AMERICAN UNIVERSITY OF BEIRUT

INVESTIGATING THE DYNAMIC INTERACTION BETWEEN CX43 AND TET2 IN INFLAMMATORY BOWEL DISEASE AND COLORECTAL CANCER

by
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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Anatomy, Cell Biology, and Physiological Sciences
of the Faculty of Medicine
at the American University of Beirut

Beirut,Lebanon
January 2024

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ABSTRACT

OF THE THESIS OF

Abed El Rahman Obaji for Master of Science
Major: Human Morphology

Title: Investigating The Dynamic Interaction Between CX43 and TET2 in Inflammatory Bowel Disease and Colorectal Cancer

Inflammatory Bowel Disease (IBD) is comprised of two disorders, Ulcerative Colitis and Chron's disease; both result in chronic inflammation of the gastrointestinal tract accompanied by repetitive acute inflammatory flare ups. Statistically this has shown to significantly increase the risk for the development of colorectal cancer (CRC). Gap junction proteins such as Connexin 43 (Cx43) are thought to be tumor suppressor genes and have been observed to be altered in pathological conditions including IBD and CRC. There is a current interest in studying the role of epigenetic mechanisms such as DNA methylation in the context of disease. In one such instance, the DNA demethylase Ten-Eleven-Translocation-2 (TET2) is implicated in altering the methylation status of various cancer–promoting, as well as tumor-suppressor genes. Our interest lies in interrogating whether TET-2 contributes to the alterations in the tumor suppressor gene Cx43's expression and function and whether this signaling axis contributes to CRC development in an IBD model. To mirror the inflammatory environment of IBD, activated monocyte supernatants were added to HT-29 human colorectal adenocarcinoma cells. Furthermore, HT-29 cells were stably transfected to either overexpress or knock out Cx43 or TET2. These cells were then assayed to assess Cx43 and TET2 expression in a reciprocal fashion by looking at RNA expression, relative protein expression, and sub-cellular localization by immunofluorescence. The addition of activated monocyte supernatant resulted in an increase in the expression of Cx43. However, TET2 overexpression resulted in Cx43 downregulation, and, somewhat surprisingly, Cx43 overexpression resulted in a downregulation of TET2 expression. This seemingly paradoxical finding opened the possibility of a two-way interaction between TET2 and Cx43 rather than a unilateral effect of TET2 on Cx43 gene methylation. These findings indicate that there is a dynamic relationship between TET2 and Cx43 in the setting of inflammation and opens the doors to investigate how this relationship results in the progression from an inflammatory state to the development and progression of colorectal cancer.

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ABBREVIATIONS

Inflammatory bowel disease	IBD
Colorectal cancer	CRC
Ulcerative Colitis	UC
Chron's disease	CD
Connexin 43	Cx43
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Ten-Eleven-Translocation-2	TET2
Genome-wide association studies	GWAS
Intestinal epithelial cells	IECs
Cyclo-oxygenase	COX
Non-steroidal anti-inflammatory drugs	NSAIDs
Gastrointestinal	GI
Antigen presenting cells	APCs
Interleukin	IL
Tumor Necrosis Factor alpha	TNF-a
CpG island methylator phenotype	CIMP
DNA mismatch repair mechanisms	dMMR
Microsatellite instability	MSI
Chromosomal instability	CIN
Microsatellite-stable	MSS
Colitis-associated cancer	CAC
Bacteroides fragilis toxin	BFT

Polyketide synthetase	PKS
Fusobacterium nucleatum	Fn
Gap junction	GJ
Gap junction-mediated intercellular communication	GJIC
DNA methyltransferases	DNMTs
5-methylcytosine	5-mC
5-hydroxymethylcytosine	5-hmC
Dextran sulfate sodium	DSS
Carbenoxolone	CBX
Zonula occludens	ZO-1
N-Cadherin	N-cad

CHAPTER I

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) encompasses the long-lasting recurring inflammatory conditions known as Crohn's disease and ulcerative colitis. Having a family history increases the likelihood of developing IBD, with the highest occurrence observed during early adulthood, although people of any age can be affected. The underlying cause of IBD is believed to be an aberrant and persistent inflammatory reaction to beneficial microorganisms within the body, occurring in individuals with a genetic predisposition (1). Crohn's disease affects different segments of the digestive tract, with the terminal ileum or the perianal region being the most commonly affected areas. The involvement is typically non-continuous, meaning there are skip areas between the affected portions. Unlike ulcerative colitis, Crohn's disease often leads to complications such as strictures (narrowing of the intestine), abscesses, and fistulas (abnormal connections between organs or tissues) (2). On the other hand, ulcerative colitis is characterized by inflammation that is restricted to the colon. It starts in the rectum and spreads in a continuous pattern towards the upper parts of the colon. It frequently involves the periappendiceal region, which is the area around the appendix (3). Genome-wide association studies (GWAS) have achieved significant progress in the field of inflammatory bowel disease (IBD). These studies have identified 99 distinct genetic risk regions, which do not overlap, in relation to IBD. Among these regions, 28 are common to both Crohn's disease and ulcerative colitis, highlighting shared genetic factors between the two conditions (4, 5). The development of inflammatory bowel disease (IBD) has been linked to a wide range of over 201 genetic mutations, and evidence from genome-

wide association studies strongly indicates that IBD is a complex process involving multiple genes (6). Approximately 80% to 90% of the identified genetic locations associated with IBD are noncoding segments and are believed to serve as epigenetic markers, microRNAs, and noncoding RNAs (6). The intestinal epithelial cells (IECs) line the colon luminal surface. Due to the constant exposition of the colon to food antigens, commensal microbes, and other cellular insults, IECs are in continuous renewal and migration every 4-5 days (7). Enterocytes make the primary cellular component of the colon, they play significant role nutrient absorption and immunoglobulins secretion. The intestinal epithelium is classified as simple columnar epithelium. Around 10% of the IECs are goblet cells. Goblet cells function lies in lubricating the intestinal wall for easy passaging of digested food and protection from the digestive enzymes (8). Neurological responses can be induced by hormones or peptides into the bloodstream by neuroendocrine cells in response to stimulation (9). Also known as chemoreceptors, neuroendocrine cells initiate digestion process, detect harmful substances and activating protective responses (10). The underlying causes of IBD are not yet fully comprehended. The current understanding of these complex interactions is still incomplete, but it is acknowledged that a range of genetic, environmental, and host-related factors collectively contribute to the onset of gut inflammation in IBD (11). The risk of developing IBD is significantly elevated, up to five times higher, among first-degree relatives of individuals already diagnosed with IBD (6).

A. Risk factors

1. Smoking

In a meta-analysis, it was found that smoking is linked to a two-fold increase in the risk of developing Crohn's disease (12). Similarly, former smoking is associated with a similar increased risk of ulcerative colitis. However, the risk is not observed for current smokers, as they showed a significant inverse association with ulcerative colitis (12, 13). Both early life exposure to smoke and passive smoking exhibit similar effects (14). However, despite smoking being a widely recognized risk factor, variations in susceptibility based on gender and ethnicity indicate the involvement of complex gene-environment interactions. Furthermore, it should be noted that not all research cohorts have consistently observed the influence of smoking on IBD (13).

2. Appendectomy

In a substantial cohort of 212,963 individuals who had appendectomy before the age of 50, a notable decrease in the incidence of ulcerative colitis was observed in those who underwent appendectomy due to perforated or non-perforated appendicitis and mesenteric lymphadenitis compared to those with nonspecific abdominal pain (15). This suggests that the presence of inflammation in the appendix, rather than just the removal of the organ, may be responsible for this protective association (13). This influence is specifically observed in cases of appendectomy performed before the age of 20 years. Conversely, within the same group of patients, there was an elevated risk of Crohn's disease for up to 20 years following the appendectomy (16). However, when individuals underwent the procedure before the age of 10 years, the risk of developing Crohn's disease was actually diminished (16).

3. Antibiotics

The gut microbiota undergoes dynamic changes and displays a wide range of variations in early childhood (17). Any disruptions or imbalances in the microbial community during this crucial phase could have implications for the gut's immune response and potentially increase the likelihood of developing IBD (13). A detailed investigation conducted within the University of Manitoba IBD cohort revealed that 58% of pediatric patients who later developed IBD had been administered antibiotics during their initial year of life, while in the control group, this percentage was significantly lower at 39% (18). The connection between antibiotic use and IBD is more significant in Crohn's disease than in ulcerative colitis (19). This link has been observed with various classes of antibiotics and is particularly pronounced when exposure occurs during the first year of life compared to later use (20, 21). The risk of disease shows a dose-response relationship, with multiple courses of antibiotics contributing to a greater increase in disease risk compared to a single course (21). Most of the studies examining the link between antibiotics and IBD have been conducted in Western populations with minimal exposure to early life infectious agents or good sanitation (13). Surprisingly, a large population-based study in Asia found that antibiotic exposure was associated with a protective effect against both Crohn's disease and ulcerative colitis (21).

4. Medications

IBD has been linked to the use of other medications, such as aspirin, non-steroidal anti-inflammatory drugs (NSAIDs, oral contraceptives, and postmenopausal hormone therapy (22-26). The relationship between NSAIDs and IBD showed greater significance when higher doses were taken for an extended period of time, this association was

consistent for both Crohn's disease and ulcerative colitis (23). Up to 33% of NSAID users may experience relapses as a result (13). The impact could be attributed to the nonselective blocking of cyclo-oxygenase (COX) enzymes; as selective COX-2 inhibitors have been linked to a lower relapse rate (27). Additionally, oral contraceptive usage is associated with a heightened risk of Crohn's disease, but this effect diminishes after discontinuation of use (24, 25).

