INVESTIGATING THE ROLE OF ERBB2 IN MOUSE COLON TUMORS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Investigating the Role of ERBB2 in Mouse Colon Tumors

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Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States and with the incidence of early-onset CRC on the rise, the CRC burden is rapidly shifting towards a younger population of individuals. Previous research has found that only 15% of CRC patients respond to epidermal growth factor receptor (EGFR) targeted therapies with the majority of CRCs initiating and growing faster through an EGFR-independent mechanism. This is especially the case when the cancer cells are resistant to tyrosine kinase inhibitors. ERBB2 is an understudied transmembrane growth factor receptor that is commonly upregulated in human CRC, and its precise mechanism in CRC remains largely unexplored. In our study, we obtained Gene Set Enrichment Analysis (GSEA) data to identify if there is enrichment or differential expression in a set of genes between wild type littermate control and ERBB2-deficient mice. Results have shown differential gene expression between the experimental and control mouse colon epithelial tissue, specifically enrichment in epithelial-mesenchymal transition (EMT) signaling characterized by a loss of epithelial cell markers and gain of mesenchymal markers. Subsequently, quantitative polymerase chain reaction (qPCR) results were used to validate the

level of expression of EMT markers that are active in the epithelial colon tissue wild type littermate control and ERBB2-deficient mice. Determination of the mechanism of action of continued ERBB2-deficient tumor growth is an integral part to identifying potential therapeutic targets to optimize human CRC patient outcomes.

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Contributors

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NOMECLATURE

CRC	Colorectal cancer
EGFR	Epidermal growth factor receptor
ERBB2	Epidermal growth factor receptor 2
ERBB3	Epidermal growth factor receptor 3
ERBB4	Epidermal growth factor receptor 4
APC	Adenomatous polyposis coli
CTNNB1	β-catenin
EMT	Epithelial Mesenchymal Transition
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
TNF-α	Tumor necrosis factor alpha
TWIST1	Class A basic helix-loop-helix protein 38
CDH1	E-cadherin
CDH2	N-cadherin
VIM	Vimentin
ZO-1	Zona occludins-1 or tight junction protein-1

1. INTRODUCTION

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in both men and women in the United States with the incidence of early-onset CRC in individuals under the age of 50 increasing rapidly (Loomans-Kropp & Umar, 2019; Siegel et al., n.d.). CRC ranks third in terms of incidence and second in terms of mortality among cancer types (Sung et al., 2021). It has been estimated that in the year of 2023, 153,020 individuals will be diagnosed with CRC and more than half of these individuals will die from the disease, including 19,550 cases and 3750 deaths in individuals younger than 50 years of age (Siegel et al., n.d.). In addition to the increasing number of individuals with early onset on CRC, the risk of CRC increases with age; within a span of four years from 2011 to 2019, CRC rates increased by 1.9% per year in individuals who are younger than 50 years of age and in those aged 50-54 years (Siegel et al., n.d.). As the CRC burden shifts to younger individuals, the incidence of CRC continues to be related to the interplay among several levels ranging from genetic or heredity to environmental factors. Unfortunately, about 20% of individuals with CRC are metastatic at presentation of the disease, and about 25% of CRC patients who initially present with in situ tumors develop CRC metastases during follow-up appointments (Biller & Schrag, 2021). Because of this, progress against CRC could be made by uncovering the etiology CRC and understanding the molecular pathways and mechanisms of CRC as this may encourage the development of therapeutic targets that are effective for specific types of CRC to optimize human CRC patient care and decrease the mortality rate of CRC.

1.2 Epidermal Growth Factor Receptor (EGFR) Family

The epidermal growth factor receptor (EGFR) family has been heavily studied and implicated in a variety of cancers. The EGFR family consists of four transmembrane proteins, EGFR/HER1, ERBB2/HER2, ERBB3/HER3, and ERBB4/HER4, which have garnered a lot of interest due to their aberrant expression and involvement in tumorigenesis (Arteaga & Engelman, 2014). EGFR receptors exist as monomers on the surface of cells where they are able to dimerize upon ligand binding (Iqbal & Iqbal, 2014). Specifically, ERBB2 is an oncogene tyrosine kinase receptor that functions as a mediator of cell proliferation and differentiation, and it is unique in that it acts as an orphan receptor, meaning that it cannot homodimerize or bind to EGF-like ligands by itself for activation (Cho et al., 2003). Heterodimers that contain ERBB2 have a high ligand binding and signaling potency as it exists in an open conformation, making it the preferred dimerization partner among the EGFR family members (Iqbal & Iqbal, 2014). Thus, ERBB2 must be complexed by heterodimerization with another member of the EGFR family to respond to ligand stimulation which can cause concurrent aberrant expression of other EGFR family members (Serova et al., 2019). ERBB2 is highly overexpressed in human malignancies such as, prostate (Carlsson et al., 2013), lung (Hirsch et al., 2009) , breast (Bose et al., 2013), and colorectal cancer (CRC) (Siena, 2018).

There has been a substantial clinical focus on EGFR overexpression in patients with CRC; however, 85% of CRC patients are unresponsive to *Egfr* inhibitory treatments (Yang et al., 2014). Mice with EGFR ablation presented with intestinal and colonic polyps that were fewer in number but larger in size (Mantilla-Rojas, 2021). Our lab has previously found that the absence of EGFR triggers ERBB2 upregulation but to a lower degree than ERBB4 upregulation and in mice with ERBB4 ablation, there was a significant decrease in polyp size and number in

intestinal and colon tumors. In ERBB2-driven cancers, ERBB3 aids in activating downstream signaling pathways, specifically the phosphatidylinositol 3-kinase (PI3K) and AKT pathway that play an integral role in tumor cell growth and proliferation (Burgering & Coffer, 1995; Chan et al., 1999). Mice with ERBB3 ablation presented with polyps that were smaller in size but larger in quantity due to increased EGFR expression (Rojas et al., 2021).

Previous research has found that mutations in ERBB2 occur in about 7% if CRCs (Pectasides & Bass, 2015). In the past, there has been substantial advancement in CRC treatment that have led to clinically meaningful improvement in the survival of patients with both metastatic and localized CRC. Although surgical care combined with anti-EGFR treatment has given rise to CRC survival, the prognoses for some patients remains poor. Thus, more research needs to be done on the molecular mechanisms that allow for the aberrant expression of ERBB2 in CRCs as the mechanism of action of ERBB2-driven CRC remains highly of interest and largely unexplored. To further understand the role of ERBB2 in tumorigenesis in the colon, we intend to investigate the involvement of ERBB2 in CRC tumorigenesis in $Apc^{Min/+}$, $Erbb2^{f/f}$, *Tg(Vil1-Cre)* mice with an ERBB2 conditional knockout allele in the intestinal epithelia.

