MicroRNA-Mediated Migration of Colon Cancer Cells

Algaber, Anwar

2021

Document Version:
Publisher's PDF, also known as Version of record

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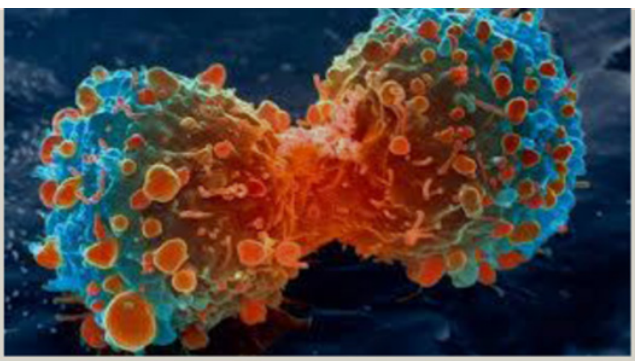
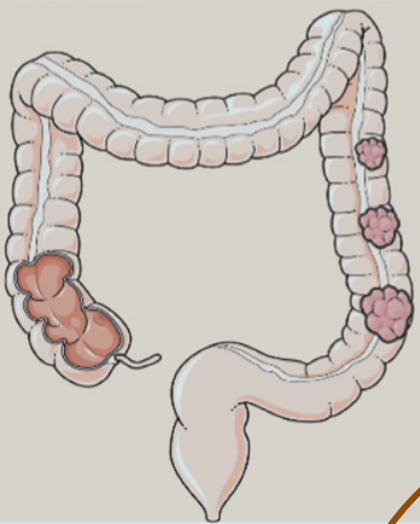
ANWAR ALGABER
DEPARTMENT OF CLINICAL SCIENCE, MALMÖ | LUND UNIVERSITY





MicroRNA-Mediated Migration of Colon Cancer Cells

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Anwar Algaber

Faculty of Medicine
Section of Surgery

DOCTORAL DESSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden. To be defended at Surgical Clinic, Carl-Bertil Laurells gata 9, floor 3, room 3050, Malmö and will be available for public via Zoom on the 10th of June 2021 at 13:00 pm.

Faculty opponent

Professor: Karin Strigård
Department of Surgical and Perioperative Sciences
Umea University, Sweden

Organization LUND UNIVERSITY	Document name: **DOCTORAL DISSERTATION**
	Date of issue: 5th of May 2021
Author: **Anwar Algaber**	Sponsoring organization

Title: MicroRNA-Mediated Migration of Colon Cancer Cells

Abstract

Colorectal cancer (CRC) is the second most common cancer in women and third most common cancer in men worldwide. The cause of the majority of death related to CRC is believed to be the migration of cancer cells to distant organs which is known as cancer metastasis. The mechanism behind cancer cell metastasis is not fully understood but accumulating studies suggest that it could be due to enhanced tumor cell motility due to overexpression of metastasis related proteins. It is believed that microRNAs (miRNAs) play a significant role in the tumorigenesis and metastasis of cancer by regulating oncogenes. The aim of this thesis is to investigate the mechanism of miRNA-mediated colon cancer cell invasion and migration as well as possible targets genes of miRNAs. We found that knockdown of miR-155-5p by antagomiR reduces the expression of HuR mRNA and migration of colon cancer cells. Our data also showed that miR-155-5p is involved in positive regulation of HuR protein under stress conditions. Notably, this positive regulation is regulated by direct binding of miR-155-5p at AU rich element region in 3′-UTR of HuR mRNA. In addition, it was found that miR-340-5p is also involved in colon cancer cell invasion and migration by regulating RhoA and FHL2 mRNA expression. Bioinformatics analysis revealed that both RhoA and FHL2 mature mRNA have conserved binding sites from 2 to 8 base positions for miR-340-5p. The seed region of miR-340-5p directly binds with the target sites of RhoA and FHL2 mRNA and negatively regulate their expression under stress conditions. We found that the inhibition of RhoA and FHL2 expression by the use of mimic miR-340-5p reduced colon cancer cells invasion and migration. In addition, it was found that inhibition of FHL2 reduces cancer cells proliferation and increases E-cadherin expression in colon cancer cells, suggesting that targeting FHL2 and RhoA by miR-340-5p might be a useful approach to antagonize colon cancer cells metastasis. The results of our studies not only show diverse mechanisms of colon cancer cells migration, but also provided valuable information that miRNAs can be an important target to develop new and effective therapeutics against colon cancer cells metastasis. Taken together, our data uncovered several new mechanisms for better understanding the mechanism of colon cancer cells metastasis and suggest that targeting miRNAs function could be a useful strategy to prevent colon cancer metastasis.

Key words: MicroRNA, Colon cancer, metastasis, HuR, RhoA, FHL2

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language: English**
ISSN and key title : 1652-8220		**ISBN: 978-91-8021-078-2**
Recipient's notes	**Number of pages: 94**	**Price N/A**
	Security classification	

Signature:  **Date: 5th of May 2021**

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Anwar Algaber

Department of Clinical Science, Malmö
Section of Surgery
Skåne University Hospital
Lund University, Sweden 2021

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Faculty of Medicine
Department of Clinical Science, Malmö
Lund University

ISBN 978-91-8021-078-2
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2021

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MADE IN SWEDEN

قال الله تعالى:

(يَرْفَعِ اللَّهُ الَّذِينَ آمَنُوا مِنكُمْ وَالَّذِينَ أُوتُوا الْعِلْمَ دَرَجَاتٍ وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ (11))

سورة المجادلة

(Allah will exalt those of you who believe and those who are given knowledge, to high degrees; and Allah Aware of what you do). Quran chapter Al-Mujadila verse no. 11

To my family

Acknowledgment

First and foremost, I would like to thank the **God** for giving me the power and confidence to continue this long journey. I wish to express my great pleasure to all people who supported me during the period of my studies. Also, express my gratitude to prophet **Mohamed** as well as **Fatima AL-Zahra**, also thank her husband, her sons, and the secret that is deposited in her.

I would like to express my deepest appreciation to my principal supervisor, Professor **Henrik Thorlacius** for giving me this golden opportunity, for his patience, enthusiasm, and for providing invaluable support during my studies. This work has given more focus and clarity by his expertise in clinical and scientific work. I have been extremely lucky to have him as a supervisor, and for the patient guidance, encouragement and advice that I have received throughout my time as his PhD student.

I am deeply grateful and thankful to my co-supervisor, **Milladur Rahman**, for his unlimited support and wise guidance over the past four years. It would never have been possible for me to make this work without your unlimited guidance and feedback during the lab work and thesis writing; your helpfulness is highly appreciated.

Deepest thanks and gratitude extend to **Anne-Marie Rohrstock** for all help that I have got from the first day when I joined the Lab. My appreciation also extends to my laboratory colleagues at the Clinical Research Centre (CRC) for all help during my studies, **Amr Al-Haidari** for facilitating all the requirements, I always got the answer from him, **Raed Madhi, Avin Hawez, Yongzhi Wang, Dler Taha, Nader Algethami, Zhiyi Ding, Feifei Du, Johan Linders, Lubna Mehdawi, Israa Mohammed Al-Amily**, **Manal Ali, and Florin George Bocean, Kifah Qaddorah and Omeyme Naqchi,** it has been a great pleasure to work with all of you.