B. Epidemiology

In Western countries, especially those in northern Europe and North America, IBD is most commonly observed. In the United States alone, approximately 1.6 million people are affected by IBD, with 785,000 individuals diagnosed with CD and 910,000 individuals diagnosed with UC (11). UC and CD exhibit comparable trends in terms of age and gender distribution, typically showing initial symptoms in individuals during their twenties or forties, without any distinct inclination towards a specific gender (28). CD tends to affect individuals at a relatively younger age, with most cases developing between 15 and 25 years old. Conversely, UC is more likely to manifest between the ages of 25 and 35 (11). Historically, these conditions were predominantly observed in individuals of white ethnicity, particularly those with Ashkenazi Jewish background. However, there has been a notable rise in the incidence of these diseases among Asian and Hispanic populations over the past decade. Furthermore, studies indicate that individuals who migrate from regions with low prevalence to regions with high prevalence face an increased risk of developing IBD, particularly their offspring who are born in areas with high prevalence. The industrialization of countries has been associated

with an escalation in IBD cases. Similarly, individuals residing in urban areas are more prone to these diseases compared to those living in rural settings (11, 28).

C. Symptoms

The classical hallmarks of CD consist of experiencing abdominal pain, having frequent episodes of watery diarrhea, and unintentional weight loss (29, 30). CD has the ability to involve any segment of the gastrointestinal (GI) tract, ranging from the mouth all the way to the anus (11). The abdominal pain is usually cramp-like in nature and can persist for a significant number of years prior to receiving a diagnosis. At times, this pain can be sudden and severe, resembling symptoms commonly seen in cases of appendicitis (11). CD is commonly characterized by a watery consistency, it follows and episodic pattern and can intermittently continue for several years before being diagnosed, as although it can also contain blood. The occurrence of diarrhea is most frequently associated with the involvement of the colon and rectum (31). Severe bleeding, although rare, can manifest in approximately 1% to 2% of patients (32). Patients with CD tend to lose weight due to malabsorption, persistent diarrhea and anorexia (11).

The manifestations and progression of UC can vary significantly, as the severity of symptoms is closely tied to the degree of inflammation within the body (33). Due to the fact that inflammation in UC is confined to the colonic mucosa, the range of symptoms observed is comparatively narrower when compared to CD (11). The predominant manifestation of UC is diarrhea, which arises due to the accelerated passage of intestinal contents through the inflamed colon, alongside tenesmus and abdominal pain (11). The intensity of diarrhea correlates with the degree of inflammation. As the disease advances, blood becomes incorporated into the stool, leading to the presence of bloody diarrhea.

Approximately 10% of patients experience severe bleeding, with 1% to 3% of individuals with UC encountering at least one episode of significant hemorrhage necessitating surgical intervention (11). Colonic strictures, observed in around 5% to 10% of individuals with an extended duration of the disease, can result in obstruction and discomfort (34). Abdominal discomfort can range from minor colic to intense cramping as the condition advances. Moreover, systemic symptoms such as fatigue, fever, and weight loss may also be present (11).

D. Pathophysiology

Dendritic cells and other antigen presenting cells (APCs) set off a series of pro-inflammatory and anti-inflammatory signaling molecules when antigens and microbial byproducts cross the intestinal barrier. These signaling molecules direct local and circulating lymphocytes to the site of inflammation (6). Leukocytes migrate to the inflamed sights by the interaction between integrins which are present on leukocyte surface with the surface adhesion molecules on endothelial cells (35). Integrins are heterodimeric transmembrane glycoproteins consisting of α and β, the circulating leukocytes express αLβ2, α4β1, α4β7, and αEβ7 on their surface which allow them to interact with different adhesion molecules specific for leukocyte migration to the inflamed intestines (35, 36). Advanced therapies that aim to manage inflammation in individuals with CD and UC are currently undergoing evaluation in clinical trials. These innovative treatments specifically focus on either blocking endothelial cell adhesion molecules or hindering the migration of lymphocytes from lymph nodes (37-39). In both CD and UC, there is an imbalance between anti-inflammatory and pro-inflammatory

signals in the intestinal mucosa. This disruption leads to the migration of white blood cells to the mucosa and is sustained by an excessive T-cell immune response (6).

In the context of UC, there is a correlation between elevated production of IL-5, a cytokine specific to Th2 cells, and enhanced activation of B cells, leading to a more robust immune response compared to the Th1 cell response observed in CD (38, 40). Remarkably, UC-specific Th-cells exhibit minimal IL4 production, implying that they do not exhibit all the characteristics typically associated with classic Th2 cells (41, 42). Th2 cells play a significant role in driving the immune response in UC, causing an elevated activation of B cells and natural killer T cells. The secretion of IL-5 and IL-13 molecules further supports and facilitates this immune response (6). Instead, UC is linked to the existence of nonclassic natural killer T cells that are CD1d-restricted. These cells are responsible for the production of IL-13, which is found in higher levels in the intestinal lining of UC patients compared to those with CD (40, 42). In clinical trials, there has been conflicting success in utilizing Anti-IL-13 inhibitors for the treatment of patients with UC (43).

In CD, the secretion of IL-12, IL-18, IL-23, and transforming growth factor b by antigen-presenting cells (APCs) and macrophages triggers the process of differentiating between Th1 and Th17 cell types (6). As a result, Th1 and Th17 cells release pro-inflammatory cytokines, including IL-17, interferon g, and tumor necrosis factor a. These cytokines contribute to a self-reinforcing amplification loop, stimulating antigen-presenting cells (APCs), macrophages, fibroblasts, and endothelial cells to produce TNF-a, IL-1, IL-6, IL-8, IL-12, and IL-18(40, 44, 45). Experimental colitis is not developed in mice lacking the IL-12 p40 subunit and IL-23, highlighting the significance of these cytokines in the inflammatory pathway specific to CD (46, 47). Currently, in clinical

practice, therapeutic drugs that specifically target the common p40 subunit of IL-12 and IL-23 are used to alleviate intestinal inflammation. Simultaneously, novel therapeutic approaches that specifically focus on the p19 subunit of IL-23 are being evaluated in clinical trials (48, 49).

CHAPTER II

COLORECTAL CANCER

A. Epidemiology

Globally, colorectal cancer makes up approximately 10% of the total yearly reported cancer diagnoses and cancer-related fatalities (50). In terms of cancer diagnoses, it ranks as the second most prevalent cancer in women and the third most frequent in men. Interestingly, women have a comparatively lower incidence and mortality rate, approximately 25% less than their male counterparts (51). As developing countries continue to make strides in progress, it is projected that the worldwide occurrence of colorectal cancer will escalate to approximately 2.5 million new cases by the year 2035 (50, 52). Stabilization and reduction in colorectal cancer rates are typically observed in highly developed nations exclusively. These positive trends are largely credited to nationwide screening initiatives and an overall increase in colonoscopy utilization, although lifestyle and dietary modifications may also play a role (53). Conversely, there is a troubling trend of rising cases of colorectal cancer in patients below the age of 50, with a notable focus on rectal cancer and left-sided colon cancer (54-56). While genetic factors, lifestyle choices, obesity, and environmental elements may exhibit some correlation, the precise causes behind this escalation in colorectal cancer cases are not fully comprehended (51).

B. Pathogenesis

The majority of cancer cases originate from a polyp, commencing with an abnormal crypt that transforms into a pre-cancerous lesion called a polyp. Over an estimated span of 10 to 15 years, this polyp can advance into colorectal cancer (51). The healthy colon lining comprises crypts, which house a diverse population of cells. Located at the base of these crypts are colonic stem cells that undergo rapid division (57). From the colonic stem cells, precursor cells emerge and eventually transform into specialized cells with distinct physiological functions. These functions encompass the roles of enterocytes, which facilitate nutrient uptake, goblet cells that produce mucus, and enteroendocrine cells that secrete hormones (Stem cell dynamics in homeostasis and cancer of the intestine). It is currently believed that the primary cell of origin for the majority of colorectal cancers is likely a stem cell or a cell population with stem-cell-like characteristics, the prevailing belief is that most colorectal cancers are likely to arise from either a stem cell or a cell population that shares characteristics resembling those of stem cells (58, 59). Cancer stem cells develop due to the gradual accumulation of genetic and epigenetic changes, causing the inactivation of tumor-suppressor genes and the activation of oncogenes (51). Within the "stem cell niche" located at the base of the colonic crypts, the colonic stem cells find their residence. They receive support from pericryptal myofibroblasts, which generate signaling factors essential for maintaining the stemness and functionality of these colonic stem cells (60). These unique cells are found at the base of the colonic crypts and are crucial for initiating and sustaining tumor formation (58, 59).

Sporadic colon tumors located in the proximal region of the colon displays distinct features. These tumors often show an advanced TNM stage upon diagnosis and possess

a pattern characterized by CpG island methylator phenotype (CIMP), with heightened levels of genome-wide promoter hypermethylation. Moreover, they exhibit microsatellite instability (MSI) due to impaired DNA mismatch repair mechanisms (dMMR), experience more frequent mutations in KRAS and BRAF genes, and are associated with a poorer prognosis in terms of survival (61, 62). On the other hand, colorectal tumors found in the distal region are more inclined to display chromosomal instability (CIN) and are generally associated with a more favorable outlook (63, 64). In the majority of colorectal cancer (CRC) cases (70%-90%), the conventional adenoma-carcinoma-metastasis model is commonly observed (57). The changes in these features are correlated with the gradual accumulation of distinctive genetic events known as the "APC-KRAS-TP53" signature, which is recognized as the Vogelstein model (65). The emergence of adenoma aligns with the occurrence of inactivating mutations or deletion of the APC gene. In the case of adenocarcinoma, the sustenance of this cancer involves inactivating mutations or deletion of the TP53 gene, coupled with telomere dysfunction and double-stranded DNA breakage, which drive chromosomal instability (CIN) (66, 67). Alternatively, approximately 10% of CRCs can follow a different path known as the serrated neoplasia pathway, which presents in one of two progression patterns: [1] The sessile serrated pathway involves the evolution of a microvesicular hyperplastic polyp into a sessile serrated adenoma. Subsequently, this can lead to the development of either a microsatellite instability (MSI) or a microsatellite-stable (MSS) carcinoma. [2] The traditional serrated pathway, on the other hand, begins with a goblet cell-rich hyperplastic polyp that progresses into a traditional serrated adenoma. Ultimately, this can lead to the formation of an MSS carcinoma (68). These serrated neoplasms show a greater frequency of activating mutations in the BRAF and KRAS genes, along with an increased

prevalence of CIMP (CpG island methylator phenotype), hypermutation rates, but barely APC mutations (51).