1.3 APC Gene Mutation and APC Mouse Models

The intestinal epithelial cells derived from both the mouse and human colon proliferate at an extremely rapid rate(Sato et al., 2011). The adenomatous polyposis coli (APC) gene is a tumor suppressor gene that is a key Wnt/β-catenin signaling factor that plays a central role in the etiology in most patients with familial colorectal cancer (Nishisho et al., 1991; Zeineldin & Neufeld, 2013). Mutations that inactivate *APC* in humans are found in about 80% of all human colon tumors (Kwong & Dove, 2009). The loss of APC function in the presence of active WNT signaling is a predominant initiating mutation in humans that results in increased nuclear levels

of transcriptional coactivator of the β-catenin gene, *Ctnnb*, that stimulates the cell cycle, initiating the development of hundreds to thousands of tumors in the intestinal epithelia (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991; Mann et al., 1999; Nishisho et al., 1991). Furthermore, Intestinal tumors in APC mutant mice demonstrate a similar tumor phenotype as humans with germline *APC* mutations and serve as a mouse model to recapitulate human CRC (Gaspar et al., 2008).

1.4 Epithelial Mesenchymal Mransition (EMT)

Normal epithelial cells are typically asymmetrical and are composed of an apical surface and basal surface which contributes to the polarity of the epithelial cells. Healthy epithelial cells are held together by cell junctions, such as tight junctions, zonula adherens, and desmosome markers that function to link cells together, regulate homeostasis required for critical cell processes, and allow for normal cell proliferation (Itoh & Bissell, 2003). Epithelial cells within epithelial tissue establish and maintain communication amongst each other through gap junctions which are communication junctions (Thiery et al., 2009). Conversely, mesenchymal cells are multipotent stromal cells that have the capacity to self-renew, differentiate, and migrate throughout the body (Kalluri & Weinberg, 2009). The loosely organized nature of mesenchymal cells in the extracellular matrix adjacent to the epithelia attribute to and allow for its increased mobility.

Epithelial to mesenchymal transition (EMT) is a critical process during embryogenesis that is later physiologically silenced in adult life in normal conditions. However, in later adult life certain circumstances may induce the reactivation of EMT, such as acute injuries, scars, collagen synthesis, and cancer (Thiery et al., 2009). EMT is a biological process in which epithelial cells undergo a series of biochemical changes that allow it to assume a mobile spindle

mesenchymal cell phenotype, enhancing its migratory capacity, invasiveness, increased resistance to apoptosis, and increased extracellular matrix components such as fibroblasts during growth and development (Kalluri & Neilson, 2003). EMT is highly involved and expressed in several pathologies, such as breast, lung, thyroid, prostate, and colon cancer (Vu & Datta, 2017). During the process of EMT in colorectal cancer, intestinal epithelial cells lose their aforementioned epithelial characteristics, cell polarity, and cell-to-cell contact, and gain mesenchymal properties, such as increased motility (Vu & Datta, 2017). In cancer, the transition from epithelial to mesenchymal cells is the assumed basis for tumor progression and metastasis as it allows cells to migrate from the primary tumor into the blood vessels (Thiery et al., 2009). Once cancer cells with mesenchymal properties migrate to their new destination, they colonize the area by reverting back to their epithelial phenotype (Nieto et al., 2016). The plasticity of carcinoma cells works in their advantage to escape apoptosis or cell death during the stages of tumor progression. The adoption of a mesenchymal-like phenotype allow for the cells to be well adapted for cell deformation and drug resistance (Nieto et al., 2016).

EMT has a very stepwise and transient nature in which it allows for epithelial cells to transition into highly mobile, spindle-shaped mesenchymal cells and back to epithelial cells in its new destination in a process called mesenchymal epithelial transition (MET), which highlights the sheer plasticity of these epithelial and mesenchymal cells (Lamouille et al., 2014). In addition, some epithelial cells may not lose all epithelial characteristics in the progression of EMT while some will, where they may demonstrate some epithelial and mesenchymal characteristics, depending on the interactions between and signaling of the different molecules and pathways involved in the process, indicating partial EMT (Huang et al., 2022).

During EMT regulation in CRC, there are certain EMT markers with altered levels of expression throughout the progression of EMT. EMT is largely mediated by a distinct set of transcription factors that function to activate EMT, including SNAIL, SLUG, TWIST, ZEB1, and ZEB 2 (Stemmler et al., 2019). These transcription factors are largely responsible for the EMT program that results in the dissemination of cellular adhesion and tight junctions that are characteristic to the epithelial phenotype and manifestation of the motile mesenchymal phenotype (Nieto et al., 2016). Active EMT is characterized by the downregulation of epithelial markers, most notably E-cadherin, and upregulation of mesenchymal markers, particularly vimentin, as well as an increase in migratory and invasive capacity (Micalizzi et al., 2010). Epithelial and mesenchymal markers, E-cadherin and N-cadherin respectively, are type-I classical cadherins in which the downregulation of E-cadherin and upregulation of N-cadherin is considered the hallmark of EMT (Loh et al., 2019; Micalizzi et al., 2010). E-cadherin is a potent tumor suppressor that functions in the maintenance of the epithelial phenotype and regulation of homeostasis of tissues by mediating different signaling pathways (Berx & van Roy, 2009). Different germline or somatic mutations, chromosomal deletions, and transcriptional silencing of the CDH1 promoter have been evidenced to interfere with the functionality of E-cadherin and cause the induction of EMT through the induction of mesenchymal cadherins, such as N-cadherin (Berx & van Roy, 2009). Overexpression of N-cadherin indicates ongoing EMT as the junctions between epithelial cells dissociate, leading to the loss of epithelial integrity and progression towards the mesenchymal phenotype (Lamouille et al., 2014). E-cadherin expression is required for the stability of the tight junction protein, ZO-1. The function of ZO-1 is to maintain the organization of the epithelia and form tight junctions (Kyuno et al., 2021). E-cadherin and ZO-1 are positively correlated, meaning that the loss of E-cadherin in EMT results

in the dissociation of the intestinal tissue expressing ZO-1 (Xiao et al., 2010). The presence of EMT-inducing transcription factors, such as TWIST, SNAIL, and zinc-finger E-box-binding (ZEB), function to regulate expression of various genes by inducing the mesenchymal state and repressing the expression of epithelial markers or tight junction proteins and subsequently, the epithelial state (Kyuno et al., 2021). These transcription factors often mediate and control the expression of each other to cooperatively silence *CDH1* or the gene that encodes E-cadherin (Peinado et al., 2007). Most notably, TWIST has the capacity to induce the expression of mesenchymal marker, N-cadherin (Alexander et al., 2006). Lastly, TNF-α is cytokine and when it is induced, it causes the cancer cells to acquire mesenchymal properties with a distinct spindle-like morphology while increasing the expression of mesenchymal marker, N-cadherin, and decreasing the expression of E-cadherin and ZO-1 (Wang et al., 2013).