My appreciation also extends to my classmates in Iraq at Al-Zahra's high school for distinguished students in Nasiriya city; **Amani Shakir Aswad who** is the director of the school**, Ferid Hamed Sulaiman, Mohammed Abed Hussein, Saoud Khalaf Atshan, Abbas Harbi Al-badri, and Khuider Al Salihy.**

My biggest appreciation and love belong to my family for their great help and thankfulness for their unlimited support, patience and encouragement especially to my wife; **Surur Al-Omari** and to my two angels in my life **Mohammed** and **Adain** for their love, patience, and understanding of my research work especially during the most time when I was at work.

I also dedicate this thesis to those behind the scene who were my power of successfulness; my Mother; **Safia Mohammed** and my Father; **Abd Al-Abbas Mohammed Algaber** and all my brothers and sisters. no words will be enough to express my appreciation to you. At last, I would like t to thank many other people at the faculty of medicine and all my friends in Sweden and Iraq whose names have not mentioned here, I never forget their help. Thank you all from the bottom of my heart!

My appreciation also extends to my best friends in the Sweden; **Naim Al-Ebrahimiy, Imad Akeab, Mohammad Yaseen, Hussain Alomari, Wadhah Alajeli, Dihaa Alomari, Hazeem Gharawi, Yahya Almshrfawi, Meki Al-umari, Fadil Al-Zubeidi, Rashid Al-Omari, Fatma Al-omari, Fawzi Al-maksusi, Isa Naser, Sherwan Rahman, Hassan Lilak, Ali Alhameedi, Mohsin Al-omari, Qasim Jubarah, Naeem Alsaedi, Wohid Al-umari, Adel Hameed, Jafar Al-audaa, Abdallah Al-Umari, Ali Al-Janabi,** and **Shakir Al-mnahi.**

Deepest thanks and gratitude extend to my friends in Iraq; **Ali Al-Omary, Riad Al-Omari, Abdulkalik Alshabib, Diyaa Aldeen Alomar, Abdulkhaleq Ibrahim, Osama Alshabib, Nazaer-Alomari, Muhammad Muwaffaq Alshabib, Sajad Alshabib, Ali Adnan Khudhair, Karrar Al-Naseri, Hasam Alshabibi, Ahmed Alshabib, Haider Ali, Mohamed Joma, Mohanad Alfahad, Hayder Alomary, Abbas Oudah, Adnan Al-Naseri, Ahmed A. Alibrahimi, Ali Badr Roomi, Saad H. Al-Badry, Khairullah Alhadidi, Hassan Abdel Wahab, Hadi Ali, Wisam Talib Ali, Muthanna shareef tahibul, Jasim Al-kharsan, Hussain Ali Al-omari, Marwan Al-khafaji and Azhar Al-salehy.**

I am deeply thankful to Faculty of Science, **Thi-Qar University** and **Ministry of education**, Thi-Qar of Education Directorate, Iraq.The studies included in this thesis were supported by Cancerfonden (190428 Pj), **Swedish Medical Research Council** (2012-3685**), Einar och Inga Nilssons stiftelse**, Greta och Johan Kocks stiftelser, Magnus Bergvalls stiftelse, Mossfelts stiftelse, Malmö University Hospital Cancer Foundation, Malmö University Hospital and Lund University.

Table of Contents

Abbreviations

CRC	Colorectal cancer
Ago2	Argonaute 2
AREs	AU-rich elements
DMEM	Dulbecco's Modified Eagle Medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
mRNA	Messenger RNA
PBS	Phosphate buffered saline Phycoerythrin
RIP	RNA immunoprecipitation
RNA	Ribonucleic acid
TSB	Target site blocker
UTR	Untranslated region
AKT	Protein kinase B
COX-2	Cyclooxygenase 2
BSA	Bovine serum Albumin
LNA	Locked Nucleic Acids
APC	Adenomatous Polyposis Coli
Bcl2	B-cell lymphoma 2
BSA	Bovine serum Albumin
BRAF	B-Raf murine sarcoma viral oncogene homolog B
CIN	Chromosomal instability
CSC	Cancer stem cell
EMT	Epithelial–mesenchymal transition
ECM	Extracellular matrix
ERK	Extracellular signal–regulated kinase
FAP	Familial adenomatous polyposis
FIT	Fecal immunochemical test
HuR	Human antigen R
IBD	Irritable bowel disease
NK	Natural killer cells
MAPK	Mitogen-activated protein kinase

miRNAs	MicroRNAs
miRNPs	Microribonucleoprotein
MMPs	Matrix metalloproteinases
MSI	Microsatellite instability
NFKB	Nuclear factor kappa-light-chain-enhancer of activated B cells
QRT-PCR	Quantitative Reverse transcription polymerase chain reaction
RBPs	RNA binding proteins
Rho	Ras homolog protein
RIP	RNA Immunoprecipitation
RISC	RNA-induced silencing complex
ROCK	Rho-associated protein kinase
siRNA	Small interference RNA
SNAI1	Snail Zinc fingerprotein
TNFα	Tumor necrosis factor alpha
TS	Target site
TSB	Target site blocker
TTP	Tristetraprolin
Wnt	Wingless-related integration site
FHL2	Four and a half LIM domains
E-cad	E-cadherin

List of Original Papers

I. Paper I

MiR-155-5p controls colon cancer cell migration via posttranscriptional regulation of Human Antigen R (HuR). Amr A. Al-Haidari, **Anwar Algaber,** Raed Madhi, Ingvar Syk, and Henrik Thorlacius. Cancer Lett. (2018) 421:145-151

II. Paper II

MicroRNA-340-5p inhibits colon cancer cell migration via targeting of RhoA expression. **Anwar Algaber**, Amr Al-Haidari, Raed Madhi, Milladur Rahman, Ingvar Syk and Henrik Thorlacius. Scientific Reports. (2020) 10 (1):16934.

III. Paper III

Targeting FHL-2-E-cadherin axis by miR-340-5p attenuates colon cancer cell migration and invasion**. Anwar Algaber**, Raed Madhi, Avin Hawez, Carl-Fredrik Frimand, Milladur Rahman. Oncology Letters (2021), In Press.

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The aims of this thesis:

1. The aim of study I was to understand how miR-155-5p regulates colon cancer cell migration via regulation of HuR protein in stress conditions.

2. The aim of study II was to understand how miR-340-RhoA axis plays a role in cancer cell migration and invasion in colon cancer.

3. The aim of study III was to examine whether miR-340-5p attenuates colon cancer cell migration and invasion by targeting FHL2-E-cadherin axis.