Colitis-associated cancer (CAC), a specific subtype of CRC, is most commonly observed in patients with IBD. CAC accounts for approximately 2% of all colorectal cancer cases (57). Fortunately, there is evidence suggesting a reduction in the prevalence of CAC over time, which is likely the result of improved medical therapies and the more widespread use of colonoscopic screening and surveillance (69, 70). A meta-analysis involving 44 studies and 31,287 patients with ulcerative colitis (UC) in Asian countries reported that the pooled prevalence of colorectal cancer (CRC) was 0.85%. The cumulative risk of CRC development was found to be 0.02% at 10 years, 4.8% at 20 years, and 13.9% at 30 years (71).

C. Pathogenesis of Colitis-Associated CRC

Colorectal cancer (CRC) that originates from inflammatory bowel disease (IBD) is commonly seen as a prime example of inflammation-induced carcinogenesis (72). The persistent inflammation triggers oxidative stress, leading to DNA damage, which, in turn, activates genes that promote tumor growth while deactivating genes that suppress tumor formation (73). As the inflammation-dysplasia-carcinoma sequence unfolds, the levels of markers indicating oxidative damage and DNA double strand breaks increase steadily (74). This progression is affected by factors arising from the host's immune response, along with contributions from the gut microbiome and its byproducts, which collectively contribute to the process of inflammation and carcinogenesis. Consequently, a series of events is set in motion, leading to genetic changes (such as mutations) and epigenetic modifications (e.g., methylation), this ultimately leads to the expansion of somatic

epithelial cells, guided by the influence of surrounding stromal and immune cells (72). Sporadic dysplastic polyps (adenomas, sessile serrated polyps) usually manifest as individual, visible lesions that occur in relatively low numbers. However, when it comes to IBD, the situation differs. In IBD, extensive regions of chronically inflamed mucosa are susceptible to undergoing neoplastic transformation, a phenomenon referred to as "field cancerization" (72). The very same major molecular pathways that are involved in the emergence of sporadic colorectal cancer (sCRC) play a significant role in the development of CAC (73). In CAC, certain gene mutations are more commonly detected than in sCRC. However, the particular genes and their frequencies differ across various studies (75-77).

In the progression of CAC, the occurrence of APC gene mutation or loss is less common and tends to take place later in the dysplasia-carcinoma sequence. In contrast, p53 mutation or loss happens frequently and very early in the process, even preceding the development of dysplasia (78). Chronic inflammation leads to sudden molecular alterations that promote tumor growth in preexisting clonal cell populations, rather than gradually accumulating due to microenvironmental influences (72).

Various experimental methodologies, including in vitro cell culture, intestine organoid models, and mice models of inflammation, have been employed in studies to uncover the role of bacteria in the carcinogenic process. Although translational research has utilized human colorectal biopsies and feces, the focus has primarily been on individuals with sporadic colorectal neoplasms, It is unrealistic to expect that a single or two organisms would be identified as the sole causative agent of CAC or sCRC (72). It is more plausible that CAC emerges due to dysbiosis within a community of commensal bacteria, resulting in reduced microbial diversity, which is often observed in cases of

colitis (72). While research has revealed a connection between specific tumor-promoting bacterial species and CRC, it is also possible that the absence of protective bacterial strains plays a significant role, although investigating this aspect may present more challenges (72). The process of human colorectal carcinogenesis has been linked to three specific bacterial species: Fusobacterium nucleatum (Fn), Escherichia coli containing pathogenic polyketide synthetase (pks) islands, and Bacteroides fragilis expressing B. fragilis toxin (BFT) (79).

The intestinal microbiota of individuals diagnosed with IBD demonstrates an increased abundance of Enterobacteriaceae/E.coli. Moreover, patients who have both IBD and CRC show a higher prevalence of mucosa-associated E. coli compared to those who do not have IBD or CRC (80). E. coli strains containing the pks gene cluster have been discovered more frequently in CRC biopsies (67%) and IBD biopsies (40%) than in healthy individuals (21%) (72). The pks island contains the colibactin gene, which produces a genotoxin responsible for inducing a distinct mutational pattern in targeted cells (81). Despite carrying the pks island, E. coli Nissle 1917 is employed as a beneficial probiotic. As a result, the specific involvement of pks+ E. coli, specifically colibactin, in human CAC needs additional research and clarification. The enterotoxigenic Bacteroides fragilis secretes the pathogenic B. fragilis toxin BFT. When this toxin binds to a specific receptor on colonic epithelial cells, it triggers the activation of Wnt and NF-kB signaling pathways (82). This activation, in turn, results in increased cell proliferation, the release of pro-inflammatory mediators by the epithelial cells, and the occurrence of DNA damage. In the mucosa of individuals with sporadic colorectal neoplasia, the BFT gene sequences have been found in 90% of cases, while they were present in 55% of controls. Moreover, these gene sequences have been observed in the stool of approximately 14%

of patients with IBD (82, 83). BFT causes acute and chronic colitis in mice, and it promotes IL-17-dependent colon carcinogenesis in the $Min^{Apc+/-}$ mouse model (82). In the APC^{Min} model, the presence of BFT results in the activation of IL-17 and Stat3 across the colon, contributing to enhanced carcinogenesis in the distal colon. This effect is achieved through the activation of NF-kB in distal colonic epithelial cells (84). Unlike enterotoxigenic Bacteroides fragilis, the non-enterotoxigenic variant, classified as a commensal bacterium, does not exhibit the same potential for causing carcinogenesis (72).

CHAPTER III

GAP JUNCTION

Gap junction channels facilitate crucial intercellular communication, essential for coordinating cellular functions in the tissues of multicellular organisms. By enabling the direct exchange of ions and small molecules, including second messengers like Ca2+, IP3, cyclic nucleotides, and oligonucleotides, these channels play a fundamental role in cellular coordination (85). Through gap junction-mediated coupling, cells can collectively respond in sync to ligands, even when only a small subset of cells express the ligand and receptor. During development, gap junctions establish communication compartments, promoting coordinated differentiation of coupled cells, while those not connected adopt distinct developmental fates (85). Six Connexins form a Connexon, which acts as a hemi-channel. This Connexon then joins with another hemi-channel from neighboring cells to create a Gap junction. Gap junctions can be made up of six identical Cx subunits (Homomeric) or six different Cx subunits (Heteromeric). These Gap junction channels can be either homotypic, with two identical connexons coming together, or heterotypic, where two different connexons assemble between adjacent cells (Figure 1) (86, 87).

A. Connexins

In invertebrates, gap junctions are established by proteins belonging to a family referred to as connexins (Cx). Humans and mice exhibit the expression of twenty different connexins (85) (Table 1). For identifying the corresponding genes, a symbol starting with

"GJ" (indicating gap junction) is used, whereas the widely adopted protein nomenclature utilizes an abbreviation starting with "Cx" (representing connexin), followed by a number that signifies the molecular mass of the predicted polypeptide, measured in kilo daltons (85).

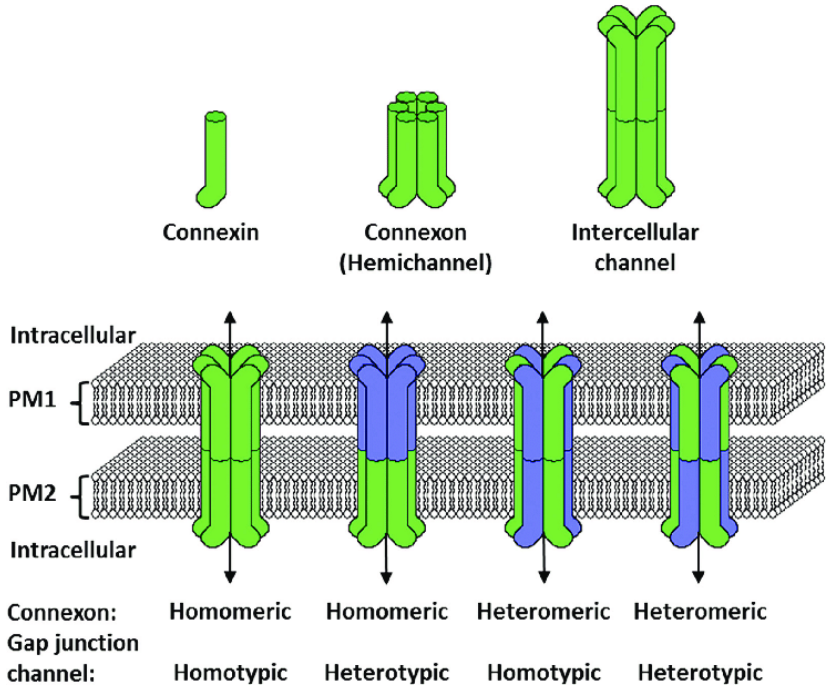


Figure 1 The illustration illustrates connexins, connexons (hemichannels), and the formation of gap junction (GJ) intercellular channels. PM1 and PM2 refer to the plasma membranes of two neighboring cells. Blue and green represent two distinct types of connexin family members (87).

Table 1 The Connexin protein and gene families in humans and mice.