In EMT regulation, there is crosstalk between different molecules and pathways that can modulate EMT, specifically between the SMAD and NF-κB pathways (Borthwick et al., 2011). Interestingly, transforming growth factor-beta (TGF-β), a key growth factor in EMT regulation, acts as a tumor suppressor during the early stages of tumor growth and development by repressing proliferation and inducing apoptosis; however, upon later stages of tumor development, it takes on an opposite function and serves as a tumor promoter by inducing EMT and suppressing antitumor immune responses (Marcucci et al., 2016). It has been found that TGF-β driven EMT has a synergistic relationship with and is accentuated by TNF-α, further promoting the progression of EMT driven cancers (Borthwick et al., 2011; De Craene & Berx, 2013). Similarly, the NF-κB family of transcription factors possesses a dual function where they are mediated by tumor suppressors, causing the NF-κB subunits to inhibit the expression of tumor promoting genes during early stages of tumor development; however, during later stages

of tumor development, NF-κB exhibits oncogenic functions, actively contributing to tumorigenesis (Perkins, 2004). Additionally, TGF-β also induces NF-κB activity during EMT by activating the transcription of NF-κB target genes (Khatami, 2017). TGF-β acts as a key driver in the induction of EMT, leading to activation of additional molecules and pathways (Deshmukh et al., 2021). The progression of EMT signaling is highly complex and relies on the interactions between different molecules and crosstalk between different pathways in the tumor microenvironment.

2. METHODS

2.1 Animal Experiments

All animal studies were conducted under protocols approved by the Texas A&M University Institution Animal Care and Use Committee guidelines. *Apc$^{Min/+}$*, *Egfr$^{f/f}$*, *Tg(Vil1-Cre)* mice (n=16) were obtained from The Jackson Laboratory (Bar Harbor, ME). *Tg(Vil1-Cre)* was selected since it results in extensive target gene deletion throughout the intestinal and colonic epithelial with minimal expression elsewhere and was used in previous studies with EGFR and ERBB3 (Lee et al., 2009). The mice were housed five per cage, fed the Purina Mills Lab Diet 2919 *ad libidum* and maintained at 22ºC under a 12-hr light cycle. Prior to tissue collection, the mice were euthanized by CO_2 asphyxiation. Tumor tissue and adjacent normal tissue was collected for downstream analysis.

2.2 Genotyping

Mice were genotyped for the *Erbb2^{tm1Mll}* allele as previously described (Leu et al., 2003). Mice were genotyped for the *Apc^{Min}* allele as previously described (Roberts et al., 2002). The *Tg(Vil1-Cre)* allele was identified using PCR primers:

Cre-F, 5'-GCGGTCTGGCAGTAAAAACTATC-'3

Cre-R, 5'-GTGAAACAGCATTGCTGTCACTT-'3.

2.3 Colon Tumor Organoid Isolation and Culture

Fresh intestinal crypts from the colon of 100-day-old *Apc$^{Min/+}$*, *Erbb2$^{f/f}$*, *Tg(Vil1-Cre) and Apc$^{Min/+}$*, *Erbb2$^{f/f}$* mice were isolated using the Stemcell Technologies™ mouse intestinal crypt isolation protocol. The colonic tumors were removed, washed with ice cold PBS, and finely minced with sterile scissors before being incubated at room temperature in PBS plus 10 mM

EDTA with gentle rocking for one hour. Crypts were mechanically separated from connective tissue by shaking before being filtered through a 70μm mesh filter into a 50 mL conical tube to remove tissue fragments. Following centrifugation, isolated crypts were suspended in Matrigel® Matrix, and 25μL of the matrigel-organoid mixture was plated into onto eight wells of a pre-warmed 24-well plate, followed by a 15-minute incubation to allow for polymerization of the Matrigel. 500 μL of room temperature IntestiCult™ Organoid Growth Medium supplemented with 100 units/mL penicillin and 100μg/mL streptomycin was added to each well containing a matrigel-organoid dome.

2.4 Organoid Maintenance

For organoid maintenance, the medium was changed every 48 hours, and the organoids were passaged by mechanical disruption one week after isolation or when they demonstrated dark, necrotic cores using the Stemcell™ Technologies organoids passing protocol.

2.5 Cryopreservation

After culturing the suspending the organoids from *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$, *Tg(Vil1-Cre) and* *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$ mice for four months, the matrigel-organoid domes were disrupted by ice-cold DPBS, washed and centrifuged at 0.3 RCF for five minutes until all the matrigel was removed, and the fragmented organoids were suspended in 1mL of CryoStor® CS10 cell cryopreservation media and placed into a cryovial. Subsequently, the organoids were placed into a freezer maintain at -80ºC using a cool-cell container for 24-hrs. The cryovials were then directly placed into liquid nitrogen for storage.

2.6 RNA Sequencing (RNAseq) and Differential Gene Expression Analysis

A total of three sequencing runs were performed to sequence 20 samples (5 tumor and 5 adjacent normal samples from both ERBB4-deficient and littermate control mice) on the

Illumina NextSeq 500 instrument in the Texas A&M Institute for Genome Sciences and Society Molecular Genomics Core using high output kit v2. A total of approximately 1.839 billion 150 bp single-end reads were checked for adaptor sequences and low-quality bases using Trimmomatic (Bolger et al., 2014), resulting in approximately 1.828 billion filtered reads (99%). RNA-Seq reads were aligned to mouse assembly mm39 using HISAT2(Kim et al., 2015) version 2.2.1 with an average of about 95.73%. Raw transcript counts were generated using HTSeq version 2.0.2 (Anders et al., 2015) while discarding ambiguous reads. Normalized read counts and differential gene expression analyses were performed using DESeq2 following guidelines recommended by the authors (Love et al., 2014). P-values were adjusted for multiple testing with the Benjamin-Hochberg false discovery rate. Ingenuity Pathway Analysis (IPA) (Qiagen) and Gene Set Enrichment Analysis (GSEA) version 4.3.2 were used to analyze differentially expressed genes between groups (Krämer et al., 2014; Subramanian et al., 2005).