Chapter 1

History of Cancer

1- History of Cancer

Cancer is a disease which has existed since the ancient times, the earliest medical records dating as far back as the times of ancient Egypt, circa 3000 BC. The Ancient Greek physician Hippocrates (460 - 375 BC) was first to document cancer like growth. He described it like a crab that attaches to the surrounding tissue with its claws. Following the studies of Hippocrates, the Roman physician Celsus (25 BC - 50 AD), who is well-known for having created the medical language in Latin, translated the word "crab" into "cancer"(1) hence, the etymological origin for the medical term for cancer. Cancer was defined by its abnormal cell growth, where it extended to surrounding nearby tissues and later on it could be metastasize to other body parts. The treatment for cancer has improved from the surgical techniques of the 19th century up to the modern techniques of chemotherapy and radiotherapy. Even though cancer treatment is an ongoing progress. it still remains the main cause of death at a global scale (2). Non-inherited cancer can be caused by various different factors, including chemical carcinogens, ionizing viruses and radiation, among others, which results from inducing genetic damage, thus leading to the identification of genes which cause cancer to develop. Such genes were classified in the 1970s into two main families: tumor suppressor genes and proto-oncogenes. Accumulated mutations in such genes are believed to provoke cellular alterations, and as a result, this can lead to the development of cancer (3). The hallmarks of cancer represent essential cellular alterations that are required for neoplastic transformation include sustained proliferative, replicative ability, induction of angiogenesis and invasion/metastasis, and evasion of growth suppression and cell death (4). In recent years, the evasion of immune destruction and reprogramming of energy metabolism have appeared as a new hallmark for cancer. Another example amongst these hallmarks include the inflammatory milieu (5). Inflammation, particularly in the case of inflammatory bowel disease (IBD), has been declared as a connection between colorectal cancer and inflammatory disease (6). In the case of colon cancer, because of oncogene mutation and tumor suppressing genes, this leads to the activation and evolution of various oncogenic pathways which deactivate as a result of (7). In this thesis, we have investigated the possible mechanisms of microRNAs such as miR-340-5p and miR-155-5p which are found to regulate genes expression such as HuR, RhoA and FHL2 in HT-29 and AZ-97 colon cancer cell lines. We have found that miR-340-5p negatively regulates colon cancer cell migration and this regulation is mediated by direct binding of miR-340-5p at 3'-UTR of RhoA and FHL-2 mRNA. We also found that miR-155-5p positively regulates colon cancer cell migration by direct binding at 3'-UTR of HuR mRNA.

Chapter 2

Structure of colon and physiology of colon cancer

Contents

1- *The intestinal tract and its function*

The large intestine, or colorectal, is the last part of the gastrointestinal tract system which is mainly composed of four parts: the cecum, the colon, the rectum, and the anal canal. The length of colon is about 1-1.5 meters and it is also divided into four sections itself: the ascending colon, the transverse colon, the descending colon, and the sigmoid colon (**Figure 1**). At the site of cecum, the large intestine meets with small intestine. The ascending colon is connected with the transverse colon near the lower part of the liver. The transverse colon extends to the splenic flexure along the abdominal wall. Next, the descending colon goes down to form S-shaped sigmoid colon before meeting with rectum

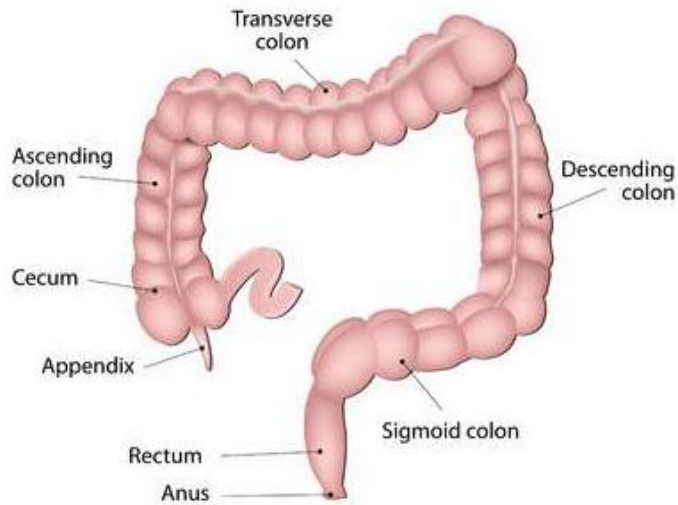

Figure 1. **Schematic illustration of the colorectal anatomy.** Adapted from Drake RL, Vogl W, Mitchell AWM, et al. Gray's Atlas of Anatomy; 2008.

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2- *Physiological function of the colon*

Colon performed important physiological and metabolic functions of the body by absorbing water and nutrients from the stool (8). It collects chloride and sodium from the stool by exchange of bicarbonate and potassium, which is important for intestinal hemostasis. Colon is important to absorb vitamin K which is produced by the gut bacteria (9). Moreover, as the intestinal lumen contains many bacterial floras, this makes it less likely to suffer from a high level of inflammation (10). To maintain the immunological balance colon harbors one of the biggest immune systems known as the Gut-associated lymphoid tissue (GALT). Because of complex immune cells interactions in lamina propia, colon epithelium is protected from external pathogens and microorganisms. Various types of immune cells, such as, macrophages, lymphoid and dendritic cells, present in GALT and play an important role in immune defense. However, there is also a risk of predispositions of inflammatory bowel diseases (IBDs) as a result of impairment of the GALT or an imbalance of gut microflora.

3- Histology of the colon

The colon is composed of four tissue layers: the mucosa, the submucosa, the muscularis, and the serosa (**Figure 2**) (11). The mucosa is located in the innermost layer and composed of column like epithelial cells arranged in a way to form the luminal surface or lining. The mucosa contains goblet cells, which are more predominant in the colon than in the small intestine, and these cells can produce mucus that lubricates the inner wall in order to allow colonic content to pass through easily. The difference between small intestine and mucosa of the colon is that it lacks villi structures. The next layer is submucosa, which is composed of dense connective tissues filled with blood vessels, lymphatic and nerve plexuses. The muscularis has two layers of its own: the inner circular and the outer longitudinal layers. The muscularis provides rhythmic waves that contract in order to move the food through the colon. The last layer of the colon is serosa, which is made of connective tissues. Same as submucosa, serosa is filled with blood vessels, lymphatics and nerves, however, covered by the visceral peritoneum.