Human		Mice	
Gene symbol	Protein name	Gene symbol	Protein name
GJB1	Cx32	Gjb1	Cx32
GJB2	Cx26	Gjb2	Cx26
GJB3	Cx31	Gjb3	Cx31
GJB4	Cx30.3	Gjb4	Cx30.3
GJB5	Cx31.1	Gjb5	Cx31.1
GJB6	Cx30	Gjb6	Cx30
GJB7	Cx25	Gja1	Cx43
GJA1	Cx43	Gja3	Cx46
GJA3	Cx46	Gja4	Cx37
GJA4	Cx37	Gja5	Cx40
GJA5	Cx40	Gja6	Cx33
GJA8	Cx50	Gja8	Cx50
GJA9	Cx59	Gja10	Cx57
GJA10	Cx62	Gjc1	Cx45
GJC1	Cx45	Gjc2	Cx47
GJC2	Cx47	Gjc3	Cx29
GJC3	Cx30.2/31.3	Gjd2	Cx36
GJD2	Cx36	Gjd3	Cx30.2
GJD3	Cx31.9	Gjd4	Cx39
GJD4	Cx40.1	Gje1	Cx23

Mutations in the genes that encode connexins have been associated with a diverse array of genetic diseases, including but not limited to deafness, neuropathies, cataracts, skeletal abnormalities, and skin disorders (88-91). GJs form when two adjacent cells contribute hemi-channels (consisting of connexin hexamers) that dock together. This arrangement facilitates the direct exchange of ions and small signaling molecules (<2 kDa) between the cells (85). Cellular proliferation, differentiation, and function heavily rely on connexins, making them pivotal in these processes. Additionally, they are considered as potential tumor-suppressor genes (92-94). For many years, a library of connexin-related illnesses has been growing, and it now contains many different types of

syndromes and disorders, such as inflammatory diseases and cancer. Various investigations have indicated that connexins and gap junction-mediated intercellular communication (GJIC) play a significant role in the inflammatory response (95). Changes in the expression of Cx43 in the gastrointestinal tract have been associated with conditions such as IBD, gastrointestinal infections, and reduced motility (96). In previous studies, we observed modifications in Cx43 expression and distribution, as well as direct communication between intestinal epithelial cells and macrophages. These findings indicate that these changes could potentially contribute to the development of inflammatory bowel disease (IBD) (97).

Altered Cx43 expression, whether upregulated or downregulated, can play a role in the initiation and advancement of cancer. The patterns of Cx43 expression are specific to different types of cancer and can vary depending on the stage of the cancer (98). Through an immunohistochemical analysis of 117 gastric cancer samples, it was observed that reduced expression of Cx43 and E-cadherin contributed to the formation of primary gastric cancer. On the other hand, increased expression of Cx43 and E-cadherin was linked to lymph node metastasis in the same samples (99). Increased Cx43 expression in primary urothelial bladder cancer was found to be linked with an adverse patient prognosis (100). By conducting an *in silico* analysis of gene expression profiles in breast cancer tissues, researchers found that heightened expression of Cx43 and Cx26 in primary breast cancers was linked to increased recurrence rates and poorer patient survival (101). However, in a tissue microarray study involving 483 cases of invasive breast cancer, none of the connexin markers (Cx26, Cx32, and Cx43) showed any significant correlation with patient survival or tumor grade (101).

CHAPTER IV

DNA METHYLATION AND DEMETHYLATION

In the case of complex diseases such as IBD and colon adenocarcinoma, the influence of their onset extends beyond heritability, as environmental and epigenetic factors (e.g., DNA methylation and demethylation, histone modifications, higher-order chromatin structure, etc.) are also believed to contribute significantly (102). The dynamic state of DNA methylation is governed by the intricate interplay between DNA methyltransferases (DNMTs) and demethylating enzymes, including Ten-Eleven Translocation (TET) proteins (103). DNA methylation is considered a highly stable and heritable epigenetic system, meeting strict criteria for both mitotic (cell division) and meiotic (cell division in germ cells) inheritance. This process involves the addition of a methyl group to the C5 position of cytosine, resulting in the formation of 5-methylcytosine (5mC), it predominantly occurs in CpG-rich regions, specifically referred to as "CpG islands," which are usually devoid of methylation (104, 105) (Figure 2). Approximately, around 70% of these CpG islands are found within human gene promoters, which subsequently results in the silencing of the associated genes (104). Comparing the DNA methylation profiles between individuals with IBD and their healthy counterparts reveals notable alterations in the former group (106). Additionally, the DNA methylation status in colon adenocarcinoma related to IBD exhibited dissimilarities compared to sporadic colon adenocarcinoma, leading to distinct patterns of gene expression (107).

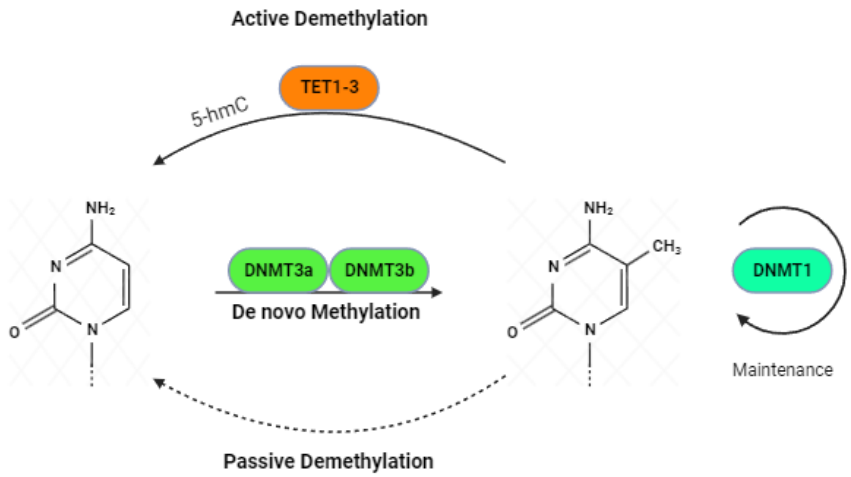


Figure 2 DNA methylation and demethylation process - *created with Biorender.com*

Demethylation processes, facilitated by TET enzymes (TET-1, TET-2, TET-3), serve as a counterbalance to DNA methylation, leading to the activation of gene transcription (108). 5-methylcytosine (5-mC), which was previously methylated, is transformed into 5-hydroxymethylcytosine (5-hmC) and other oxidized derivatives. Subsequently, these modified forms are identified and excised through the thymine-DNA glycosylase/base enzyme repair (TDG/BER) pathway, resulting in the recovery of an unmethylated cytosine residue, This entire process is mediated by TET enzymes (109). A range of diseases and cancers involves mutations in TET genes. In the context of inflammatory diseases, TET-2 assumes a pivotal function in inhibiting the pro-inflammatory cytokine, interleukin-6 (IL-6) (103, 110). Tumors of the digestive system exhibit varying levels of TET enzymes, which are being investigated as possible prognostic markers (111). The expression reduction of TET-1, TET-2, and TET-3 has been observed in gastric cancers, coinciding with the loss of the 5-hydroxymethylcytosine

(5-hmC) mark (112). This downregulation has also been documented in colorectal carcinoma.

TET-2 transcriptional levels may serve as predictors of therapy outcome and illness recurrence (112). A recent study found that the human gene responsible for TET-2 encodes three promoter elements that undergo diverse regulation across various tissues and during different stages of development (113). Several studies have consistently shown that the Cx43 promoter undergoes hypermethylation as the transition from inflammation to cancer occurs (114, 115). In a previous study conducted with the HT-29 human colorectal adenocarcinoma cell line, it was demonstrated that inflammation leads to elevated expression levels of both Cx43 and TET-2 in HT-29 cells (103). These finding were supported by in vivo analysis, induced inflammation in mice by DSS results in an upregulation of Cx43 and TET-2 expression (103). The administration of Carbenoxolone (CBX) has been shown to have a reversing effect on this particular phenomenon (103).

CHAPTER V

AIM OF THE STUDY

The DNA demethylase Ten-Eleven-Translocation-2 (TET2) is believed to play a role in modifying the methylation status of multiple cancer-promoting and tumor-suppressor genes. We are particularly interested in investigating whether TET-2 is involved in the changes observed in the tumor suppressor gene Cx43's expression and function, and whether this signaling pathway contributes to the development of colorectal cancer in an inflammatory bowel disease (IBD) model.

CHAPTER VI

MATERIALS AND METHODS

A. Cell lines and culture conditions

For the purpose of simulating human intestinal epithelial cells (IECs), the HT-29 and Caco-2 cell lines were selected, both of which were derived from tissues afflicted with colorectal adenocarcinoma. These cells are frequently utilized to mimic intestinal transport and to investigate inflammatory processes in pathological contexts. When cultured in a monolayer configuration, they undergo increased differentiation, resembling intestinal epithelial cells (IECs) in terms of their characteristics (97, 116, 117). To create viral supernatant for transduction, human embryonic kidney cells (specifically designated as packaging HEK 293T cells) were harnessed in the process (103). Furthermore, the human monocytic cell line THP-1 was employed as an in vitro representation of activated macrophages to generate a conditioned medium with an inflammatory profile. (97, 118). We have previously showed that after being subjected to to phorbol myristate acetate (PMA) and lipopolysaccharide (LPS), THP-1 cells undergo activation, adhering to the culture vessel and exhibiting elevated transcriptional levels of connexins, Toll-Like Receptor (TLR)-2, and TLR-4 expressions (97). Furthermore, they display heightened expression levels of NF-κB p65 and COX-2, along with inflammatory cytokines TNF-α and IL-1β (97). In the scope of this study, THP-1 cells in suspension were stimulated using 50 ng/ml PMA (Sigma, Missouri, USA) for a duration of 24 hours. This was followed by exposure to 1 μg/ml of LPS (Sigma, Missouri, USA) for an additional 4 hours. After the activated THP-1 cells adhered to the surface of the cell culture plate, they

were cleansed using media free of PMA and LPS. Subsequently, these cells were allowed to grow for a period of 72 hours (103). The conditioned inflammatory media (CM) produced was then harvested, subjected to filtration, and subsequently applied in an in vitro context to colon cell lines, creating a simulated environment of inflammation.

The cells were cultured in full RPMI-1640 medium (Sigma, Missouri, USA) for HT-29 and THP-1 cells, while HEK 293T cells were cultured in Dulbecco's Modified Eagle's medium (DMEM AQ, Sigma, Missouri, USA). The culture media were enriched with 10% FBS (Sigma, Missouri, USA), 100 U/ml penicillin G, and 100 µg/ml streptomycin (Sigma, Missouri, USA). Cell cultivation took place at 37°C in a humidified incubator with an atmosphere containing 5% CO2.

