexpressing HT29 cell line but is being suppressed, which is seen in the lack of a complete shift towards a mesenchymal profile. This difference in the two cell lines may be due to their different characteristics and should be further investigated.

Despite being identified as a bad prognostic marker in cancers such as triple negative breast cancer (D. Mittal et al., 2016), our prognostic analyses has signified that high A2BR expression leads to a significantly better overall survival as well as recurrence-free survival in CRC patients compared to low A2BR expression. Despite the belief that A2BR antagonists could be used as anti-cancer drugs, high A2BR expression may be a positive prognostic marker in CRC and its inhibition in a clinical setting could negatively affect patient survival (Z.-G. Gao & Jacobson, 2019).

We examined that increase in A2BR expression decreased cell proliferation and tumor growth, inhibited migratory abilities, and shifted the EMT marker profile to a more epithelial one. Furthermore, pharmacological inhibition of A2BR enhanced the proliferative capacity as well as cell motility in terms of migration and invasion. These *in vitro* findings, suggesting an increase in the metastatic ability upon A2BR inhibition, should be further supported by *in vivo* metastasis experiments such as visualization of luminescent cancer cells under an in vivo imager to observe the ability of the cells to accumulate in the lung where they are filtered through after intravenous tail vein injections. More so, prognostic analyses indicated a better prognosis in CRC patients with high A2BR expression.

Overall, our results suggest that A2BR showcases a tumor suppressive effect in colorectal cancer intrinsically and pharmacological inhibition of A2BR activity leads to a more aggressive CRC phenotype. However, the mechanistic insights of these results should be further investigated to fully understand the impact of A2BR in colorectal cancer, perhaps by initally focusing on the context-dependent activation of ERK1/2 pathway through interactions with Gs and Gi proteins.

REFERENCES

Abbracchio, M. P., Burnstock, G., Boeynaems, J.-M., Barnard, E. A., Boyer, J. L., Kennedy, C., Knight, G. E., Fumagalli, M., Gachet, C., Jacobson, K. A., & Weisman, G. A. (2006). International Union of Pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacological Reviews*, *58*(3), 281–341. https://doi.org/10.1124/pr.58.3.3

Antonio, L. S., Stewart, A. P., Varanda, W. A., & Edwardson, J. M. (2014). Identification of P2X2/P2X4/P2X6 heterotrimeric receptors using atomic force microscopy (AFM) imaging. *FEBS Letters*, *588*(12), 2125–2128. https://doi.org/10.1016/j.febslet.2014.04.048

Baker, S. J., Fearon, E. R., Nigro, J. M., Hamilton, S. R., Preisinger, A. C., Jessup, J. M., vanTuinen, P., Ledbetter, D. H., Barker, D. F., Nakamura, Y., White, R., & Vogelstein, B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science (New York, N.Y.)*, *244*(4901), 217–221. https://doi.org/10.1126/science.2649981

Battaglin, F., Naseem, M., Lenz, H.-J., & Salem, M. E. (2018). Microsatellite Instability in Colorectal Cancer: Overview of Its Clinical Significance and Novel Perspectives. *Clinical Advances in Hematology & Oncology : H&O*, *16*(11), 735–745.

Bazzichetto, C., Milella, M., Zampiva, I., Simionato, F., Amoreo, C. A., Buglioni, S., Pacelli, C., Le Pera, L., Colombo, T., Bria, E., Zeuli, M., Del Bufalo, D., Sperduti, I., & Conciatori, F. (2022). Interleukin-8 in Colorectal Cancer: A Systematic Review and Meta-Analysis of Its Potential Role as a Prognostic Biomarker. *Biomedicines*, *10*(10), 2631. https://doi.org/10.3390/biomedicines10102631

Berg, K. C. G., Eide, P. W., Eilertsen, I. A., Johannessen, B., Bruun, J., Danielsen, S. A., Bjørnslett, M., Meza-Zepeda, L. A., Eknæs, M., Lind, G. E., Myklebost, O., Skotheim, R. I., Sveen, A., & Lothe, R. A. (2017). Multi-omics of 34 colorectal cancer cell lines—A resource for biomedical studies. *Molecular Cancer*, *16*(1), 116. https://doi.org/10.1186/s12943-017-0691-y

Botteri, E., Borroni, E., Sloan, E. K., Bagnardi, V., Bosetti, C., Peveri, G., Santucci, C., Specchia, C., van den Brandt, P., Gallus, S., & Lugo, A. (2020). Smoking and Colorectal Cancer Risk, Overall and by Molecular Subtypes: A Meta-Analysis. *The American Journal of Gastroenterology*, *115*(12), 1940–1949. https://doi.org/10.14309/ajg.0000000000000803

Bull, C. J., Bell, J. A., Murphy, N., Sanderson, E., Davey Smith, G., Timpson, N. J., Banbury, B. L., Albanes, D., Berndt, S. I., Bézieau, S., Bishop, D. T., Brenner, H., Buchanan, D. D., Burnett-Hartman, A., Casey, G., Castellví-Bel, S., Chan, A. T., Chang-Claude, J., Cross, A. J., … Gunter, M. J. (2020). Adiposity, metabolites, and colorectal cancer risk: Mendelian randomization study. *BMC Medicine*, *18*(1), 396. https://doi.org/10.1186/s12916-020-01855-9

Burnstock, G. (2006). Purinergic signalling. *British Journal of Pharmacology*, *147 Suppl 1*(Suppl 1), S172-181. https://doi.org/10.1038/sj.bjp.0706429