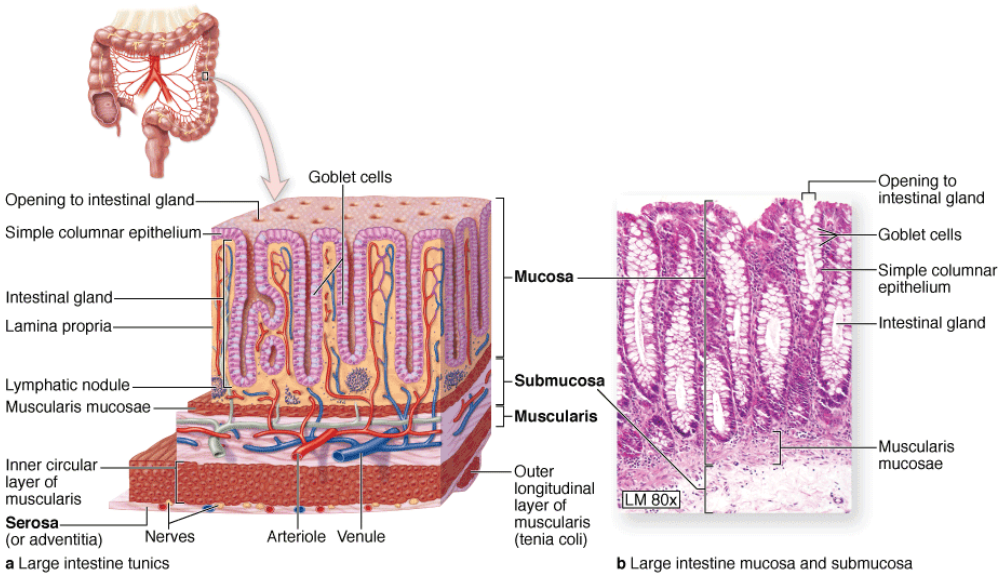

Figure 2. Schematic illustration of colon tissue layers. Adapted from: Mescher AL: Junqueria´s Basic Histology: Text and Atlas, 12th Edition: http://www.accessmedicine.com.

4- *Inflammation and cancer*

It is widely held that inflammation plays one of the most important malignant transformation factors during cancer development (12). More than 150 years ago, Virchow noticed that tumors on the site of chronic inflammation which had heavily accompanied by inflammatory cells (13). These observations have led to the conclusion that the inflammations are a driven force of neoplastic disease. Thereafter, connection of inflammation with cancer was well appreciated by significant epidemiological researchers and approximately 25% of all cancers are shown to be associated with chronic inflammation (14-16). Persistent inflammation is strongly associated with carcinogenesis, such as, chronic inflammatory conditions, for example, IBD; (Crohn's disease and ulcerative colitis) is implicated to CRC (Colorectal cancer) (17), chronic reflux esophagitis is implicated in Barrett's esophagus and esophageal carcinoma (18), viral hepatitis C and B are implicated in hepatocarcinoma (19), human papillomavirus in cervix is implicated in cervical cancer (20).

The tissue injury which are resulted from chemical, physical, biological as well as by infectious stimuli is known to trigger sequential events of highly orchestrated inflammatory response. Unresolved inflammation conditions may create a favorable tumor microenvironment to facilitate changes in tumor suppressor genes or oncogenes as well as post-translational modifications involved in key cell signaling, apoptosis and DNA repair (21, 22).

Chapter 3

Colorectal cancer

Contents

1- Colorectal cancer

Colorectal cancer, also known as CRC, can be observed through abnormal growth in different parts of the large intestine. In the early stages, it starts off as a proliferative growth known as polyps (23). During this stage, polyps tend to grow histologically at a slower rate, which is commonly referred to as dysplastic adenoma. The hyperplastic stage, which follows, may take several years to develop due to the slow development rate of a polyp. In the meantime, different forms of mutation start to accumulate throughout the different stages of development over time, until the malignant carcinoma is shaped. Although around 25% of patients have family genetic history, most of the colon cancer cases are sporadic, meaning no family genetic connection (24). The Adenomatous polyposis coli (APC) gene is a tumor suppressor, and mutations have been detected and documented in APCs in colon cancer. Moreover, it is claimed that such mutations represent a likelihood of early stages of colon tumorigenesis (25). More than 35% of colon cancer cases have occurred in the sigmoid part of the colon, that is why it is known as colorectal cancer (26). The colon cancer's metastatic potential is defined by the ability of colon cancer cells to communicate and interact with its tumor microenvironment (27). During the growth stage, malignant cells gain some characteristics, which help them to metastasize. These characteristics include: increased cancer cells adhesion to endothelial cells, increased cell migration towards chemotactic agents released by target organs, and a higher response to stimuli growth (28). Inside the tumor microenvironment, and specifically during the tumorigenesis and metastasis phases, the chemokines and their receptors play a significant role (29, 30). The discovery of chemokines, and especially the roles played by their receptors, has proven to have been very helpful within the field of cancer biology, as this has helped to offer concrete evidence on their role in metastasis generally, as well as in site-specific metastasis (31, 32). Moreover, further studies on the reorganization of cytoskeletal cells, in the process of cancer cell movement, have provided better insights in regards to molecular aspects of the metastatic biology of cancer. For example, the Rho GTPases family has a very important role in cancer cell metastasis (33, 34). Different cancer cells express different types of chemokine, as well as chemokine receptors, and according to the shape pattern of this expression, one could possibly identify clues related to the metastatic development and behavior of cancer cells (35, 36). Moreover, the discovery of microRNAs (miRNAs) in cancer biology has proven to revolutionize the scientific understanding of various complex mechanisms that regulate cancer cell metastasis.

2- Epidemiology

CRC is identified as one of the most common causes for cancer related deaths in both men and women, especially in the industrialized/developed world. In Europe, CRC is the third most common type of cancer diagnosed, and also it is the second largest cause of cancer which is related to higher deaths (37-39). The most common reason of CRC mortality (about 90%) were due to the spread of primary cancer to other distant organs. The complex multi-step process through which cancer cells migrate from primary source to distant place is known as metastasis, which causes the organs to stop functioning as they should (40, 41). If metastasis is taking place, the 5-year survival rate drops from 95% to less than 10% after surgical intervention (39). In recent years, various screening programs have been implemented on a wider scale in order to detect and prevent the development of CRC at an early stage in patients who are at a higher risk of developing CRC. This has resulted in the reduction of CRC related deaths on a global scale. The screening programs include tests in order to detect pre-cancerous colorectal polyps or even the development of early-stage cancer, even before the symptoms have appeared or before the disease has had a chance to grow and spread. Moreover, this also makes treatment easier and more feasible to implement, not to mention that it offers a higher chance of success of preventing and treating. The most popular test screening for CRC is the Faecal Occult Blood Test (FOBT) or Faecal Immunochemical Test (FIT). If positive results appear, sigmoidoscopy or colonoscopy can be used to confirm the presence of cancerous or inflammatory findings. On the other hand, such tests have their limitations in regards to their sensitivity, invasiveness, and low specificity (42). In spite of this, world-wide CRC cases are predicted to increase over 2.2 million new cases, as well as 1.1 million deaths, by 2030 (43).

Table 1. Summaries of most common and updated screening tests recommended for CRC.

Screening methods	Benefits	Limitations	Time interval
Fecal occult blood	-Low cost -Sampling is done at home -No bowel preparation required -Non-invasive -Does not required sedation	-Low sensitivity and specificity -Multiple samples are required -Colonoscopy is indicated upon the positive results	Annual
Fecal immunochemical tests	-Low cost -Sampling is done at home -No bowel preparation required -Non-invasive -Does not required sedation	-Low sensitivity and specificity -Multiple samples are required -Colonoscopy is indicated upon the positive results	Annual
Stool DNA test	-Sampling is done at home -Only single sample is needed -No bowel preparation required -Non-invasive -Does not required sedation	-Low sensitivity and specificity -High cost -Colonoscopy is indicated upon the positive results	Uncertain
Double-contrast Barium Enema	-Can usually visualize all the colon samples -No need to sedation	-Full bowel	5 years
Colonoscopy	-High sensitivity and specificity -Can remove polyps, obtain biopsies as well as detect other diseases -Can visualize the entire colon	- Full bowel preparation is required - sometime required sedation -High cost	5 years

3- Etiology

It is claimed that potential mutations at oncogenes, tumor suppressor genes, and also genes involved in the process of repairing DNA structures are primary causes for CRC (44). Such mutations are as a result of either loss-of-function or gain-of-function of important proteins which play a role in regulating important cellular processes including, apoptosis, proliferation, and cell migration (45, 46). Approximately 25-30% of cases are related to those with a family history of CRC cases, while approximately 70-75% of CRC cases are sporadic (non-inherited genetic mutations) (24).