2.7 Gene Set Enrichment Analysis (GSEA)

Gene Set Enrichment Analysis (GSEA) was used to identify enrichment in a gene set between tumor tissue and adjacent normal tissue of ERBB2-deficient (n=5) and littermate control (n=5) mice in a group of gene markers ranked by their relevance with a phenotype of interest. GSEA was carried out by utilizing the GSEA version 4.3.2, obtained from the Broad Institute. Overlaps with the epithelial mesenchymal transition (EMT) hallmark gene gets were computed. GSEA enrichment plots were plotted to determine enriched gene sets.

2.8 RNA Extraction and Quality Determination

Total 16 samples of RNA from ERBB2 knockout (n=4) and control (n=4) mice were isolated from flash frozen tumors for downstream analysis. Total RNA for tumors was isolated

using Maxwell (Promega AS1280), and RNA quality was determined using the TapStation kit (Agilent G2991-90121) according to the manufacturer's instructions.

2.9 Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Total RNA from tumors and adjacent normal tissue was isolated using Maxwell LEV simply RNA Tissue Kit (Promega AS1280) according to the manufacturer's instructions. Total RNA was converted to cDNA using the Transcriptor First Strand cDNA Kit (Roche 04896866001). Quantitative reverse transcription PCR reactions were set on a 384 well plate and samples were run in triplicate using cDNA with LightCycler® 96 Thermocycler using LightCycler 480 Sybr Green I Master Mix (Roche 04896866001) on a LightCycler® 96 System (Roche). Specific primers were designed to amplify a fragment from the target gene, and fold expression differences were calculated using the $2^{-\Delta\Delta Ct}$ method relative to *Gapdh* expression. Quantitative reverse transcription PCR reactions were performed by an investigator blinded to genotype. Quantitative reverse transcription PCR was performed using the following primers:

GAPDH-F, 5'-AGGTCGGTGTGAACGGATTTG-3'

GAPDH-R, 5'- GGGGTCGTTGATGGCAACA-3'

TNF-α-F, 5′-GGTGCCTATGTCTCAGCCTCTT-3′

TNF-α-R, 5′-GCCATAGAACTGATGAGAGGGAG-3′

TWIST1-F, 5'-GCCCGTGGACAGAGATTC-'3

TWIST1-R, 5'- CTATCAGAATGCAGAGGTGTGG-'3

CDH1-F, 5'-GCCGGAGAGGCACCTGGAGA-3'

CDH1-R, 5'-GCGCGGACGAGGAAACTGGT-3'

CDH2-F, 5'-TGACAATGACCCCACAGCTC-'3

CDH2-R, 5'-GTCCTGCTCACCACCACTAC-'3

VIM-F, 5’-CGGAAAGTGGAATCCTTGCAGG-3’

VIM-R, 5’-AGCAGTGAGGTCAGGCTTGGAA-3’

ZO-1-F, 5'-ACTCCCACTTCCCCAAAAAC-3'

ZO-1-R, 5'-CCACAGCTGAAGGACTCACA-3'

2.10 Statistical analysis

The fold of expression of each EMT signaling marker was quantified by $2^{-\Delta\Delta Ct}$ method. The difference between the means among three or more groups was compared using a one-way analysis of variance (ANOVA) with post-hoc Tukey’s honestly significant difference (HSD) test. P-values of less than 0.05 were considered statistically significant. Statistical details and p-values can be found in the figure legends. All data are represented as mean ± SEM. Statistical analysis and figure generation were performed by Microsoft 365 Excel. Statistical analysis was performed by an investigator blinded to genotype.

3. RESULTS

3.1 Intestinal Epithelial Cell-specific *Erbb2* Ablation Increases Colon Tumor Size

ERBB2 is commonly overexpressed and exhibits altered expression in a multitude of cancer types, including breast (Bose et al., 2013), lung (Hirsch et al., 2009), and colorectal cancer (CRC) (Siena, 2018). Despite this, the role of ERBB2 in the growth and proliferation of tumors in mouse colon tissue remains sparsely explored. Based on the overexpression of ERBB2 in CRC, we interrogated how the absence of ERBB2 allows for the continued tumor growth and progression in the intestinal epithelia, using the $Apc^{Min/+}$ mouse model with an intestinal epithelium specific deletion of *Erbb2* ($Apc^{Min/+}$, $Erbb2^{f/f}$, *Tg(Vil1-Cre)*), using *Vil*-Cre and conditional knockout allele of *Erbb2* and wild type littermate controls ($Apc^{Min/+}$, $Erbb2^{f/f}$). To evaluate the effect of EGFR in intestinal tumorigenesis at 100 days of age, it was evident that all $Apc^{Min/+}$ mice (n = 30 ERBB2 wild type control; n = ERBB2-deficient) developed visible polyps (>0.5 mm in diameter) in the small intestine regardless of genotype. It is known that most tumors in the $Apc^{Min/+}$ mouse model occur in the small intestine with less tumors developing in the colon(Shoemaker et al., n.d.) (Figure 1A); however, although the tumors that develop in the colon are lesser in quantity, they are larger and grow more aggressively in comparison to the development of tumors in the small intestine (Figure 2A and 2B). This ERBB2-dependent increase in size polyps was observed throughout regions of the small intestine and trended in the same direction in the colon. The polyps in mice with an ERBB2 specific ablation are significantly larger in size compared to the wild type littermate control mice, specifically in larger polyps that were at least >1.0 mm in diameter (Figure 2B). Taken together, these results demonstrate that epithelial-specific ERBB2 signaling is important in intestinal tumorigenesis in

$Apc^{Min/+}$ mice and suggest that there is an alternate mechanism of contributing to the increased tumor size and more aggressive growth and progression in the absence of *Erbb2* expression.

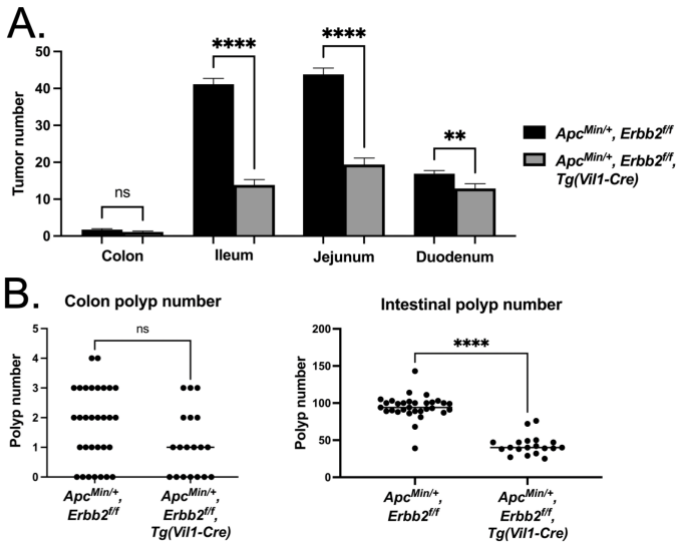

Figure 1: Polyp number of wild type ERBB2 compared to ERBB2-deficient in mouse intestinal epithelium. *(A) Overall intestinal polyp number; (B) Polyp number separated between the colon and small intestine. Each dot represents polyp number in each 100-day old mouse. Ns denotes no significant difference. ****p-value<0.0001.*