B. Generation of elevated Cx43 gene expression in HT-29 cells

The construct containing Cx43-pDendra2N (Evrogen, Russia) was previously cloned into pCSCW lentiviral vectors (97). The plasmid was employed for the transformation of DH5α competent E. coli bacteria, initiating their growth. Subsequently, the plasmid was separated and refined using the EndoFree Maxi plasmid purification kit (Qiagen, Germany). This purified plasmid, combined with additional plasmids (gag/pol/env), was used in the transfection of HEK 293T cells (103). This transfection process was conducted to generate viral supernatant, carrying the Cx43-pDendra2 chimeric proteins. The viral supernatant was applied to introduce changes in HT-29 cells through transduction. Following this process, HT-29 cells were cultivated and expanded. Cells exhibiting a strong positive response (referred to as HT-29 Cx43D cells from then on) were isolated using the BD FACSAria™ III sorter (BD Biosciences, USA). The viral supernatants were acquired from HEK 293T cells and utilized to modify HT-29 cells.

These cells, known as HT-29 Cx43D, were then sorted using a BD Fluorescence-Activated Cell Sorting (FACS) Aria SORP cell sorter, focusing on individual cells. The presence of green fluorescence in HT-29 Cx43D cells confirms the successful enhancement of exogenous Cx43 within these cellular entities.

C. Generating HT-29 cells with reduced Cx43 expression using the CRISPR/Cas9 system

HT-29 Cells with reduced Cx43 expression, recognized as HT-29 Cx43- Cells, were formed utilizing the CRISPR/Cas9 genetic modification approach. Following the incorporation of the Cx43 target sequence (20 bp) into the guide RNA scaffold of the pX330-CRISPR vector, bacterial transformation was conducted as previously described, and 10-15 colonies were selected for verifying the accurate integration of the guide RNA through sequencing (103). HT-29 cell transfection was conducted using verified positive clones, followed by puromycin selection (1 μg/ml) until separate colonies were established. Subsequently, multiple positive clones were examined to validate the decrease in Cx43 expression, as assessed through RNA and protein evaluations.

D. Cell growth assay

In 24-well plates, parental HT-29 cells, as well as HT-29 Cx43D and HT-29 Cx43- cells, were seeded at a density of 40000 cells/cm^2. Following incubation for 24 hours, 48 hours, and 72 hours, the cells were washed with PBS, treated with trypsin, and subsequently assessed for viability through trypan blue exclusion staining.

E. RNA isolation

Cells were washed with PBS and total RNA was extracted using TRIzol® Reagent (Ambion) as per manufacturers' instructions.

F. qRT-PCR

In the initial step, one microgram of total RNA was subjected to reverse transcription, utilizing the SensiFast™ cDNA Synthesis Kit (Bioline, Taunton, MA, USA). For rt-PCR, a customized SYBR green mixture was utilized within the CFX96 system (Bio-Rad, USA). The specific target sequences, including Cx43, TET-2, N-cad, ZO-1 and GAPDH, were amplified using designed primers. The fluorescence threshold cycle (Ct) value was determined for each gene and normalized against the corresponding GAPDH Ct value. The PCR conditions were as follows: an initial pre-cycle at 95°C for a duration of 5 minutes, succeeded by 40 cycles. Each cycle encompassed heating at 95°C for 10 seconds, annealing at 52-62°C for 30 seconds, and extension at 72°C for 30 seconds. A concluding extension step at 72°C was performed for an additional 5 minutes. Ensuring methodological precision, all experiments were performed with technical duplicates and independently repeated at least three times.

G. Protein extraction and Western blot

Cells were washed with PBS and scraped while situated on ice, utilizing a lysis buffer. This buffer, enriched protease and phosphatase inhibitors, included a mixture of constituents: 0.5M Tris-HCl buffer at pH 6.8, 2% SDS, and 20% glycerol.

After subjecting the lysate to sonication, it was processed further. The protein content, once quantified, was loaded onto gels containing SDS-polyacrylamide (10%) for

electrophoresis. As these proteins moved, they were transferred onto PVDF membranes (provided by BioRad, USA). These membranes were rendered protein-resistant by using a mixture of 5% fat-free milk in PBS. Human or mouse primary antibodies were then introduced to these membranes, where they incubated for either 3 hours at room temperature or for a full night at 4°C. This step was followed by the introduction of secondary antibodies conjugated with horseradish peroxidase, which interacted with the primary antibodies on the membranes. The resulting blots were then visualized through the application of an enhanced chemiluminescence detection kit. In order to establish baseline levels, GAPDH (from Abnova, Taipei, Taiwan) and β-actin (from Sigma, Missouri, USA) were employed as markers of loading control. The quantification of the visible bands on the blots was executed using the ImageJ software (US National Institutes of Health, USA).

H. Immunofluorescence microscopy

Cultured cells were placed onto coverslips, subsequently fixed using 4% paraformaldehyde (PFA), and subjected to permeabilization with 0.05% Triton X-100 for 20 minutes.

I. Statistical analysis

Numerical values were expressed as mean ± SEM or mean ± SD. The *P* value was determined and considered significant for $P < 0.05$. Differences between experimental groups were assessed using Student *t*-test, one-way analysis of variance (ANOVA) or by two-way ANOVA.

CHAPTER VII

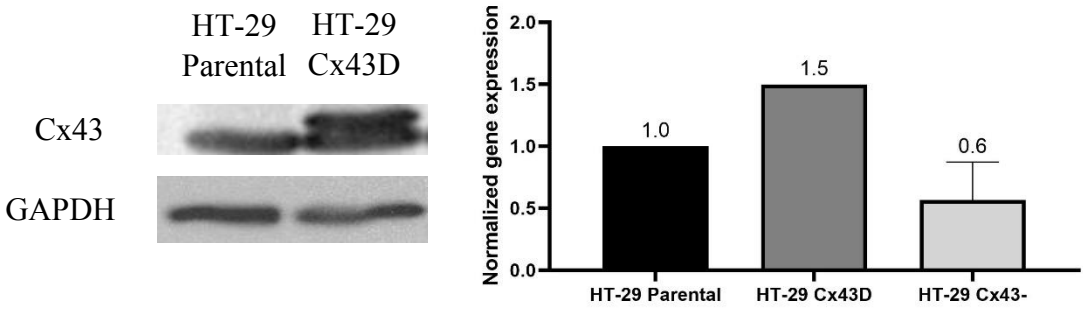
RESULTS

A. Characterization of Genetically Modified Cell Lines

1. Characterization of the HT-29 cell model employed in this study

To explore the impact of TET-2 modulation with varying levels of Cx43 expression in HT-29 cells, Cx43 was either elevated (resulting in HT-29 Cx43D cells) or reduced (yielding HT-29 Cx43- cells). Increase and decrease in the levels of Cx43 were manifested at the translational level, as evidenced by western blot analysis. Figure 4A Demonstrates an increase in exogenous Cx43 protein levels in HT-29 Cx43D and a reduction in endogenous Cx43 protein expression in HT-29 Cx43- cells, in comparison to the parental HT-29 cells. Increased expression of Cx43 is observed in HT-29 Cx43D cells, as depicted in (Figure 4B). This increased expression is notable at the cell periphery, where gap junction plaques are formed. Conversely, there is a decline in Cx43 levels in HT-29 Cx43- cells. In contrary, to study the impact of Cx43 alteration in different TET-2 expression levels in HT-29 cells, TET-2 was down regulated yielding HT-29 shTET2 cells. Verification of TET-2 protein expression was conducted through rt-PCR in HT-29 parental and HT-29 shTET2 cells (Figure 4C).

A



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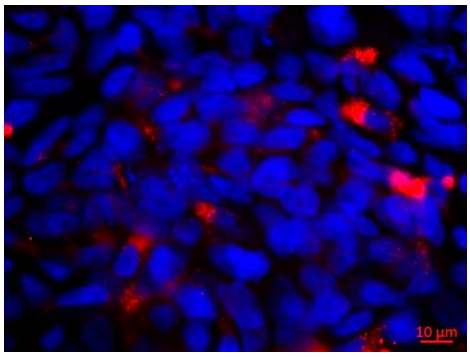
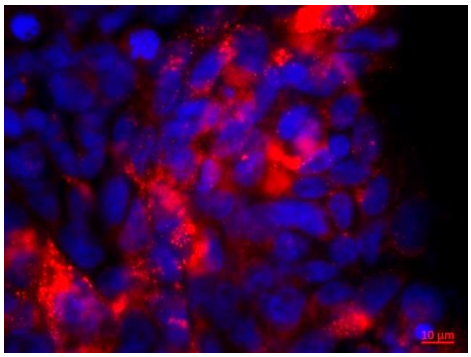
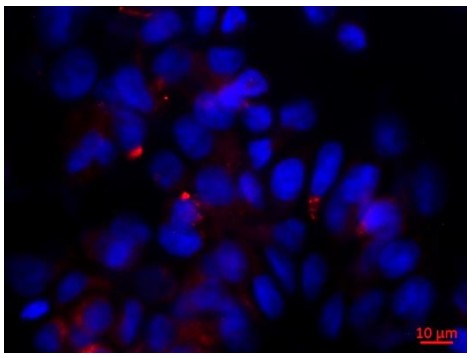