4- Genetic and risk factors

Approximately 20-25% of CRC cases are caused by genetic factors. Among these factors, one can observe Familial adenomatous polyposis (FAP) as well as the Lynch syndrome, or as known by the medical community hereditary non-polyposis colon cancer (HNPCC) (47). For the risk factors, there are two major categories that CRC factors can be classified into: genetic and non-genetic.

5- Non-genetic factors

In the instance for non-genetic factors, these include an array of aspects such as: age, life-style habits; intake of red meat, low-fiber intake, heavy consumption of alcohol and tobacco, low physical exercise, and obesity (48). Patients suffering from inflammatory chronic conditions such as IBD, among which Crohn's disease and ulcerative colitis, pose a higher risk (2 – 15 folds) of developing CRC. For this particular reason, such patients are advised to be screened for CRC symptoms more frequently than usual, disregard their age (49-51).

6- Clinical features and staging of colon cancer

Symptoms of CRC are less common and visible in early phases, but pronounced once the disease is already at intermediate and advanced stages (52-54). Most common reported symptoms of CRC are summarized in (**Table 2**). CRC histological staging is the standard staging system that helps surgeons, clinicians, and oncologists to evaluate the extent of the disease (**Figure 3**) (55). Lockhart-mummery was the first to propose staging CRC in 1926, which was based on operative findings in patients suffering from rectal cancer (56, 57). In 1932, Dukes improved the staging by providing a more detailed staging that was focused on the relation between the patient's survival and the degree to which the tumor has penetrated within the lymph nodes and intestinal walls (58).

Such staging was even further developed and also modified by Kirkland, Astler and Coller (59) whose model had been heavily used worldwide for a long period of time until 1973, when the American joint committee for cancer (AJCC) established the Tumor, node, and metastasis (TNM) staging system. The contribution of such new system was based on distant metastasis, primary tumors, and regional lymph nodes (**Figure 3**) (60). Moreover, this method has also become the most popular system for staging in the field of clinical practice at a global scale to this day, as it also helps to offer critical aspects as to which certain therapeutic prognosis and decisions should be taken.

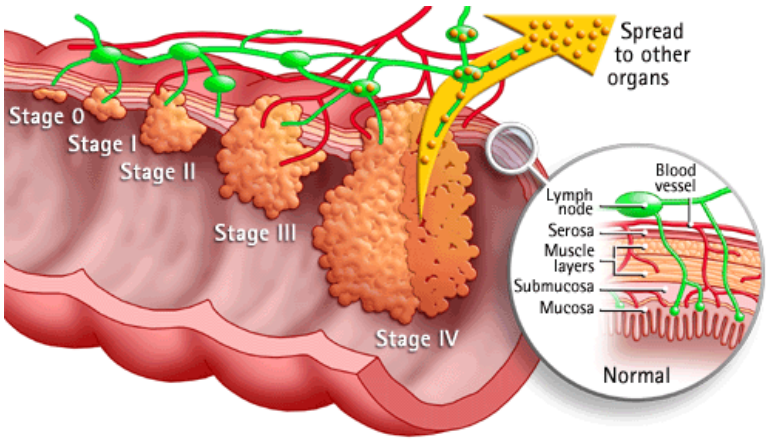

Figure *3*. Schematic illustration of Histological staging in colorectal cancer. As colorectal cancer progresses from Stage 0 to Stage IV the cancer cells grow through the layers of the rectum wall and spread to lymph nodes and other organs. Source: https://www.webmd.com/colorectal-cancer/ss/slideshow-colorectal-cancer-overview.

Table 2. Common Colorectal Cancer clinical features
Symptom duration
Early CRC < 4 weeks (33%), > 4 weeks (77%) **Advanced CRC < 4 weeks (19%), > 4 weeks (81%)**
Hematological observations
Rectal bleeding **Fecal blood** **Anemia†**
Physical observations
Weight loss **Abdominal pain** **Decreased appetite** **Anorexia**
Change in bowel habits
Diarrhea **Constipation** **Altered stools** **Mucus in stool**
Others
Nausea or vomiting **Rectal pain** **Fatigue and General malaise** **Obstruction**

† Anemia: hemoglobin of, 13.4 g/dl (male) and, 12.3 g/dl (female).

7- Colon cancer therapy

As state previously, due to the fact that CRC is a heterogeneous disease, the modalities for therapy vary and the choice for the most effective treatment depends on many factors. However, the major factor for the critical therapeutic decision highly depends on the stage of the disease at the time of the diagnosis. Generally, the best choice of intervention is surgery and the removal of cancer in the early stages, on the other hand, as the phases of cancer advances further on, the surgical removal of cancer starts to be more and more challenging. If the CRC progressed to stage II, III, or IV, neoadjuvant preoperational chemotherapy might be used as a treatment in order to shrink the tumor as well as to help make surgical removal of cancer and selected margins better and less invasive. Another option for therapy is adjuvant chemotherapy. Such drugs which are usually administered as single or in combination regimens, and are also applied after surgery in the instance of advanced stages, or also in no respectable mCRC, which is vital for killing tumor cells and improving symptoms, as well as increasing the survival rate (61). Another therapeutic modality is radiotherapy, and it is often applied in the case of rectal cancer (62). Due to recent advancements in the field of colon cancer treatment, personalized medicine has been introduced. Such type of targeted therapy depends on the molecular profile of every patient suffering from cancer, for example, BRAF or RAS mutation and MSI status in CRC patients (63). Within such context, it is important to point out that CRC patients who suffer from RAS mutations do not actually benefit from anti-EGFR targeted therapy. Therefore, the RAS status can help directing and helping with the therapeutic algorithm towards another treatment regimen.