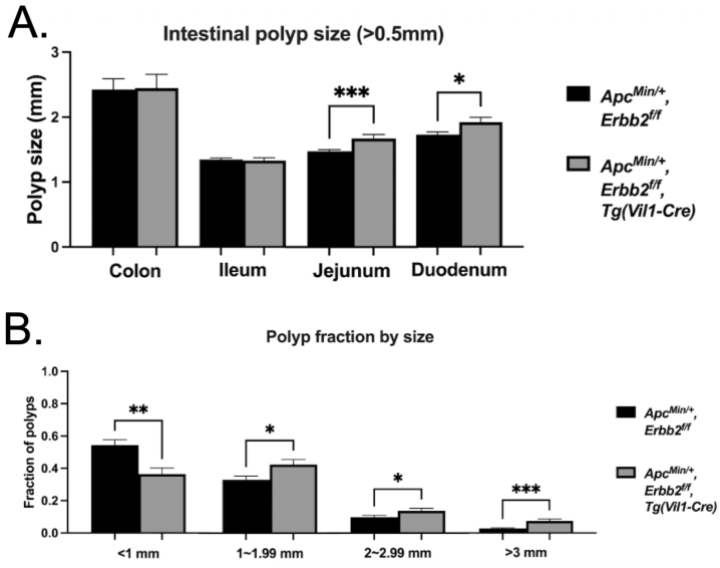

Figure 2: Intestinal polyp size in mice lacking ERBB2 compared to wild type ERBB2 in intestinal epithelium*. (A) Average polyp size through the intestine; (B) Polyp fraction by size. Black bars represent mean polyp size in Erbb2 wild type mice and gray bars represent mean polyp size in Erbb2 intestinal epithelial-specific knockout mice. *p-value<0.05, **p-value<0.01, ***p-value<0.001.*

3.2 Abnormal Branching Morphology in ERBB2-deficient Organoids

ERBB2 ablation did not *Erbb2* did not generate overt differences in gross appearance of polyps in vivo; however, organoid culture revealed abnormal morphological differences between the wild type (*Apc*$^{Min/+}$, *Erbb2*$^{f/f}$) and ERBB2-deficient (*Apc*$^{Min/+}$, *Erbb2*$^{f/f}$, *Tg(Vil1-Cre)*) mice with an ERBB2 conditional knockout allele in the intestinal epithelia. The ERBB2 knockout organoids displayed darker necrotic cores and demonstrated a distinct branching morphology from the organoids of the wild type littermate control tumors (Figure 3), suggesting epithelial mesenchymal transition (EMT) and increased cell proliferation. Compared to the ERBB2 knockout organoids, the wild type littermate control organoids appeared to be lighter in color, indicating less rapid cell proliferation, and they displayed a more traditional spheroid phenotype.

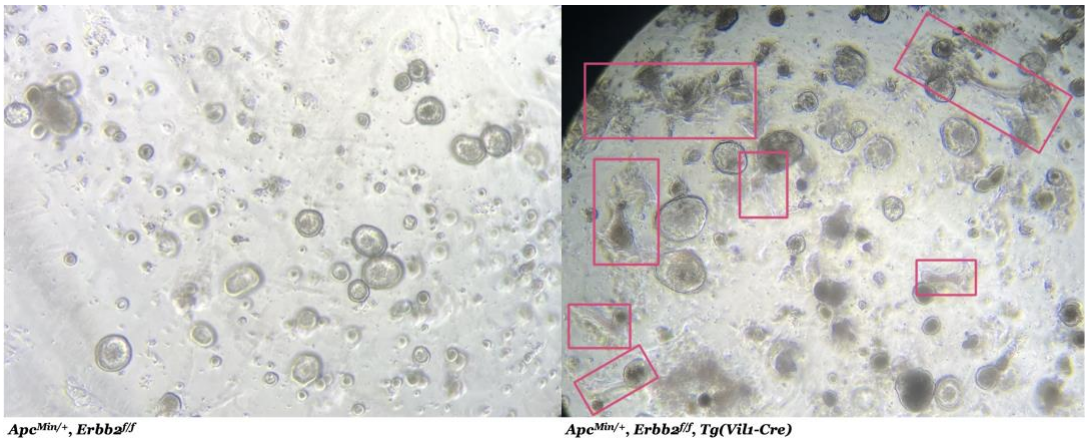

Figure 3: *Abnormal branching of* ERBB2-deficient *(Apc*$^{Min/+}$*, Erbb2*$^{f/f}$*, Tg(Vil1-Cre) organoids compared to wild type littermate control mice (Apc*$^{Min/+}$*, Erbb2*$^{f/}$*)*

3.3 Transcriptomic Analysis of ERBB2-deficient Colonic Tumors Reveals Alternate Mechanisms for Continued ERBB2-deficient Tumor Growth and Progression

To identify potential molecular pathways and mechanisms associated with increased polyp size in *Erbb2*-deficient CRC tumor growth, we conducted RNA sequencing (RNAseq) of

colon tumors developing with and without functional *Erbb2*. Differential gene expression analysis between tumors and adjacent normal tissue samples revealed 1157 differentially expressed genes that are characteristic to the progression of *Erbb2*-deficient colon tumors (Figure 4). Transcriptomic analysis conducted through Ingenuity Pathway Analysis (IPA) predicted significant activation in upstream regulator that is known to be involved in EMT, TGF-β1 (Table 1).