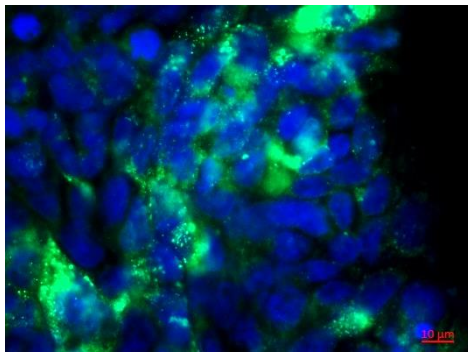
HT-29 Parental



HT-29 Cx43D endogenous



HT-29 Cx43-



HT-29 Cx43D exogenous

C

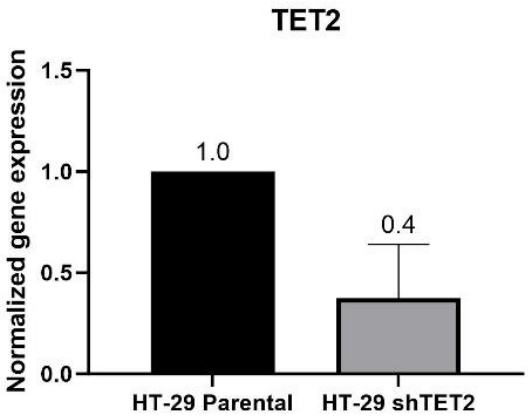
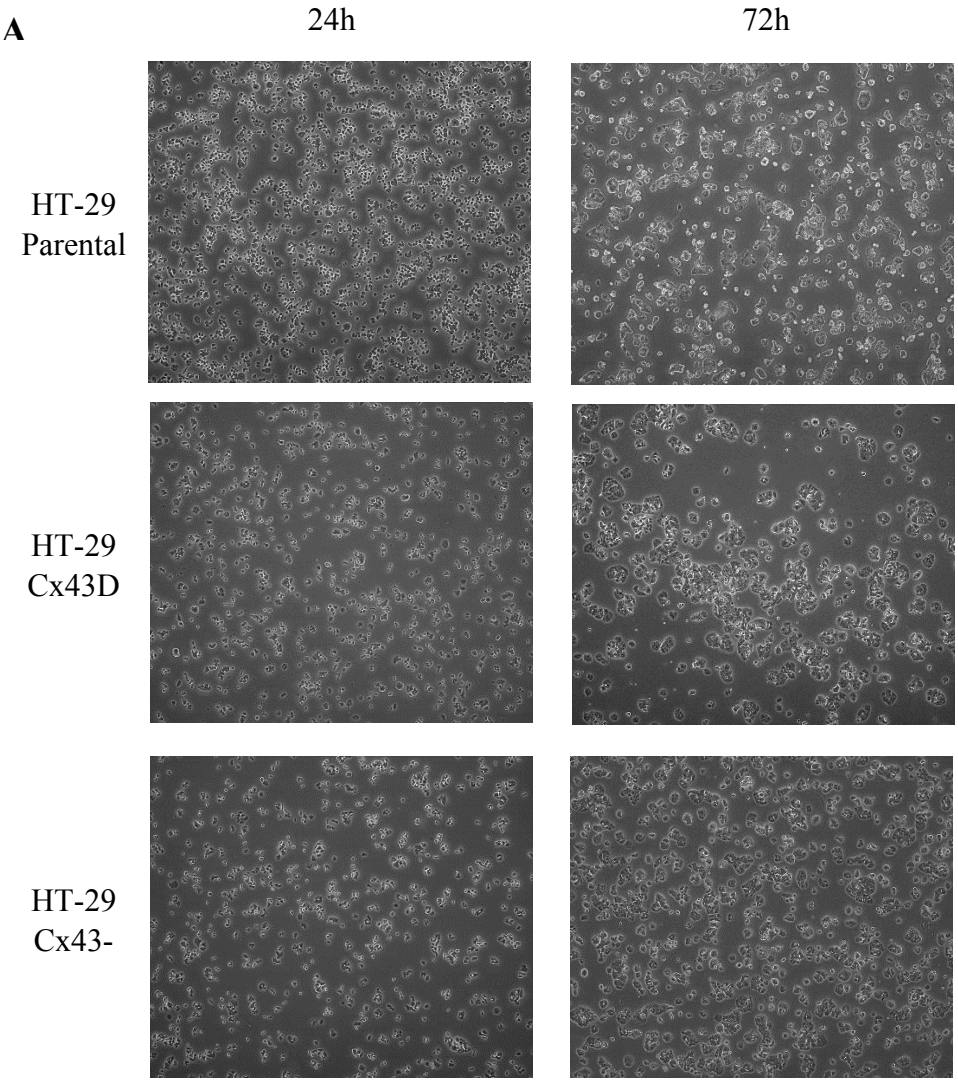


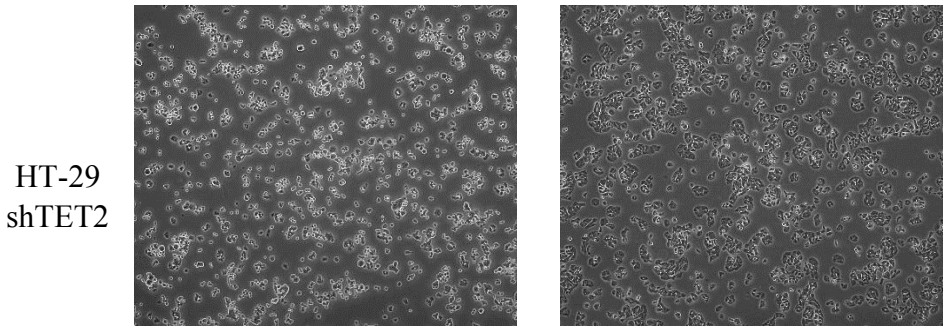
Figure 3 Characterization of the HT-29 cell model employed in this study**. A:** Western blott of connexin 43 (Cx43) protein expression levels in the following: parental HT-29, and HT-29 Cx43D cells. The bar graphs represent the mean densitometric analysis derived from three separate experiments, where protein expression was normalized to GAPDH; **B:** Immunofluorescence micrographs showing the expression of Cx43 in parental HT-29 cells, HT-29 Cx43D and HT-29 Cx43- cells (red: endogenous Cx43, green: exogenous Cx43). Scale bar 10 μm; **C:** Histograms show normalized gene expression TET2 in parental HT-29, HT-29 shTET2.

2. Assessment of Cell Morphology and Proliferation

To investigate the impact of modulating Cx43 or TET2 on the growth and morphology of HT-29 cells, cell counts were conducted at 24, 48, and 72 hours. Compared to HT-29 Parental cells, HT-29 Cx43- and HT-29 shTET2 cells exhibited a higher proliferation rate. However, HT-29 Cx43D showed slower proliferation rate compared to HT-29 Parental as depicted in (Figure 5 A and B). Additionally, cell morphology was evaluated using light microscopy at the same time points. Notably, the HT-29 Cx43 CRISPR cells demonstrated a significantly lower doubling time (32.39 hours), while the HT-29 shTET2 and CX43-Dendra cells exhibited a slight decrease in doubling time (40.90 hours and 43.78 hours, respectively) compared to the wild-type HT-29 cells (46.47 hours) (Figure 5C).

HT-29 Cx43D cells exhibited bigger islands seen at10x magnification and more compact morphology noticeable at 40x magnification compared to HT-29 Parental cells figure 6. However, both HT-29 Cx43- and HT-29 shTET2 cell showed less compact and dense islands seen at 10x and 40x magnification. These findings align with the tumor-suppressive function of Cx43, markedly restraining the proliferation of HT-29 cells.





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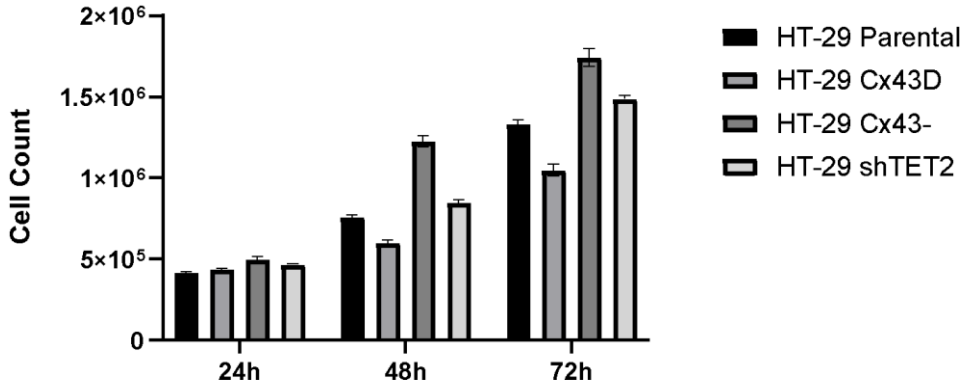

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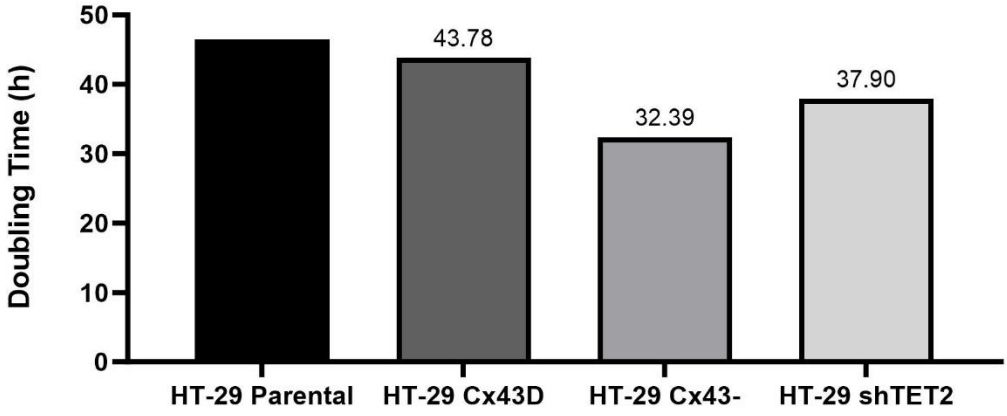
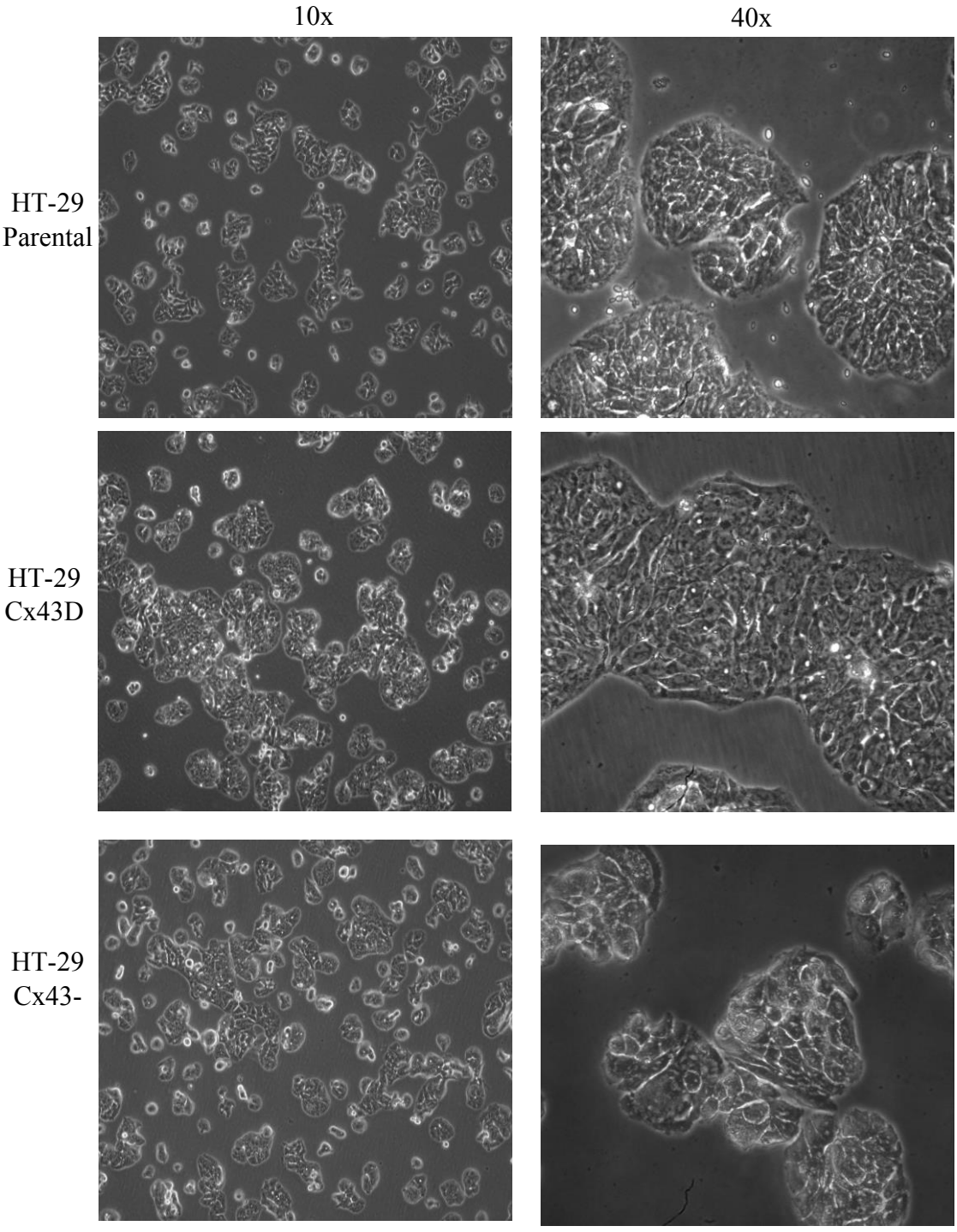