Burnstock, G. (2018). Purine and purinergic receptors. *Brain and Neuroscience Advances*, *2*, 2398212818817494. https://doi.org/10.1177/2398212818817494

Burnstock, G., & Verkhratsky, A. (2009). Evolutionary origins of the purinergic signalling system. *Acta Physiologica (Oxford, England)*, *195*(4), 415–447. https://doi.org/10.1111/j.1748-1716.2009.01957.x

Cai, S., Li, Y., Ding, Y., Chen, K., & Jin, M. (2014). Alcohol drinking and the risk of colorectal cancer death: A meta-analysis. *European Journal of Cancer Prevention: The Official Journal of the European Cancer Prevention Organisation (ECP)*, *23*(6), 532–539. https://doi.org/10.1097/CEJ.0000000000000076

Campos-Contreras, A. del R., González-Gallardo, A., Díaz-Muñoz, M., & Vázquez-Cuevas, F. G. (2022). Adenosine Receptor A2B Negatively Regulates Cell Migration in Ovarian Carcinoma Cells. *International Journal of Molecular Sciences*, *23*(9), Article 9. https://doi.org/10.3390/ijms23094585

Cekic, C., Sag, D., Li, Y., Theodorescu, D., Strieter, R. M., & Linden, J. (2012). Adenosine A2B receptor blockade slows growth of bladder and breast tumors. *Journal of Immunology (Baltimore, Md.: 1950)*, *188*(1), 198–205. https://doi.org/10.4049/jimmunol.1101845

Cheng, J., Chen, Y., Wang, X., Wang, J., Yan, Z., Gong, G., Li, G., & Li, C. (2015). Meta-analysis of prospective cohort studies of cigarette smoking and the incidence of colon and rectal cancers. *European Journal of Cancer Prevention: The Official Journal of the European Cancer Prevention Organisation (ECP)*, *24*(1), 6–15. https://doi.org/10.1097/CEJ.0000000000000011

Coddou, C., Yan, Z., Obsil, T., Huidobro-Toro, J. P., & Stojilkovic, S. S. (2011). Activation and regulation of purinergic P2X receptor channels. *Pharmacological Reviews*, *63*(3), 641–683. https://doi.org/10.1124/pr.110.003129

Coppedè, Coppedè F, Lopomo A, Spisni R, Migliore L. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. World J Gastroenterol 2014; 20(4): 943-956 [PMID: 24574767 DOI: 10.3748/wjg.v20.i4.943], Coppedè F, Lopomo A, Spisni R, Migliore L. Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. World J Gastroenterol 2014; 20(4): 943-956 [PMID: 24574767 DOI: 10.3748/wjg.v20.i4.943], Coppedè, F., & Angela Lopomo, R. S. (2014). Genetic and epigenetic biomarkers for diagnosis, prognosis and treatment of colorectal cancer. *World Journal of Gastroenterology*, *20*(4), 943–956. https://doi.org/10.3748/wjg.v20.i4.943

Dai, Z., Coker, O. O., Nakatsu, G., Wu, W. K. K., Zhao, L., Chen, Z., Chan, F. K. L., Kristiansen, K., Sung, J. J. Y., Wong, S. H., & Yu, J. (2018). Multi-cohort analysis of colorectal cancer metagenome identified altered bacteria across populations and universal bacterial markers. *Microbiome*, *6*(1), 70. https://doi.org/10.1186/s40168-018-0451-2

Darashchonak, N., Koepsell, B., Bogdanova, N., & von Versen-Höynck, F. (2014). Adenosine A2B receptors induce proliferation, invasion and activation of cAMP response element binding protein (CREB) in trophoblast cells. *BMC Pregnancy and Childbirth*, *14*, 2. https://doi.org/10.1186/1471-2393-14-2

Dekker, E., Tanis, P. J., Vleugels, J. L. A., Kasi, P. M., & Wallace, M. B. (2019). Colorectal cancer. *The Lancet*, *394*(10207), 1467–1480. https://doi.org/10.1016/S0140-6736(19)32319-0

Di Virgilio, F. (2012). Purines, Purinergic Receptors, and Cancer. *Cancer Research*, *72*(21), 5441–5447. https://doi.org/10.1158/0008-5472.CAN-12-1600

Di Virgilio, F., & Adinolfi, E. (2017). Extracellular purines, purinergic receptors and tumor growth. *Oncogene*, *36*(3), 293–303. https://doi.org/10.1038/onc.2016.206

Di Virgilio, F., Sarti, A. C., Falzoni, S., De Marchi, E., & Adinolfi, E. (2018). Extracellular ATP and P2 purinergic signalling in the tumour microenvironment. *Nature Reviews. Cancer*, *18*(10), 601–618. https://doi.org/10.1038/s41568-018-0037-0

Dinu, D., Dobre, M., Panaitescu, E., Bîrlă, R., Iosif, C., Hoara, P., Caragui, A., Boeriu, M., Constantinoiu, S., & Ardeleanu, C. (2014). Prognostic significance of KRAS gene mutations in colorectal cancer—Preliminary study. *Journal of Medicine and Life*, *7*(4), 581–587.