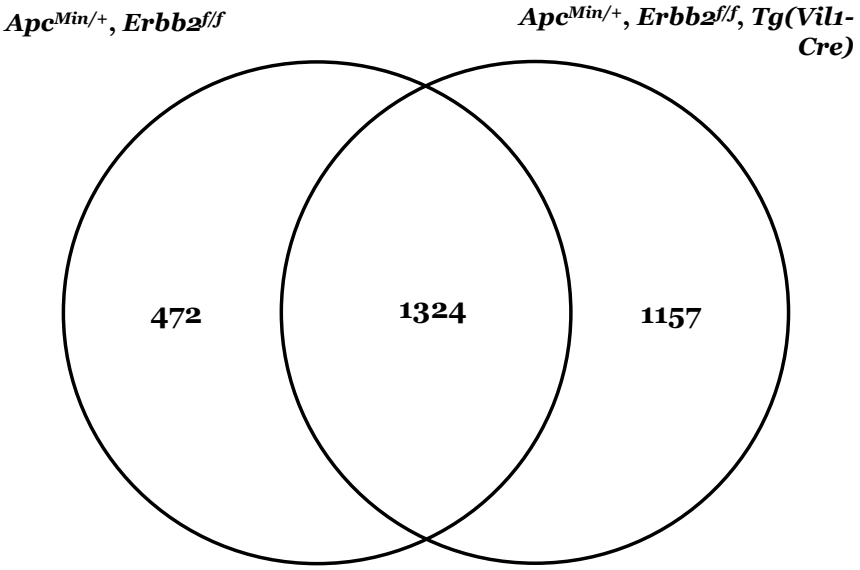

Figure 4: Transcriptomic analysis of wild type and ERBB2-deficient colonic polyps. *Venn diagram of differentially expressed genes between wild-type and ERBB2-deficient colonic polyps compared to adjacent normal epithelium.*

Table 1: *Top activated and inhibited upstream regulators involved in progression of ERBB2-deficient polyps predicted by Ingenuity Pathway Analysis (IPA)*

Upstream regulator	Molecule type	Predicted activation state	Activation z-score	p-value of overlap
EGFR	Kinase	Activated	3.822	3.22E-06
AGT	Growth factor	Activated	3.756	2.25E-06
Kianic acid	Chemical toxicant	Activated	3.647	3.59E-06
TGFB1	Growth factor	Activated	3.597	5.75E-12
NPM1	Transcription regulator	Activated	3.153	7.28E-06
SP600125	Chemical drug	Inhibited	-3.331	1.23E-04
NR3C1	Ligand-dependent nuclear receptor	Inhibited	-3.306	3.29E-07
PTEN	Phosphatase	Inhibited	-3.296	5.18E-04
HNF4A	Transcription regulator	Inhibited	-2.969	7.09E-04
Immunoglobulin	Complex	Inhibited	-2.88	6.75E-03

EGFR, epidermal growth factor receptor; AGT, angiotensinogen; TGFβ1, transforming growth factor beta 1; NPM1, nucleophosmin 1, NR3C1, nuclear receptor subfamily 3 group C member 1; PTEN, phosphatase and tensin homolog; HNF4A, hepatocyte nuclear factor 4 alpha

3.4 Gene Set Enrichment Analysis (GSEA) Indicates Potential EMT Signaling in ERBB2-deficient Tumor Tissue

To further validate potential epithelial mesenchymal transition (EMT) due to the observed abnormal branching morphology of the ERBB2-deficient organoids, we investigated the hallmark epithelial mesenchymal transition (EMT) associated with *Erbb2*-deficient CRC tumor growth and progression. GSEA revealed differential expression of EMT between wild type littermate control and ERBB2-deficient mice. In comparison to the intestinal specific deletion of ERBB2, the enrichment plot for the $Apc^{Min/+}$, $Erbb2^{f/f}$ wild type mice demonstrate no evidence of significant EMT marker activity in both the adjacent normal tissue and the tumor tissue with a nominal p-value of 0.48 (Figure 5). In contrast, the enrichment plot for the $Apc^{Min/+}$, $Erbb2^{f/f}$*, Tg(Vil1-Cre)* indicates potential EMT marker activity, particularly in the tumor tissue

(Figure 6). This is alluded to the possibility of the mechanism of continued tumor growth and progression in the absence of ERBB2 to be caused by EMT signaling.

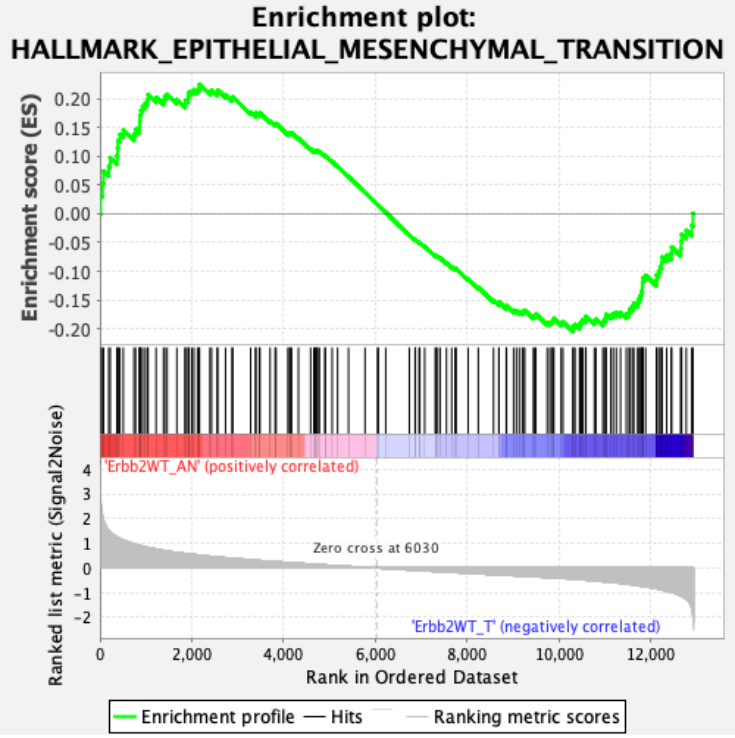

Figure 5: *Hallmark Epithelial Mesenchymal Transition (EMT) enrichment plot for* $Apc^{Min/+}$, $Erbb2^{f/f}$

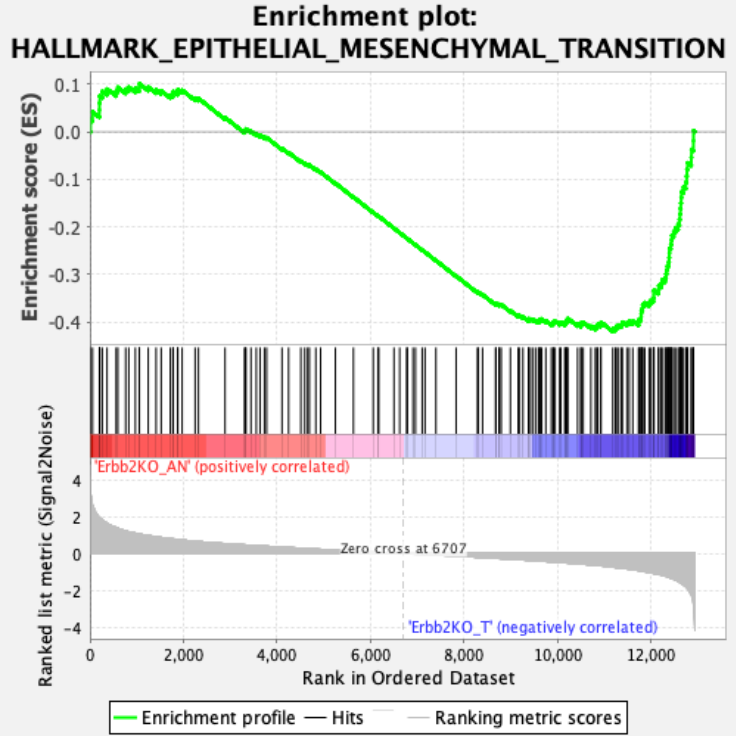

Figure 6: *Hallmark Epithelial Mesenchymal Transition (EMT) enrichment plot for* $Apc^{Min/+}$, $Erbb2^{f/f}$, *Tg(Vil1-Cre)*