Figure 4 **A:** Light microscopy images obtained using a 5x magnification lens at three different time points (24 and 72h). **B:** Bar graphs showing cell numbers of the different utilized cell lines (HT-29 WT, HT-29 Cx43D, HT-29 Cx43-, and HT-29 shTET2) seeded at 400K cells and counted after 24, 48, and 72h using the trypan blue dye exclusion assay. **C**: Bar graphs showing the calculated doubling time (h) for HT-29 WT, HT-29 Cx43D, HT-29 Cx43-, and HT-29 shTET2.


10x
40x
HT-29
Parental
HT-29
Cx43D
HT-29
Cx43-

HT-29
shTET2

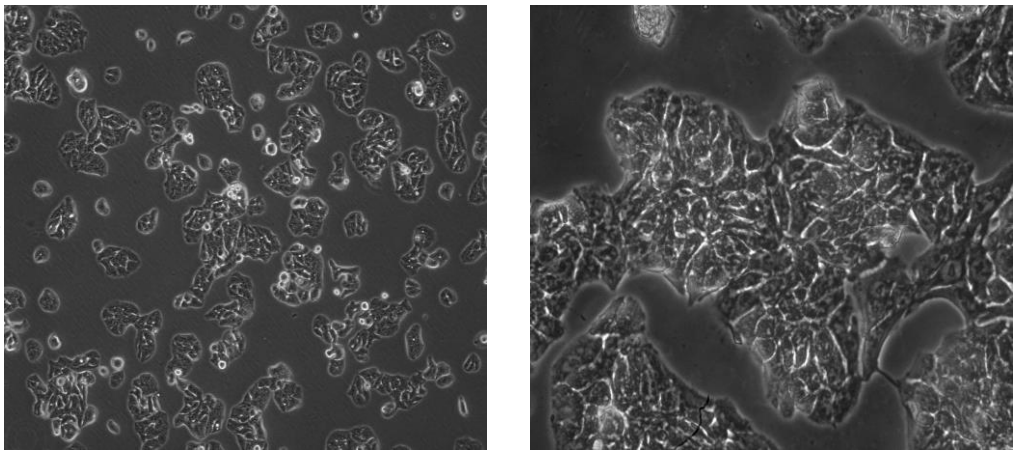


Figure 5 HT-29 WT, HT-29 Cx43D, HT-29 Cx43-, and HT-29 shTET2 cell morphology at 72h. Light microscopy images obtained using a 10x and 40x magnification lens.

B. Cx43 and TET2 interplay

To study the interaction between Cx43 and TET2, HT-29 Parental, HT-29 Cx43D, HT-29 Cx43-, and HT-29 shTET2 were screened for TET2 and Cx43 m-RNA gene expression. HT-29 Cx43D showed higher expression of Cx43, while HT-29 Cx43- and HT-29 shTET2 represented lower expression compared to HT-29 parental cells (Figure 7A). However, screening for TET2 showed a significant upregulation in HT-29 Cx43D and a slight upregulation in HT-29 Cx43-, whereas HT-29 shTET2 exhibited a low expression compared to HT-29 parental cells (Figure 7B).

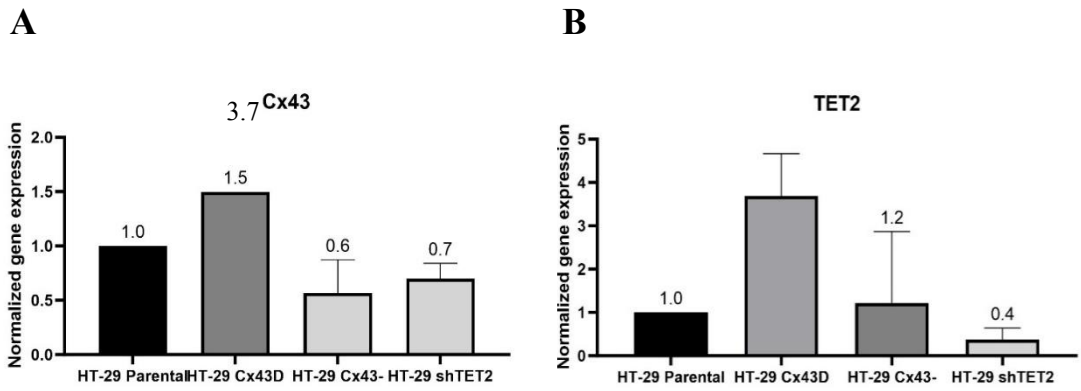


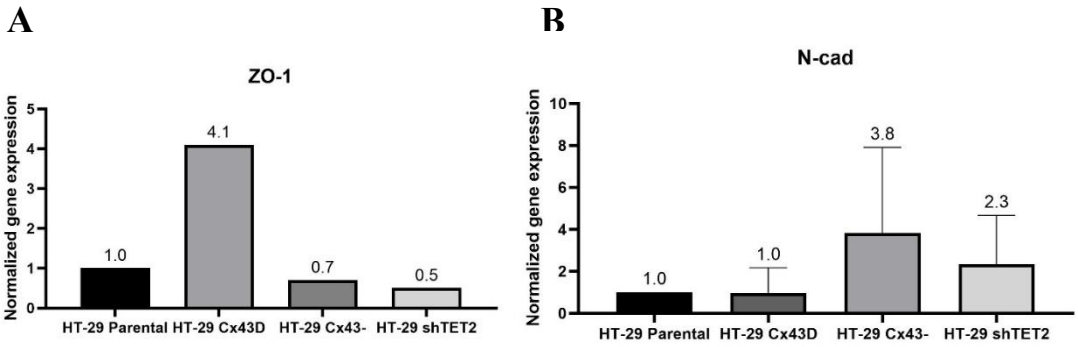
Figure 6 **A:** Bar graphs illustrate the normalized gene expression of TET2 detected through rt-PCR. HT-29 Cx43D demonstrates an upregulation of TET2, while HT-29 Cx43- exhibits a marginal increase compared to HT-29 Parental and HT-29 shTET2. **B:** The histograms present the normalized gene expression of Cx43, with Cx43 mRNA levels decreasing in HT-29 shTET2 and HT-29 Cx43-.

C. Effects of Modulating Cx43 or TET2 on ZO-1 and N-cadherin Expression Levels

To investigate the epithelial-to-mesenchymal transition, we examined HT-29 Parental, HT-29 Cx43D, HT-29 Cx43-, and HT-29 shTET2 cells for the presence of epithelial (ZO-1) and mesenchymal (N-cad) markers. This assessment involved analyzing both the transcriptional levels and the localization of these markers within the cells. In (figure 7A) elevated ZO-1 expression is evident in HT-29 Cx43D, whereas HT-29 Cx43 and HT-29 shTET2 exhibit decreased expression in comparison to HT-29 Parental. Conversely, N-cad is upregulated in both HT-29 Cx43- and HT-29 shTET2, while HT-29 Cx43D exhibits decreased N-cad expression compared to HT-29 Parental (Figure 7B).

In order to strengthen these findings, immunofluorescence imaging was performed on the cells used in this study screening for ZO-1 and N-cad. HT-29 Cx43D cells showed upregulation of ZO-1, whereas HT-29 Cx43- and HT-29 shTET2 cells exhibited downregulation compared to HT-29 Parental cells. However, (figure 7D)

showing an upregulated of N-cad in both HT-29 Cx43- and HT-29 shTET2 cells, in contrast to HT-29 Cx43D which showed downregulation.