Chapter 4

MicroRNAs and colon cancer metastasis

Contents

1. Introduction of MicroRNAs.
2. AU-rich elements (AREs).
3. Tumor cell migration biology.

1- *Introduction of MicroRNAs*

MicroRNAs, also known as miRNAs, are noncoding nucleotide RNAs and they are approximately between 22 and 25 nucleotides which regulate gene expression through a process of post-transcriptional regulation. In the human genomes, approximately 30% of protein-coding genes are held tight under regulation by miRNAs (64, 65). It is commonly considered that such short nucleotide sequences are evolutionary conserved amongst different species. They are involved in wide range of cellular functions including cell proliferation, survival, differentiation, apoptosis, and migration (66). Because of the nature roles of the miRNAs, various miRNAs in fact exert their function upon a single gene, while the single miRNA is able to act on many gene targets (67). Generally, miRNAs regulate their own mRNAs via direct target recognition, which is often applied through perfect or even imperfect base pair bindings. These can lead either to the inhibition of translation or even the complete degradation of the target. Such a process is in fact mediated by the argonaute-2 protein (Ago-2), which represents an RNA-induced silencing complex (RISC), and this represents a catalytic subunit as well as the main form of decay machinery of RISC complex (68, 69). According to recent studies, it has been reported that miR-155 is overexpressed in various types of cancers, among which colon cancer as well (70, 71). Moreover, there is also a high expression that is correlated with poor prognosis within colorectal cancer patients. According to multiple investigations, it has been reported that miR-155 plays a role in tumor cell migration as well as invasion (71-73). In addition, as supported by further published reports which shown that the miR-155-5p works during stress as an oncogenic miRNA in specific human tumors, including colon cancer (74). On the other hand, an increasing amount of evidence has revealed the fact that microRNAs, in contrast with their traditional role, could potentially increase the expression of their targets, whether through direct or indirect responses to distinct cofactors. Such cofactors include AU-rich elements (AREs) in the 3'-UTR, along with stress conditions, including pH change, nutrients shortage etc. Because of this, miRNAs can perform different regulatory functions within various types of cancers (75, 76). Evidences suggest that miR-155 could in fact upregulate tumor necrosis factor alpha (TNFα) in RAW 264.7 cells (77). In addition, it has also been proven that miR-155 can exert positive regulation via the enhancement of TNFα translation as a response to endotoxin shock (78). Thus, miRNA can perform translation activation of target gene by binding to the ARE site at 3'-UTR. For instance, one report has shown that synthetic miR-cxcr4 as well as let-7 can activate the targets translation. This is done by target sites located at 3'-UTR of a reporter gene in a serum starved HeLa and HEK293 cells

(79). Moreover, it has also been shown that miR-21 can upregulate Bcl-2 in pancreatic cancer (80) as well as miR-106b was discovered to be able to upregulate RhoA (81). MiR-340-5p was down-regulated in many cancers such as glioblastoma, prostate, breast, gastric, osteosarcoma as well as colorectal cancer cells (82-87). On a further note, according to more recent data, miR-183 has shown to positively regulate PSA in prostate cancer cells (75). RhoA, member of Rho GTPases family, involves in various vital tumor cellular functions including cytoskeletal organization, actin stress fiber formation, membrane trafficking, and proliferation (88). On a further note, RhoA has also been shown to overexpress in different tumor types, among which includes also colon cancer (89). As demonstrated by accumulated studies, the RhoA plays a crucial role in relation to active colon cancer cell migration, and the abolition of RhoA has significantly decreased metastasis cancer cells. In our previous studies, we found that miR-155 could directly affect colon cancer cell migration by regulation HuR gene expression (90). There have not been many studies to show the role of miR-155 in regulating colon cancer cell migration in the context of cellular stress. For this particular purpose, we have grown colon cancer cell line in low serum condition (to mimic the cellular stress) and evaluated the role of miR-155 in colon cancer cells migration

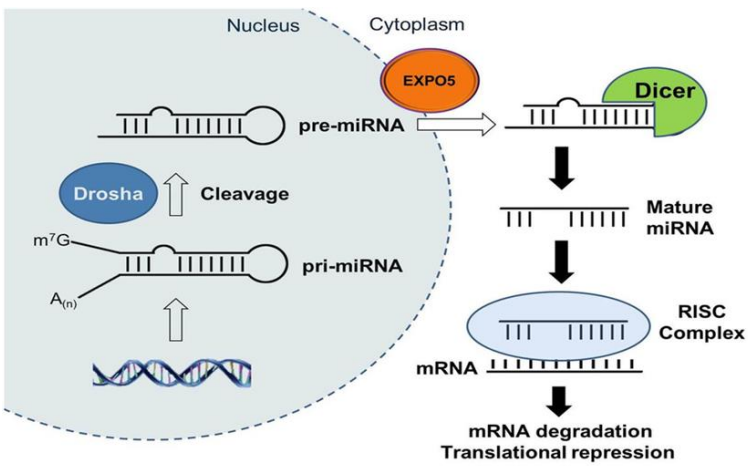

Figure 4: The biogenesis of microRNAs. MicroRNAs (miRNAs) are firstly transcribed via polymerase II (Pol II) as primary-miRNA (pri-miRNA) transcripts. These are then processed by Drosha in order to generate pre-miRNAs. Pre-miRNAs are exported from the nucleus to the cytoplasm via exportin 5 (EXPO5). The Dicer complex is recruited through pre-miRNAs in order to remove the stem loop from pre-miRNAs. Afterwards, this will mature miRNAs in which they represent one strand of the miRNA duplex, that are incorporated into RNA-induced silencing complex (RISC). Within the RISC, miRNAs bind to complementary sequences of target mRNAs in order to repress their translation or induce their degradation. Source: Jung HJ, et al. Front Genet. 2015 Jan 13; 5:472.

2- AU-rich elements (AREs)

The stability of mRNAs is represented a critical factor in regards to controlling gene expression at the posttranscriptional stage. Within the 3'-UTR of short-lived mRNAs there is one well-identified destabilizing cis-acting elements, which includes the AREs (91). On the other hand, not all AREs are involved in destabilizing functions, some functions of AREs are regulated by specific binding molecules. For example, RNA binding proteins (RBPs) or binding site for noncoding RNAs, such as, miRNAs, can regulate the fate of mRNA (92, 93). In 1986, AREs were first described as a sequence which could play the role of regulating the stability of mRNAs and also could play role as decay elements (94).

On a further note, AREs tend to be classified into three major categories: (i) Class I AREs are composed of many dispersed pentamer AUUUA, tetramer AUUA, or also nonamer UUAUUUA (U/A) (U/A) motifs, including those that can be found in c-fos mRNA. (ii) Class II AREs are composed of many overlapping AUUUA motifs that were exemplified through GM-CSF ARE. (iii) Class III AREs are composed of no AUUUA pentamer at all, including those that can be found within c-jun ARE (77, 95). In spite of the fact that these AREs can act as a machinery of decay for plenty various mRNAs, certain reports suggest that cellular conditions can play an integral and determinant role in influencing gene expressions through miRNAs and AREs interactions (76, 77). For instance, tristetraprolin (TTP) can target the TNF-α with the help of micro-Ribonucleoprotein (microRNP) during the process of destabilization. In addition, miR-16 together with Ago-protein complex can lead to TNFα degradation (77).

3- Tumor cell migration biology

In cancer pathology, cancer metastasis is still the biggest challenge as well as the most difficult consequence amongst patients who suffer from cancer. Once the cancer reaches the metastasized phase, the five-year survival rate which dramatically decreases to almost 10% (39). Metastasis is a multistep process dependent on cancer cell's ability to migrate, invade, to the tissues nearby (96). The first stage in cancer is metastasis which occurs when the cancer cell detaches from the main primary tumor. This process is mediated through the loss of E cadherin, which is consider as a tumor suppressor gene (97). The E-cadherin expresses at the junctions located between cells that maintain cell-cell adhesion (98).