Eltzschig, H. K., Sitkovsky, M. V., & Robson, S. C. (2012). Purinergic signaling during inflammation. *The New England Journal of Medicine*, *367*(24), 2322–2333. https://doi.org/10.1056/NEJMra1205750

Fang, Y., & Olah, M. E. (2007). Cyclic AMP-dependent, protein kinase A-independent activation of extracellular signal-regulated kinase 1/2 following adenosine receptor stimulation in human umbilical vein endothelial cells: Role of exchange protein activated by cAMP 1 (Epac1). *The Journal of Pharmacology and Experimental Therapeutics*, *322*(3), 1189–1200. https://doi.org/10.1124/jpet.107.119933

Fearon, E. R. (2011). Molecular Genetics of Colorectal Cancer. *Annual Review of Pathology: Mechanisms of Disease*, *6*(Volume 6, 2011), 479–507. https://doi.org/10.1146/annurev-pathol-011110-130235

Fearon, E. R., & Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. *Cell*, *61*(5), 759–767. https://doi.org/10.1016/0092-8674(90)90186-I

Feoktistov, I., & Biaggioni, I. (2011). Role of adenosine A2B receptors in inflammation. *Advances in Pharmacology (San Diego, Calif.)*, *61*, 115–144. https://doi.org/10.1016/B978-0-12-385526-8.00005-9

Feoktistov, I., Goldstein, A. E., Ryzhov, S., Zeng, D., Belardinelli, L., Voyno-Yasenetskaya, T., & Biaggioni, I. (2002). Differential expression of adenosine

receptors in human endothelial cells: Role of A2B receptors in angiogenic factor regulation. *Circulation Research*, *90*(5), 531–538. https://doi.org/10.1161/01.res.0000012203.21416.14

Fleming, M., Ravula, S., Tatishchev, S. F., & Wang, H. L. (2012). Colorectal carcinoma: Pathologic aspects. *Journal of Gastrointestinal Oncology*, *3*(3). https://doi.org/10.3978/j.issn.2078-6891.2012.030

Fredholm, B. B., IJzerman, A. P., Jacobson, K. A., Klotz, K. N., & Linden, J. (2001). International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacological Reviews*, *53*(4), 527–552.

Galván, J. A., Helbling, M., Koelzer, V. H., Tschan, M. P., Berger, M. D., Hädrich, M., Schnüriger, B., Karamitopoulou, E., Dawson, H., Inderbitzin, D., Lugli, A., & Zlobec, I. (2014). TWIST1 and TWIST2 promoter methylation and protein expression in tumor stroma influence the epithelial-mesenchymal transition-like tumor budding phenotype in colorectal cancer. *Oncotarget*, *6*(2), 874–885. https://doi.org/10.18632/oncotarget.2716

Gao, Z. G., Inoue, A., & Jacobson, K. A. (2018). On the G protein-coupling selectivity of the native A2B adenosine receptor. *Biochemical Pharmacology*, *151*, 201–213. https://doi.org/10.1016/j.bcp.2017.12.003

Gao, Z.-G., Gao, R. R., Meyer, C. K., & Jacobson, K. A. (2025). A2B adenosine receptor-triggered intracellular calcium mobilization: Cell type-dependent involvement of Gi, Gq, Gs proteins and protein kinase C. *Purinergic Signalling*. https://doi.org/10.1007/s11302-025-10070-1

Gao, Z.-G., Haddad, M., & Jacobson, K. A. (2025). A2B adenosine receptor signaling and regulation. *Purinergic Signalling*, *21*(2), 201–220. https://doi.org/10.1007/s11302-024-10025-y

Gao, Z.-G., Inoue, A., & Jacobson, K. A. (2018). On the G protein-coupling selectivity of the native A2B adenosine receptor. *Biochemical Pharmacology*, *151*, 201–213. https://doi.org/10.1016/j.bcp.2017.12.003

Gao, Z.-G., & Jacobson, K. A. (2019). A2B Adenosine Receptor and Cancer. *International Journal of Molecular Sciences*, *20*(20), 5139. https://doi.org/10.3390/ijms20205139

Gerstberger, S., Jiang, Q., & Ganesh, K. (2023). Metastasis. *Cell*, *186*(8), 1564–1579. https://doi.org/10.1016/j.cell.2023.03.003

Goulding, J., May, L. T., & Hill, S. J. (2018). Characterisation of endogenous A2A and A2B receptor-mediated cyclic AMP responses in HEK 293 cells using the GloSensor™ biosensor: Evidence for an allosteric mechanism of action for the A2B-selective antagonist PSB 603. *Biochemical Pharmacology*, *147*, 55–66. https://doi.org/10.1016/j.bcp.2017.10.013

Gram, I. T., Park, S.-Y., Wilkens, L. R., Haiman, C. A., & Le Marchand, L. (2020). Smoking-Related Risks of Colorectal Cancer by Anatomical Subsite and Sex. *American Journal of Epidemiology*, *189*(6), 543–553. https://doi.org/10.1093/aje/kwaa005

Hajizadeh, F., Masjedi, A., Heydarzedeh Asl, S., Karoon Kiani, F., Peydaveisi, M., Ghalamfarsa, G., Jadidi-Niaragh, F., & Sevbitov, A. (2020). Adenosine and adenosine receptors in colorectal cancer. *International Immunopharmacology*, *87*, 106853. https://doi.org/10.1016/j.intimp.2020.106853

Hampel, H., Frankel, W. L., Martin, E., Arnold, M., Khanduja, K., Kuebler, P., Nakagawa, H., Sotamaa, K., Prior, T. W., Westman, J., Panescu, J., Fix, D., Lockman, J., Comeras, I., & de la Chapelle, A. (2005). Screening for the Lynch syndrome (hereditary nonpolyposis colorectal cancer). *The New England Journal of Medicine*, *352*(18), 1851–1860. https://doi.org/10.1056/NEJMoa043146