3.5 Quantitative Reverse Transcription PCR (RT-qPCR) Reveals Potential EMT Regulation

To elucidate the gene set enrichment analysis (GSEA) attributing to the continued tumor growth and progression to epithelial mesenchymal transition (EMT) signaling, EMT markers known to have altered expression during EMT were validated using quantitative PCR. The epithelial markers tested were E-cadherin (CDH1) and zona occludins-1 (ZO-1). TWIST1 was also validated along with mesenchymal markers, tumor necrosis factor alpha (TNF-α), N-cadherin (CDH2), and vimentin (VIM). Altered expression of these EMT markers have been associated with CRC tumor growth, progression, and metastasis. Decreased level of expression of CDH1 and ZO-1 due to the loss of anti-invasive and anti-migratory properties of normal epithelial cells have been correlated with metastasis and EMT signaling. A high level of expression of TWIST1, TNF-α, CDH2, and VIM have been associated with increased tumor cell

survival, adoption of mesenchymal spindle-like morphology, and migration, indicating EMT regulation. Quantitative PCR revealed significant overexpression of vimentin transcripts in ERBB2-deficient tumor and adjacent normal tissue compared to the tumor tissue of the wild type littermate control mice (Figure 7). Significant overexpression of mesenchymal marker, VIM, is also accompanied by overexpression of TNF-α, which is consistent with the progression of EMT regulation (Figure 7). The opposite was noticed in TWIST1 where it is significantly overexpressed in the wild type litter mate control tumor and adjacent normal tissue rather than the tumor and adjacent normal tissue of the mice with the epithelial specific deletion of ERBB2 (Figure 7). A similar trend of overexpression of mesenchymal marker, N-cadherin, was observed as well in the wild type tumor and adjacent normal tissue compared to the ERBB2-deficient tumor and adjacent normal tissue (Figure 7). However, quantitative PCR data demonstrates that there is decreased expression of epithelial markers, E-cadherin and ZO-1, signifying the loss of epithelial cell characteristics (Figure 7). Altogether, these results show that continued tumor growth and proliferation paired with the observed abnormal branching morphology in the absence of ERBB2 in the colonic organoids may potentially be correlated with a decrease in epithelial markers and adoption of the motile mesenchymal phenotype.

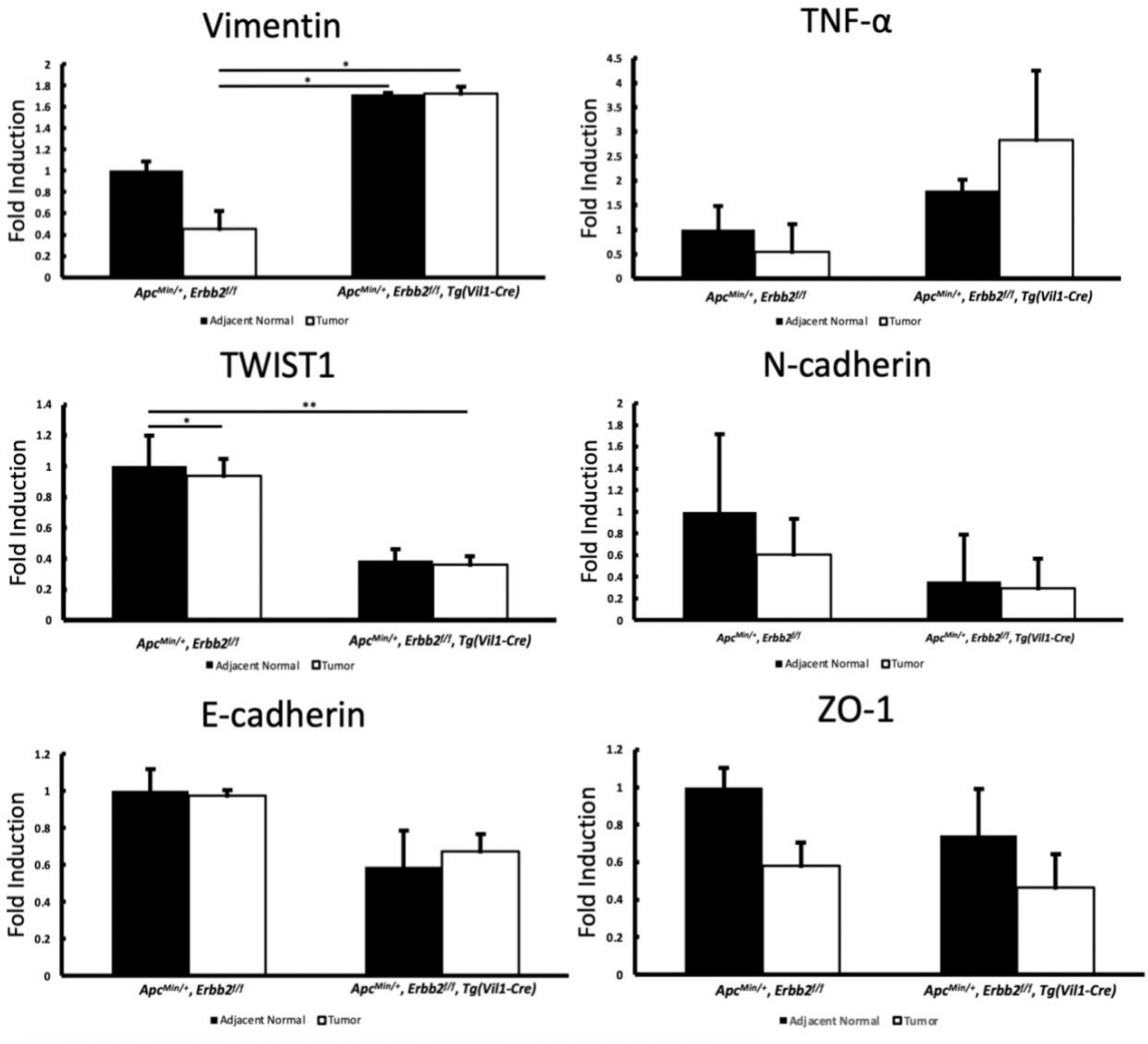

Figure 7: Validation of epithelial mesenchymal transition (EMT) markers in ERBB2-deficient colon polyps. *Black bars represent mean level of transcripts in normal tissue adjacent to polyps; white bars represent level of transcripts in the colon tumors between wild type littermate control mice and ERBB2-deficient mice. *p-value<0.05, **p-value<0.01.*