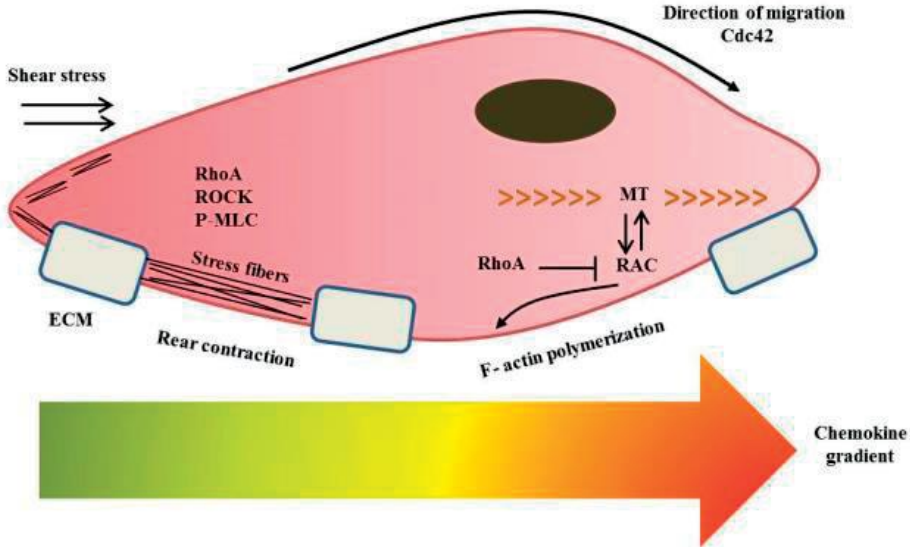

Figure 5. Basic illustration of Rho family member's interaction in directed chemotaxis. Source: Al-Haidari, A. (2018). *Chemokine-Mediated Migration of Colon Cancer Cells*. Lund University.

Lower expression of E-cadherin has been demonstrated in the cases of aggressive tumor types. In addition, E-cadherin is associated with EMT (Epithelial to Mesenchymal Transition), which helps cells to acquire an invasive phenotype. Moreover, E-cadherin is shown to switch to N-cadherin, thus promoting cell to cell-matrix adhesion instead of usual cell-cell adhesion (97). During EMT regulation, many signaling pathways have been found to involve, such as the Wnt and Ras-MAPK pathways (99). It is commonly believed that the Wnt/β-catenin signaling pathway is one of the earliest signaling in the process of cancer metastatic (100). It is also important to note that invasion is considered as a major part of tumor cell active migration. Once EMT is activated, the invasive migration capacity for tumor cells are initiated by a series of complex changes such as, reorganization of cytoskeletal filament, changes in cell-matrix adhesion. Intra-vital imaging of tumor cell migration has revealed that cell-matrix adhesion is necessary for tumor cells to adhere to the surrounding matrix (101). Cancer cells interact with ECM through cell surface receptors, also known as

integrin, which are made of heterodimers. Such heterodimers are made up of non-covalently related α and β subunits. Endothelial cells along with stroma cells is shown to interact with cancer cells, indicating broad activities within the cancer microenvironment. Additionally, the blockade of integrin signaling has been proven to inhibit tumor growth, as well as metastasis, and angiogenesis (102). Chemo-attractants mainly mediate tumor cell migration directly, and these are released from blood vessels or even by other cell types. As soon as the tumor cells reach blood vessels, they are now able to migrate and reach any distant organs easily (103). The process of tumor cells entering into blood vessel is known as intravasation, and it represents the penetration of cancer cells through the basement membrane of ECM. During the process of intravasation, cancer cells develop amoeboid-like pseudopod structures through the regulation of various gene expression that are required for active cell mobility, for example, Rho family proteins (104). According to several studies, the members of the Rho family proteins include the following: RAC, RhoA and Cdc42. Such proteins cooperate in order to regulate the cytoskeletal changes that are needed for migratory behavior within the cells. For instance, RAC is responsible with regulating membrane protrusions formation at the leading edge while Cdc42 is necessary for the process of polarity cell migration (88, 105). Such process is often accompanied by upregulation of various proteases including MMPs (metalloproteinases) as well as cathepsins which are necessary for digestion of basement membrane and migration of cells to surrounding tissue (96). Furthermore, RhoA is required for the generation of actin filaments which is involved in generating contractile force that is important for cell movement. In addition, to RhoA, downstream effector Rho-related serine/threonine Kinase (ROCK), can lead to stress fiber formation alongside the contracting point at the rear edge of the cell, thus permitting the cell body to slide forward (**Figure 5**) (106). Cancer cells migration is classified into two main groups: individual and collective tumor cell migration (107). In individual tumor cell migration, a single cell disseminates to other place using EMT phenotypic capabilities, and use amoeboid types of migration. Moreover, cell migration via EMT heavily depends on integrin and MMPs, which common in the connective tissue tumors, such as fibro sarcomas, gliomas, and epithelial carcinomas (108). In contrast to individual tumor cell migration, collective tumor cell migration uses protease-independent and integrin-dependent mechanisms in order to be able to navigate instead of degrading the ECM barrier (109, 110). Due to the deformable shapes of cell, cancer cells can migrate at 10-30-fold higher in terms of velocities than those that are observed within EMT migration system (110). Such migration type is a typical feature for various

neuroendocrine tumors, small-cell lung carcinomas, kidney, prostate, and various other hematological malignancies such as leukemic cells and lymphomas (110). Collective tumor cell migration is the most common and efficient mechanism through which epithelial carcinomas circulate and migrate within the vessels, such as colon cancer cells migration. In addition, collective migration ensures the survival and supports mechanical arrest of cancer cells inside the blood vessels of distant organs (111). Furthermore, collective migration offers the necessary autocrine signaling for pro-migratory factors, as well as protects cancer cells from any form of immune system attacks (27). Therefore, the heterogeneity of migratory cells is a significant advantage in order to help the cells to migrate as one whole functional body. Thus, the understanding of cancer cell migration mechanisms would help us to develop effective anti-metastatic agent.

Chapter 5

Methodology

Contents

1. Cell lines used in the study.
2. Cell transfection.
3. Assessment of protein and gene expression.
4. Cell proliferation assessment.
5. Chemotaxis and invasion assay.
6. Protein activation assay and western blot.
7. Bioinformatics analysis of binding sites.
8. RIP assay.
9. Confocal microscopy.
10. Statistical analysis.

1- Cell lines used in the study

In this thesis, we used two different colon cancer cell lines. One is human epithelial colon adenocarcinoma cell line HT-29, which was obtained from American Type Culture Collection (HTB-38, ATCC, Manassas, VA, USA) (112, 113). Another one is AZ-97 cell line, which was isolated from a 76-year-old female patient undergoing surgical resection in our laboratory at Skåne University Hospital, Malmö, Sweden as previously described (114). The cells were cultured at optimal growth conditions in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Stockholm, Sweden), supplemented with 10% fetal bovine serum (FBS), and antibiotics 100 U/ml penicillin, 100 µg/ml streptomycin at 37ºC and 5% CO2.