HCT116 Cell Line—A Comprehensive Guide to Colorectal Cancer Research. (n.d.). Cytion. Retrieved July 8, 2025, from https://www.cytion.com/Knowledge-Hub/Cell-Line-Insights/HCT116-Cell-Line-A-Comprehensive-Guide-to-Colorectal-Cancer-Research/

Henderson, T. O., Oeffinger, K. C., Whitton, J., Leisenring, W., Neglia, J., Meadows, A., Crotty, C., Rubin, D. T., Diller, L., Inskip, P., Smith, S. A., Stovall, M., Constine, L. S., Hammond, S., Armstrong, G. T., Robison, L. L., & Nathan, P. C. (2012). Secondary gastrointestinal cancer in childhood cancer survivors: A cohort study. *Annals of Internal Medicine*, *156*(11), 757–766, W-260. https://doi.org/10.7326/0003-4819-156-11-201206050-00002

Ho, C.-H., Cheng, K.-C., Chao, C.-M., Lai, C.-C., Chiang, S.-R., Chen, C.-M., Liao, K.-M., Wang, J.-J., Lee, P.-H., Hung, C.-M., Tai, C.-M., & Chiu, C.-C. (2020). Does radiotherapy increase the risk of colorectal cancer among prostate cancer patients? A large population-based study. *Journal of Cancer*, *11*(21), 6204–6212. https://doi.org/10.7150/jca.44726

HT-29: Human Colorectal Adenocarcinoma Cell Line (ATCC HTB-38) | Memorial Sloan Kettering Cancer Center. (n.d.). Retrieved July 8, 2025, from https://www.mskcc.org/research-advantage/support/technology/tangible-material/human-colorectal-adenocarcinoma-cell-line-ht-29

Huang, Z., Xie, N., Illes, P., Di Virgilio, F., Ulrich, H., Semyanov, A., Verkhratsky, A., Sperlagh, B., Yu, S.-G., Huang, C., & Tang, Y. (2021). From purines to purinergic signalling: Molecular functions and human diseases. *Signal Transduction and Targeted Therapy*, *6*(1), 1–20. https://doi.org/10.1038/s41392-021-00553-z

Huang, Z., & Yang, M. (2022). Molecular Network of Colorectal Cancer and Current Therapeutic Options. *Frontiers in Oncology*, *12*. https://doi.org/10.3389/fonc.2022.852927

Hughes, L. A. E., Khalid-de Bakker, C. A. J., Smits, K. M., van den Brandt, P. A., Jonkers, D., Ahuja, N., Herman, J. G., Weijenberg, M. P., & van Engeland, M. (2012). The CpG island methylator phenotype in colorectal cancer: Progress and problems. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, *1825*(1), 77–85. https://doi.org/10.1016/j.bbcan.2011.10.005

Ionescu, V. A., Gheorghe, G., Bacalbasa, N., Chiotoroiu, A. L., & Diaconu, C. (2023). Colorectal Cancer: From Risk Factors to Oncogenesis. *Medicina*, *59*(9), Article 9. https://doi.org/10.3390/medicina59091646

Ji, X., Kim, Y. C., Ahern, D. G., Linden, J., & Jacobson, K. A. (2001). [3H]MRS 1754, a selective antagonist radioligand for A(2B) adenosine receptors. *Biochemical Pharmacology*, *61*(6), 657–663. https://doi.org/10.1016/s0006-2952(01)00531-7

Kasama, H., Sakamoto, Y., Kasamatsu, A., Okamoto, A., Koyama, T., Minakawa, Y., Ogawara, K., Yokoe, H., Shiiba, M., Tanzawa, H., & Uzawa, K. (2015). Adenosine A2b receptor promotes progression of human oral cancer. *BMC Cancer*, *15*(1), 563. https://doi.org/10.1186/s12885-015-1577-2

Keum, N., & Giovannucci, E. (2019). Global burden of colorectal cancer: Emerging trends, risk factors and prevention strategies. *Nature Reviews Gastroenterology & Hepatology*, *16*(12), 713–732. https://doi.org/10.1038/s41575-019-0189-8

Kim, D.-S., & Scherer, P. E. (2021). Obesity, Diabetes, and Increased Cancer Progression. *Diabetes & Metabolism Journal*, *45*(6), 799–812. https://doi.org/10.4093/dmj.2021.0077

Kirley, S. D., D'Apuzzo ,Massimo, Lauwers ,Gregory Y., Graeme-Cook ,Fiona, Chung ,Daniel C., & and Zukerberg, L. R. (2005). The cables gene on chromosome 18Q regulates colon cancer progression in vivo. *Cancer Biology & Therapy*, *4*(8), 861–863. https://doi.org/10.4161/cbt.4.8.1894

Lan, J., Lu, H., Samanta, D., Salman, S., Lu, Y., & Semenza, G. L. (2018). Hypoxia-inducible factor 1-dependent expression of adenosine receptor 2B promotes breast cancer stem cell enrichment. *Proceedings of the National Academy of Sciences*, *115*(41), E9640–E9648. https://doi.org/10.1073/pnas.1809695115

Lao, V. V., & Grady, W. M. (2011). Epigenetics and colorectal cancer. *Nature Reviews Gastroenterology & Hepatology*, *8*(12), 686–700. https://doi.org/10.1038/nrgastro.2011.173

Le, T.-T. T., Berg, N. K., Harting, M. T., Li, X., Eltzschig, H. K., & Yuan, X. (2019). Purinergic Signaling in Pulmonary Inflammation. *Frontiers in Immunology*, *10*, 1633. https://doi.org/10.3389/fimmu.2019.01633

Lee, C. S., Song, I. H., Lee, A., Kang, J., Lee, Y. S., Lee, I. K., Song, Y. S., & Lee, S. H. (2021). Enhancing the landscape of colorectal cancer using targeted deep sequencing. *Scientific Reports*, *11*(1), 8154. https://doi.org/10.1038/s41598-021-87486-3

Liebl, M. C., & Hofmann, T. G. (2021). The Role of p53 Signaling in Colorectal Cancer. *Cancers*, *13*(9), 2125. https://doi.org/10.3390/cancers13092125

Linden, J., Thai, T., Figler, H., Jin, X., & Robeva, A. S. (1999). Characterization of human A(2B) adenosine receptors: Radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. *Molecular Pharmacology*, *56*(4), 705–713.