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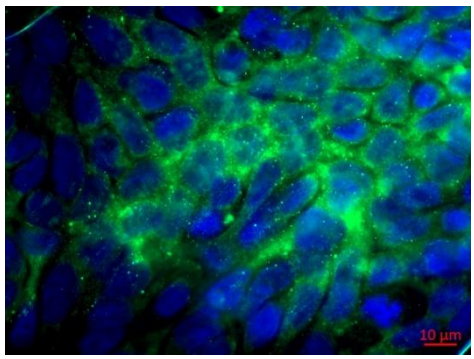
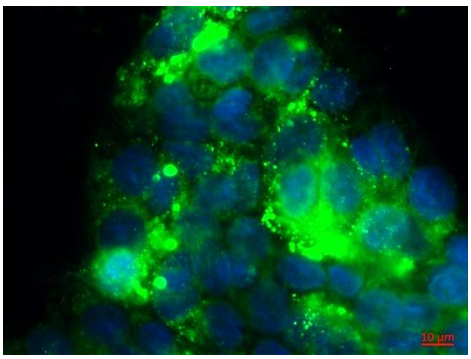
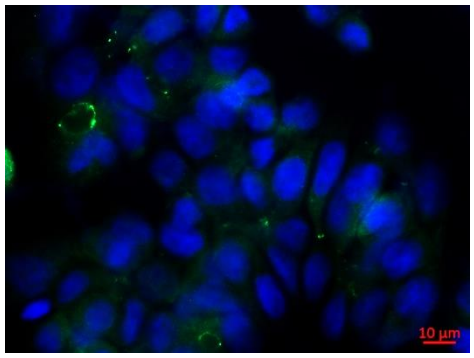


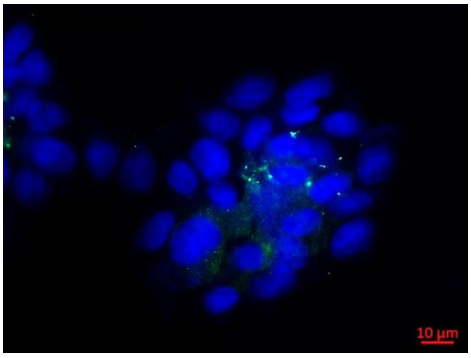
HT-29 Parental



HT-29 Cx43D



HT-29 Cx43-



HT-29 shTET2

D

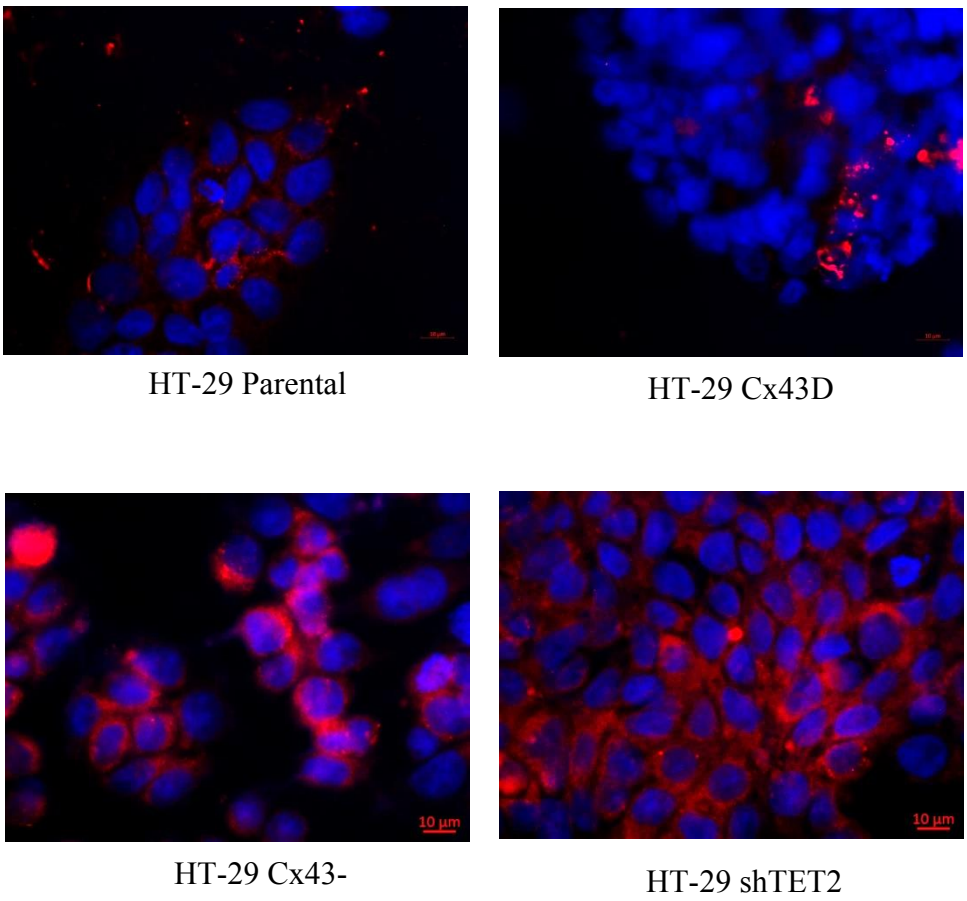


Figure 7 Epithelial and mesenchymal markers on transcriptional level and their protein localization. **A:** bar graphs illustrate the gene expression levels of the epithelial marker ZO-1**. B:** Histogram depict the gene expression levels of the mesenchymal marker N-cad. **C:** Immunofluorescence images showing ZO-1 expression in HT-29 parental, HT-29 Cx43D, HT-29 Cx43, and HT-29 shTET2. Scale bar: 10µm **D:** Immunofluorescence micrographs of parental HT-29, HT-29 Cx43D, HT-29 $Cx43^{-}$ cells, and HT-29 shTET2 showing the localization of mesenchymal marker N-cad. Scale bar: 10µm.

CHAPTER VIII

DISCUSSION

In different pathological conditions such as inflammation and cancer, changes in the expression, regulation, and interaction of junctional complexes with other proteins have been documented. Epigenetic processes involving methylation and demethylation play significant roles in the malignant transformation of cells. More precisely, the functionality of gap junction-forming Cx43 and the demethylating enzyme TET-2 has been observed to be compromised in both inflammatory responses and cancer (95-97, 110, 124). Research findings have highlighted the role of intercellular communication, mediated by connexins forming channels, in contributing to the inflammatory response. The onset of inflammation is predominantly initiated through gap junction-mediated signaling, involving the transfer of ATP among neighboring cells. Following this, ATP molecules are discharged into the extracellular milieu, serving as signaling entities that trigger purinergic receptors and escalate the inflammatory reaction (95). A study conducted in 2016 the involvement of gap junctions in the progression of IBD through the enhancement of direct communication between IECs and macrophages, facilitated by the degradation of the basement membrane (97).

The precise role of the demethylating enzyme TET-2 in IBD and colorectal adenocarcinoma remains incompletely elucidated. Recent studies showed that frequent point mutations in the TET-2 gene can result in the truncation of the protein and the subsequent loss of its enzymatic activity (125). Mutations and diminished expression of TET proteins have also been noted in solid tumors, and a reduction in the translational levels of TETs appears to be a notable feature across various cancers, including those

affecting the digestive system (126). Beyond its association with carcinogenesis, TET-2 has an anti-inflammatory function. Particularly, in the presence of inflammatory conditions, TET-2 suppresses the transcription of IL-6 in dendritic cells and macrophages (110). In a recent investigation, it was revealed that the decline in gap junctions was accompanied by an upswing in TET-2 expression. This effect was notably enhanced when inflammation was present in cells deficient in Cx43 and in mice treated with chemical inhibition of gap junctions using CBX (103). The elevation of Cx43 in inflamed colons is suggested to potentially mitigate or slow down the onset of malignancy. Hence, the absence of Cx43 in colon cancer tissues could be associated with a shift toward malignancy, deactivating tumor-suppressor genes while activating genes that promote tumors (103).

Results of this study suggest that increasing Cx43 levels in HT-29 cells leads to a reduction in the cell proliferation rate, while HT-29 Cx43- and HT-29 shTET2 cells exhibited a noticeable increase compared to HT-29 Parental cells. Within the scope of this study's aim, we examined the expression levels of both Cx43 and TET-2 in all cell subsets. The results demonstrated a significant upregulation of the TET-2 gene in HT-29 Cx43D, whereas in HT-29 Cx43- cells, there was a slight increase in expression. The increased expression of Cx43, combined with the reduction of TET-2 in HT-29 cells, may suggest that elevating Cx43 in an inflamed colon has the potential to alleviate or delay the onset of malignancy. To support this observation, we examined the expression of Cx43 in HT-29 shTET2 cells, where Cx43 was found to be downregulated, which explains the heightened proliferation rate in these cells.

To explore invasion and metastasis, we examined EMT markers, focusing on ZO-1 and N-cad. The results provided evidence for the varying proliferation rates in HT-29

Cx43D, HT-29 Cx43-, and HT-29 shTET2 cells. Cx43 upregulated cells displayed an increase in the epithelial marker ZO-1, whereas the reverse was observed in HT-29 Cx43- and HT-29 shTET2 cells. Furthermore, the mesenchymal marker N-cad showed an upregulation in HT-29 Cx43- and HT-29 shTET2 cells and a downregulation in HT-29 Cx43D cells.

Although TET-2 still not fully studied in cancers, our results gives a glimpse of the possible role of TET-2 in progression and transition of IBD to CRC.

CHAPTER IX

CONCLUSION

As noted in the literature, TET-2 and Cx43 were reported to be upregulated under inflammatory conditions (103). The data presented in this manuscript demonstrates the upregulation of TET-2 when Cx43 is upregulated in HT-29 cells, while in HT-29 cells with downregulated Cx43 is found to be downregulated. We propose that the transition from inflammatory bowel disease (IBD) to colorectal cancer (CRC) is likely to occur when intestinal cells downregulate the enzyme TET-2 which has anti-inflammatory properties. This downregulation may hinder the expression of tumor suppressor genes, including Cx43, leading to the transition from IBD to CRC. Further investigations will assess the levels of Cx43 and TET-2 in vivo using mouse models with CRC and IBD to substantiate our findings and understand the dynamic interaction between Cx43 and TET-2.

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