4. CONCLUSION

4.1 EMT Regulation in the Absence of ERBB2 in the Intestinal Epithelial is Plausible

ERBB2 is a member of the EGFR family that is commonly overexpressed and implicated in a variety of cancer types. When ERBB2 signaling pathways are activated, various metastasis associated properties can induce cancer metastasis, such as epithelial mesenchymal transition (EMT). We examined intestinal polyp development in the absence of ERBB2 activity using an intestinal epithelial-specific deletion of *Erbb2*. Although the polyps that developed in the colon are fewer in number, they are significantly larger in size and grow more aggressively in the absence of ERBB2. This result demonstrates that there must be an alternate mechanism for the continued tumor growth and progression in the absence of ERBB2 in the intestinal epithelia.

To elucidate the mechanism underlying the differential effect of ERBB2 loss on polyp size and growth phenotype, we examined the growth phenotype through organoid culture of colon tumors from $Apc^{Min/+}$, $Erbb2^{f/f}$ and $Apc^{Min/+}$, $Erbb2^{f/f}$, *Tg(Vil1-Cre)* mice. Upon culture, it was evident that there was an abnormal branching growth phenotype in the absence of ERBB2 compared to the wild type littermate control organoids, which appeared to be more spheroid in shape. Given the motile nature and high migratory capacity of epithelial cells undergoing EMT regulation, the characteristic EMT branching morphology of organoids towards a mesenchymal phenotype alluded to the possibility of EMT regulation in the absence of ERBB2 in the intestinal epithelia given the motile, spindle-shaped nature of mesenchymal cells.

In order to gain insight into whether epithelial mesenchymal transition (EMT) could be the underlying cause of the observed abnormal morphology of the ERBB2-deficient organoids, gene set enrichment analysis (GSEA) was performed. GSEA confirmed the statistically

significant differential expression of hallmark epithelial mesenchymal transition (EMT) between the *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$ and *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$, *Tg(Vil1-Cre)* mice, indicating that EMT is highly plausible and worth investigating.

To determine the presence of EMT regulation in ERBB2-deficient mice, we quantified the transcript levels of expression of six EMT markers in the intestinal and adjacent normal tissue of *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$ and *Apc*$^{Min/+}$, *Erbb2*$^{f/f}$, *Tg(Vil1-Cre)* mice by quantitative reverse transcription PCR (RT-qPCR). Based on the RT-qPCR results, we propose that EMT regulation may be involved in the increase in colon tumor size and aggressive growth phenotype in the absence of ERBB2. Vimentin is a key mesenchymal marker and its overexpression in the absence of ERBB2 is characteristic of EMT signaling. Consistent with our hypothesis, there was significant expression of this key marker, vimentin, in the tumor and adjacent normal tissue of the ERBB2-deficient mice compared to the wild type littermate control mice in the absence of ERBB2. On the same place, overexpression of mesenchymal marker, TNF-α, in the tumor tissue and adjacent normal tissue in the ERBB2-deficient mice was evident, further adding to the evidence of possible EMT signaling in the absence of ERBB2 in the intestinal epithelia. In support of our hypothesis, higher levels of expression of epithelial markers, E-cadherin and ZO-1, were observed in the tumor tissue and adjacent normal tissue of the wild type littermate control mice due to the dissemination of adherens junctions and tight junctions present prior to the induction of EMT. However, further RT-qPCR is required to confirm the occurrence of active EMT regulation in ERBB2-deficient tumors due to the observed increased expression of N-cadherin and significant overexpression of TWIST1 in the tumor and adjacent normal tissue of the wild type littermate control mice.

4.2 Future Directions

Research on molecular genomic markers underlying EMT regulation hold significant potential in identifying a subset of patients that are at highest risk of developing metastasis of CRC to other tissues. Due to the nature of the *APC*^{min} mutation and plethora of polyp growth and development throughout the intestinal epithelia, the mice had to be euthanized at an early age of 100-days. At the same time, the nature of EMT that it is a stepwise process in which different molecules and pathways interact with each other causing altered or aberrant expression of various EMT markers depending on the stage of EMT. Thus, reduced expression of mesenchymal markers, N-cadherin and TWIST1, in the tumor and adjacent normal tissue of the ERBB2-deficient mice can be due to the fact that EMT had not had enough time to fully manifest itself, specifically in the switch from epithelial marker, E-cadherin to mesenchymal marker, N-cadherin, and in the crosstalk between the SMAD and NF-κB pathways that allow for TGF-β induced EMT. However, partial EMT may also be plausible as some epithelial cells may not lose all epithelial characteristics or adopt the mesenchymal phenotype to its completion as seen in the conflicting trend of levels of expression of mesenchymal markers Vimentin, N-cadherin, and TWIST1. Follow up studies include utilizing an inducible CRC mouse model in which the mice develop fewer tumors, allowing for the mice to humanely live longer while allowing for the manifestation of EMT in the intestinal epithelia of the mice. Follow up quantitative PCR on EMT markers will then be performed again to validate the EMT targets. Lastly, we plan to perform a western blot to separate and identify other proteins that may be involved in the continued tumor growth and progression in the absence of ERBB2.

The precise mechanism leading to the aggressive growth phenotype and tumors that develop greater in size in the absence of ERBB2 is a key follow-up question. It is still unclear

whether EMT is the precise mechanism for this tumor growth and development. Ingenuity pathway analysis (IPA) revealed other potential upstream regulators that could be involved in the increased size and aggressive growth phenotype in the absence of ERBB2, such as EGFR and TGF-β1 that we intend to investigate. Due to the synergistic nature of TGF-β1 and TNF-α and crosstalk between different pathways and transcription factors, other EMT targets involved in these processes will be investigated. Nonetheless, this research provides evidence of potential EMT regulation in the tumor growth and development in the absence of *Erbb2* expression. Although the continued investigation of other potential mechanisms is warranted, our findings may act as a foundation to determine the most efficient route to completely identify the exact mechanism of the increased tumor size and aggressive tumor growth phenotype in the absence of ERBB2.

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