Loh, C.-Y., Chai, J. Y., Tang, T. F., Wong, W. F., Sethi, G., Shanmugam, M. K., Chong, P. P., & Looi, C. Y. (2019). The E-Cadherin and N-Cadherin Switch in Epithelial-to-Mesenchymal Transition: Signaling, Therapeutic Implications, and Challenges. *Cells*, *8*(10), 1118. https://doi.org/10.3390/cells8101118

Longhi, M. S., Robson, S. C., Bernstein, S. H., Serra, S., & Deaglio, S. (2013). Biological functions of ecto-enzymes in regulating extracellular adenosine levels in neoplastic and inflammatory disease states. *Journal of Molecular Medicine (Berlin, Germany)*, *91*(2), 165–172. https://doi.org/10.1007/s00109-012-0991-z

Lu, J., Kornmann, M., & Traub, B. (2023). Role of Epithelial to Mesenchymal Transition in Colorectal Cancer. *International Journal of Molecular Sciences*, *24*(19), 14815. https://doi.org/10.3390/ijms241914815

Ma, D.-F., Kondo, T., Nakazawa, T., Niu, D.-F., Mochizuki, K., Kawasaki, T., Yamane, T., & Katoh, R. (2010). Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. *Human Pathology*, *41*(11), 1550–1557. https://doi.org/10.1016/j.humpath.2010.04.008

Malki, A., ElRuz, R. A., Gupta, I., Allouch, A., Vranic, S., & Al Moustafa, A.-E. (2020). Molecular Mechanisms of Colon Cancer Progression and Metastasis: Recent Insights and Advancements. *International Journal of Molecular Sciences*, *22*(1), 130. https://doi.org/10.3390/ijms22010130

Massimi, M., Cardarelli, S., Galli, F., Giardi, M. F., Ragusa, F., Panera, N., Cinque, B., Cifone, M. G., Biagioni, S., & Giorgi, M. (2017). Increase of Intracellular Cyclic AMP by PDE4 Inhibitors Affects HepG2 Cell Cycle Progression and Survival. *Journal of Cellular Biochemistry*, *118*(6), 1401–1411. https://doi.org/10.1002/jcb.25798

McNabb, S., Harrison, T. A., Albanes, D., Berndt, S. I., Brenner, H., Caan, B. J., Campbell, P. T., Cao, Y., Chang-Claude, J., Chan, A., Chen, Z., English, D. R., Giles, G. G., Giovannucci, E. L., Goodman, P. J., Hayes, R. B., Hoffmeister, M., Jacobs, E. J., Joshi, A. D., … Peters, U. (2020). Meta-analysis of 16 studies of the association of alcohol with colorectal cancer. *International Journal of Cancer*, *146*(3), 861–873. https://doi.org/10.1002/ijc.32377

Merighi, S., Simioni, C., Gessi, S., Varani, K., Mirandola, P., Tabrizi, M. A., Baraldi, P. G., & Borea, P. A. (2009). A(2B) and A(3) adenosine receptors modulate vascular endothelial growth factor and interleukin-8 expression in human melanoma cells treated with etoposide and doxorubicin. *Neoplasia (New York, N.Y.)*, *11*(10), 1064–1073. https://doi.org/10.1593/neo.09768

Mittal, D., Sinha, D., Barkauskas, D., Young, A., Kalimutho, M., Stannard, K., Caramia, F., Haibe-Kains, B., Stagg, J., Khanna, K. K., Loi, S., & Smyth, M. J. (2016). Adenosine 2B Receptor Expression on Cancer Cells Promotes Metastasis. *Cancer Research*, *76*(15), 4372–4382. https://doi.org/10.1158/0008-5472.CAN-16-0544

Mittal, V. (2018). Epithelial Mesenchymal Transition in Tumor Metastasis. *Annual Review of Pathology*, *13*, 395–412. https://doi.org/10.1146/annurev-pathol-020117-043854

Mizukoshi, K., Okazawa, Y., Haeno, H., Koyama, Y., Sulidan, K., Komiyama, H., Saeki, H., Ohtsuji, N., Ito, Y., Kojima, Y., Goto, M., Habu, S., Hino, O., Sakamoto, K., & Orimo, A. (2020). Metastatic seeding of human colon cancer cell clusters expressing the hybrid epithelial/mesenchymal state. *International Journal of Cancer*, *146*(9), 2547–2562. https://doi.org/10.1002/ijc.32672

Mojarad, E. N., Kuppen, P. J., Aghdaei, H. A., & Zali, M. R. (2013). The CpG island methylator phenotype (CIMP) in colorectal cancer. *Gastroenterology and Hepatology From Bed to Bench*, *6*(3), 120–128.

MRS1754 | A2BR antagonist | Probechem Biochemicals. (n.d.). Retrieved July 8, 2025, from https://www.probechem.com/products_MRS1754.html

Nagao, K., Koshino, A., Sugimura-Nagata, A., Nagano, A., Komura, M., Ueki, A., Ebi, M., Ogasawara, N., Tsuzuki, T., Kasai, K., Takahashi, S., Kasugai, K., & Inaguma, S. (2022). The Complete Loss of p53 Expression Uniquely Predicts Worse Prognosis in Colorectal Cancer. *International Journal of Molecular Sciences*, *23*(6), 3252. https://doi.org/10.3390/ijms23063252

Navarro, G., Cordomí, A., Zelman-Femiak, M., Brugarolas, M., Moreno, E., Aguinaga, D., Perez-Benito, L., Cortés, A., Casadó, V., Mallol, J., Canela, E. I., Lluís, C., Pardo, L., García-Sáez, A. J., McCormick, P. J., & Franco, R. (2016). Quaternary

structure of a G-protein-coupled receptor heterotetramer in complex with Gi and Gs. *BMC Biology*, *14*(1), 26. https://doi.org/10.1186/s12915-016-0247-4

Neumann, J., Zeindl-Eberhart, E., Kirchner, T., & Jung, A. (2009). Frequency and type of *KRAS* mutations in routine diagnostic analysis of metastatic colorectal cancer. *Pathology - Research and Practice*, *205*(12), 858–862. https://doi.org/10.1016/j.prp.2009.07.010

Nguyen, H. T., & Duong, H.-Q. (2018). The molecular characteristics of colorectal cancer: Implications for diagnosis and therapy (Review). *Oncology Letters*, *16*(1), 9–18. https://doi.org/10.3892/ol.2018.8679

Nguyen, T. T., Ung, T. T., Kim, N. H., & Jung, Y. D. (2018). Role of bile acids in colon carcinogenesis. *World Journal of Clinical Cases*, *6*(13), 577–588. https://doi.org/10.12998/wjcc.v6.i13.577

Nojadeh, J. N., Behrouz Sharif, S., & Sakhinia, E. (2018). Microsatellite instability in colorectal cancer. *EXCLI Journal*, *17*, 159–168. https://doi.org/10.17179/excli2017-948

Ogino, S. (2006). CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. *Gut*, *55*(7), 1000–1006. https://doi.org/10.1136/gut.2005.082933

Parfenyev, S. E., Daks, A. A., Shuvalov, O. Y., Fedorova, O. A., Pestov, N. B., Korneenko, T. V., & Barlev, N. A. (2025). Dualistic role of ZEB1 and ZEB2 in tumor progression. *Biology Direct*, *20*(1), 32. https://doi.org/10.1186/s13062-025-00604-3

Park, D. Y., Sakamoto, H., Kirley, S. D., Ogino, S., Kawasaki, T., Kwon, E., Mino-Kenudson, M., Lauwers, G. Y., Chung, D. C., Rueda, B. R., & Zukerberg, L. R. (2007). The Cables Gene on Chromosome 18q Is Silenced by Promoter Hypermethylation and Allelic Loss in Human Colorectal Cancer. *The American Journal of Pathology*, *171*(5), 1509–1519. https://doi.org/10.2353/ajpath.2007.070331

Pierantoni, C., Cosentino, L., & Ricciardiello, L. (2024). Molecular Pathways of Colorectal Cancer Development: Mechanisms of Action and Evolution of Main

Systemic Therapy Compunds. *Digestive Diseases*, *42*(4), 319–324. https://doi.org/10.1159/000538511

Pino, M. S., & Chung, D. C. (2010). The Chromosomal Instability Pathway in Colon Cancer. *Gastroenterology*, *138*(6), 2059–2072. https://doi.org/10.1053/j.gastro.2009.12.065

PSB 603 | Adenosine A2B Receptors. (n.d.). Tocris Bioscience. Retrieved July 8, 2025, from https://www.tocris.com/products/psb-603_3198

Rajagopal, M., & Pao, A. C. (2010). Adenosine Activates A2b Receptors and Enhances Chloride Secretion in Kidney Inner Medullary Collecting Duct Cells. *Hypertension*, *55*(5), 1123–1128. https://doi.org/10.1161/HYPERTENSIONAHA.109.143404

Rajput, A., Dominguez San Martin, I., Rose, R., Beko, A., LeVea, C., Sharratt, E., Mazurchuk, R., Hoffman, R. M., Brattain, M. G., & Wang, J. (2008). Characterization of HCT116 Human Colon Cancer Cells in an Orthotopic Model. *Journal of Surgical Research*, *147*(2), 276–281. https://doi.org/10.1016/j.jss.2007.04.021

Roliano, G. G., Azambuja, J. H., Brunetto, V. T., Butterfield, H. E., Kalil, A. N., & Braganhol, E. (2022). Colorectal Cancer and Purinergic Signalling: An Overview. *Cancers*, *14*(19), Article 19. https://doi.org/10.3390/cancers14194887

Roshandel, G., Ghasemi-Kebria, F., & Malekzadeh, R. (2024). Colorectal Cancer: Epidemiology, Risk Factors, and Prevention. *Cancers*, *16*(8), Article 8. https://doi.org/10.3390/cancers16081530

Rumpold, H., Niedersüß-Beke, D., Heiler, C., Falch, D., Wundsam, H. V., Metz-Gercek, S., Piringer, G., & Thaler, J. (2020). *Prediction of mortality in metastatic colorectal cancer in a real-life population: A multicenter explorative analysis | BMC Cancer | Full Text*. https://bmccancer.biomedcentral.com/articles/10.1186/s12885-020-07656-w

Sachdeva, S., & Gupta, M. (2013). Adenosine and its receptors as therapeutic targets: An overview. *Saudi Pharmaceutical Journal : SPJ*, *21*(3), 245–253. https://doi.org/10.1016/j.jsps.2012.05.011

Scheuplein, F., Schwarz, N., Adriouch, S., Krebs, C., Bannas, P., Rissiek, B., Seman, M., Haag, F., & Koch-Nolte, F. (2009). NAD+ and ATP released from injured cells induce P2X7-dependent shedding of CD62L and externalization of phosphatidylserine by murine T cells. *Journal of Immunology (Baltimore, Md.: 1950)*, *182*(5), 2898–2908. https://doi.org/10.4049/jimmunol.0801711

Schmid, R., & Evans, R. J. (2019). ATP-Gated P2X Receptor Channels: Molecular Insights into Functional Roles. *Annual Review of Physiology*, *81*, 43–62. https://doi.org/10.1146/annurev-physiol-020518-114259

Scott, P., Anderson, K., Singhania, M., & Cormier, R. (2020). Cystic Fibrosis, CFTR, and Colorectal Cancer. *International Journal of Molecular Sciences*, *21*(8), 2891. https://doi.org/10.3390/ijms21082891

Sepúlveda, C., Palomo, I., & Fuentes, E. (2016). Role of adenosine A2b receptor overexpression in tumor progression. *Life Sciences*, *166*, 92–99. https://doi.org/10.1016/j.lfs.2016.10.008

Shaikh, R., Bhattacharya, S., & Prajapati, B. G. (2024). Microsatellite instability: A potential game-changer in colorectal cancer diagnosis and treatment. *Results in Chemistry*, *7*, 101461. https://doi.org/10.1016/j.rechem.2024.101461

Shen, Y., Tang, G., Gao, P., Zhang, B., Xiao, H., & Si, L.-Y. (2018). Activation of adenosine A2b receptor attenuates high glucose-induced apoptosis in H9C2 cells via PI3K/Akt signaling. *In Vitro Cellular & Developmental Biology. Animal*, *54*(5), 384–391. https://doi.org/10.1007/s11626-018-0241-y

Shin, A. E., Giancotti, F. G., & Rustgi, A. K. (2023). Metastatic colorectal cancer: Mechanisms and emerging therapeutics. *Trends in Pharmacological Sciences*, *44*(4), 222–236. https://doi.org/10.1016/j.tips.2023.01.003

Simon, K. (2016). Colorectal cancer development and advances in screening. *Clinical Interventions in Aging*, *11*, 967–976. https://doi.org/10.2147/CIA.S109285

Sinha, D., Saha, P., Samanta, A., & Bishayee, A. (2020). Emerging Concepts of Hybrid Epithelial-to-Mesenchymal Transition in Cancer Progression. *Biomolecules*, *10*(11), 1561. https://doi.org/10.3390/biom10111561

Sinicrope, F. A. (2010). DNA mismatch repair and adjuvant chemotherapy in sporadic colon cancer. *Nature Reviews Clinical Oncology*, *7*(3), 174–177. https://doi.org/10.1038/nrclinonc.2009.235

Solomon, H., Dinowitz, N., Pateras, I. S., Cooks, T., Shetzer, Y., Molchadsky, A., Charni, M., Rabani, S., Koifman, G., Tarcic, O., Porat, Z., Kogan-Sakin, I., Goldfinger, N., Oren, M., Harris, C. C., Gorgoulis, V. G., & Rotter, V. (2018). Mutant p53 gain of function underlies high expression levels of colorectal cancer stem cells markers. *Oncogene*, *37*(12), 1669–1684. https://doi.org/10.1038/s41388-017-0060-8

Soltani, G., Poursheikhani, A., Yassi, M., Hayatbakhsh, A., Kerachian, M., & Kerachian, M. A. (2019). Obesity, diabetes and the risk of colorectal adenoma and cancer. *BMC Endocrine Disorders*, *19*(1), 113. https://doi.org/10.1186/s12902-019-0444-6

Sreekumar, R., Harris, S., Moutasim, K., DeMateos, R., Patel, A., Emo, K., White, S., Yagci, T., Tulchinsky, E., Thomas, G., Primrose, J. N., Sayan, A. E., & Mirnezami, A. H. (2018). Assessment of Nuclear ZEB2 as a Biomarker for Colorectal Cancer Outcome and TNM Risk Stratification. *JAMA Network Open*, *1*(6), e183115. https://doi.org/10.1001/jamanetworkopen.2018.3115

Stanczak, A., Stec, R., Bodnar, L., Olszewski, W., Cichowicz, M., Kozlowski, W., Szczylik, C., Pietrucha, T., Wieczorek, M., & Lamparska-Przybysz, M. (2011). Prognostic Significance of Wnt-1, β-catenin and E-cadherin Expression in Advanced Colorectal Carcinoma. *Pathology & Oncology Research*, *17*(4), 955–963. https://doi.org/10.1007/s12253-011-9409-4

Sun, S., Ning, X., Zhang, Y., Lu, Y., Nie, Y., Han, S., Liu, L., Du, R., Xia, L., He, L., & Fan, D. (2009). Hypoxia-inducible factor-1alpha induces Twist expression in tubular epithelial cells subjected to hypoxia, leading to epithelial-to-mesenchymal transition. *Kidney International*, *75*(12), 1278–1287. https://doi.org/10.1038/ki.2009.62

Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence

and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660

Uygur, B. (2021). *Characterization of extracellular purinergic signaling components in colorectal carcinoma*. http://hdl.handle.net/11693/55128

Valera, S., Hussy, N., Evans, R. J., Adami, N., North, R. A., Surprenant, A., & Buell, G. (1994). A new class of ligand-gated ion channel defined by P2x receptor for extracellular ATP. *Nature*, *371*(6497), 516–519. https://doi.org/10.1038/371516a0

Valle, L., de Voer, R. M., Goldberg, Y., Sjursen, W., Försti, A., Ruiz-Ponte, C., Caldés, T., Garré, P., Olsen, M. F., Nordling, M., Castellvi-Bel, S., & Hemminki, K. (2019). Update on genetic predisposition to colorectal cancer and polyposis. *Molecular Aspects of Medicine*, *69*, 10–26. https://doi.org/10.1016/j.mam.2019.03.001

Veettil, S. K., Wong, T. Y., Loo, Y. S., Playdon, M. C., Lai, N. M., Giovannucci, E. L., & Chaiyakunapruk, N. (2021). Role of Diet in Colorectal Cancer Incidence: Umbrella Review of Meta-analyses of Prospective Observational Studies. *JAMA Network Open*, *4*(2), e2037341. https://doi.org/10.1001/jamanetworkopen.2020.37341

Vogelstein, B., & Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nature Medicine*, *10*(8), 789–799. https://doi.org/10.1038/nm1087

Voss, J. H., Mahardhika, A. B., Inoue, A., & Müller, C. E. (2022). Agonist-Dependent Coupling of the Promiscuous Adenosine A2B Receptor to Gα Protein Subunits. *ACS Pharmacology & Translational Science*, *5*(5), 373–386. https://doi.org/10.1021/acsptsci.2c00020

Vu, T., & Datta, P. K. (2017). Regulation of EMT in Colorectal Cancer: A Culprit in Metastasis. *Cancers*, *9*(12), Article 12. https://doi.org/10.3390/cancers9120171

Wang, Y., Shi, J., Chai, K., Ying, X., & Zhou, B. P. (2013). The Role of Snail in EMT and Tumorigenesis. *Current Cancer Drug Targets*, *13*(9), 963–972. https://doi.org/10.2174/15680096113136660102

Wang, Y., Wang, P., & Shao, L. (2021). Correlation of ulcerative colitis and colorectal cancer: A systematic review and meta-analysis. *Journal of Gastrointestinal Oncology*, *12*(6). https://doi.org/10.21037/jgo-21-624

Warabi, M., Kitagawa, M., & Hirokawa, K. (2000). Loss of MHC class II expression is associated with a decrease of tumor-infiltrating T cells and an increase of metastatic potential of colorectal cancer: Immunohistological and histopathological analyses as compared with normal colonic mucosa and adenomas. *Pathology - Research and Practice*, *196*(12), 807–815. https://doi.org/10.1016/S0344-0338(00)80080-1

Watson, M. J., Lee, S. L., Marklew, A. J., Gilmore, R. C., Gentzsch, M., Sassano, M. F., Gray, M. A., & Tarran, R. (2016). The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Uses its C-Terminus to Regulate the A2B Adenosine Receptor. *Scientific Reports*, *6*(1), 27390. https://doi.org/10.1038/srep27390

Wei, Q., Costanzi, S., Balasubramanian, R., Gao, Z.-G., & Jacobson, K. A. (2013). A2B adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signalling*, *9*(2), 271–280. https://doi.org/10.1007/s11302-012-9350-3

Wong, S. H., & Yu, J. (2019). Gut microbiota in colorectal cancer: Mechanisms of action and clinical applications. *Nature Reviews Gastroenterology & Hepatology*, *16*(11), 690–704. https://doi.org/10.1038/s41575-019-0209-8

Yang, Z., Wang, X., Zhou, H., Jiang, M., Wang, J., & Sui, B. (2024). Molecular Complexity of Colorectal Cancer: Pathways, Biomarkers, and Therapeutic Strategies. *Cancer Management and Research*, *16*, 1389–1403. https://doi.org/10.2147/CMAR.S481656

Ye, P., Xi, Y., Huang, Z., & Xu, P. (2020). Linking Obesity with Colorectal Cancer: Epidemiology and Mechanistic Insights. *Cancers*, *12*(6), Article 6. https://doi.org/10.3390/cancers12061408

Yurgelun, M. B., Kulke, M. H., Fuchs, C. S., Allen, B. A., Uno, H., Hornick, J. L., Ukaegbu, C. I., Brais, L. K., McNamara, P. G., Mayer, R. J., Schrag, D., Meyerhardt, J. A., Ng, K., Kidd, J., Singh, N., Hartman, A.-R., Wenstrup, R. J., & Syngal, S. (2017). Cancer Susceptibility Gene Mutations in Individuals With Colorectal Cancer. *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology*, *35*(10), 1086–1095. https://doi.org/10.1200/JCO.2016.71.0012

Zhao, Z., Feng, Q., Yin, Z., Shuang, J., Bai, B., Yu, P., Guo, M., & Zhao, Q. (2017). Red and processed meat consumption and colorectal cancer risk: A systematic review and meta-analysis. *Oncotarget*, *8*(47), 83306–83314. https://doi.org/10.18632/oncotarget.20667

Zimmermann, H. (2000). Extracellular metabolism of ATP and other nucleotides. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *362*(4–5), 299–309. https://doi.org/10.1007/s002100000309