2- Cell transfection

Mimic-miR-340-5p, Mimic-miR-155-5p and mimic-ctrl were purchased from Life Technologies, Carlsbad, CA, USA. TransIT-TKO transfection reagent (Mirus; Madison, WI, USA) was used to evaluate the role of miR-155 and miR-340. To study the target sites of miR-155 and miR-340, target site blockers (TSBs) was purchased from Exiqon A/S (Vedbaek, Denmark). The miRCURY LNA_TSBs were designed to specifically compete with the miR-155-5p and miR-340-5p. HT-29 and AZ-97 colon cancer cells were cultured to 70-80% confluence and then were starved (0.1 % serum) overnight. On the next day, 1 × 106 the cells were plated into a 6-well culture plate. Moreover, the cells were transfected with antagomiR-155-5p (200 nM), mimic-miR-340-5p (50 nM) or Mimic-Ctrl (50 nM) or even antagomiR-ctrl for a 24 hours by using Mirus transfection reagent in Opti-MEM reduced serum media according to manufacturer's instructions. After 24 hours, the cells were harvested and then the expressions of miR-340-5p and miR-155-5p as well as the expressions of mRNA for RhoA, HuR and FHL2 were evaluated.

3- Assessment of protein and gene expression

For gene expression studies, we have used qRT-PCR. The total of RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and purified using Direct-zol RNA extraction kit (Zymo Research, Irvine, CA, USA) according to manufacturer's recommendations. The concentration and purity of total RNA was checked using Nano Drop spectrophotometer at 260nm absorbance. The cDNA was synthesized by using total RNA (0.4 µg) by Mir-XTM miRNA First-Strand Synthesis Kit. Expressions of miR-155-5p and miR-340-5p, as well as HuR, RhoA, FHL-2 and E-cad mRNAs were quantified using mir-XTM

miRNAqRT-PCR SYBR® kit (Clontech, Mountain View, CA, USA). Relative expressions were quantified to control housekeeping genes (U6 and beta actin) by using $2^{-\Delta\Delta}$ CT method. The Primer sequences used in this study are listed below:

Table 3. Primers sequence used for mRNAs gene expression	
HuR	Forward 5′-CCTCTAATGGCTGGATCCTATTT-3′ Reverse 5′-GTCCTGTCAAAGTCTCCGTTAG-3′
RhoA	Forward 5`-AGAGGTGTATGTGCCCACAGTGTT-3` Reverse 5`-AGGCGATCATAATCTTCCTGCCCA-3`
Hsa-MiR-155-5p	Forward 5'- GGGTTAATGCTAATCGTGATAGGGGT -3'
β-actin	Forward 5`-AGAGCCTCGCCTTTGCCGATCC-3` Reverse 5`-CACATGCCGGAGCCGTTGTCG-3`
E-cadherin	Forward 5′- ACAGCCCCGCCTTATGATT-3 Reverse 5′- TCGGAACCGCTTCCTTCA-3
FHL2	Forward 5'-GAA ACT CAC TGG TGG ACA AGC-3 Reverse 5'-GTG GCA GAT GAA GCA GGT CT-3
U6 snRNA	Forward 5'-GCTTCGGCAGCACATATACTA-3` Reverse 5`- CGAATTTGCGTGTCATCCTTG-3`
Hsa-miR-340-5p	Forward 5′-GGCTTATAAACGAATCACAGTCATTAAAA-3′

4- Cell proliferation assessment

We evaluated colon cancer cell proliferation under different treatment conditions. Cells were cultured with or without mimic-miR-340 (50 μM), and ctrl-mimic-miR-340 (50 μM), antagomiR-155 (25 –200nM), antagomiR-Ctrl or target site blockers for 24, 48, and 72 h at 37ºC (5% CO2). Cell proliferation assays were performed using either CCK8 colorimetric kit or fluorescence based methods. Trypan blue exclusion assay was used to measure the viability of tumor cells after microRNAs transfection. Percentage of proliferation was calculated after taking the value on wells containing antagomiR-155-5p divided by value in the antagomiR-Ctrl wells.

5- Chemotaxis and invasion assay

Migration and invasion assays were performed by using 24-well cell migration chambers with 8 μm pore size inserts (Corning Coster, Corning, NY, USA) as described previously (112). For invasion assay, each chamber was coated with 30 μm of extracellular matrix (ECM) gel (SigmaAldrich, St. Louis, MO, USA). HT-29 cancer cells were transfected with either mimic-miR-340-5p (50 nM) alone or Mimic-Ctrl (50 nM) alone or miR-340-5p (50 nM) in the presence of TSB or TSB-Ctrl for 24 h in Opti-MEM serum reduced media. Next day, transfected cells were collected and 1 × 106 cells/ml were loaded into the inserts and DMEM media containing 10% serum was added in the lower chambers and incubated for 24 h at 37ºC in 5% CO2. Non-migrated cells were removed from the upper surface of the insert using cotton swab and the cells on the lower surface of the insert membrane were fixed with 100% methanol after that they were stained with 0.5% crystal violet. Cells were counted in five different fields of each sample. Data are presented as the mean number of migrated cells per high power field.

6- Protein activation assay and western blot

For protein assays, we used ELISA-based technique and immunoblotting. In order to assay RhoA activity, RhoA-GTP activity was measured by using a G-LISA kit according to the instructions of the manufacturer. The protein concentration was determined by using Precision Red Advanced Protein Assay. 1 mg/ml of protein was used for quantitative detection of active RhoA, and the absorbance was detected at 490 nm by using ELISA microplate reader. For western blot experiments, the cells were starved for 24 hours and then transfected with the antagomiR-155-5p, antagomiR-Ctrl, target site blockers (TSB) and TSB-Ctrl for 24 hours. In

the following day, the cells were processed and fraction of 10 µg of protein cytoplasm was immunoblotted by using specific monoclonal anti-HuR antibody, as well as the anti-lamin A, which worked as an internal control in order to detect nuclear contamination. After incubation with primary antibody, membrane was again incubated with secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. Stain free total protein loads were used to normalized respective bands. Bands images were created using the BioRad ChemiDoc™ MP imaging system. After normalization, calculation of band intensity was done by Image Lab™ software version 5.2.1.

7- Bioinformatics analysis of binding sites

The RNA-hybrid based bioinformatics tool was used to predict the binding sites for miR-155-5p at 3'-UTR of HuR mRNA and miR-340-5p at 3'-UTR of RhoA mRNA and FHL2 mRNA. According to literature, miRNA binding to the AU-rich elements (AREs) regulates positive upregulation of target mRNA. In study I, we checked complementary base pairing between AREs in the 3' UTR of HuR mRNAs with miR-155-5p in the seeding region. Our analysis focused on the ARE motifs, such as, AUUUA and AUUA. In order to assess the function of the binding sites, our experiment designed two target site blockers. The blockers selectively bind with the sequence of the 3'-UTR of HuR mRNA at ARE region which should prevent the binding of miR-155-5p to its target site. Target site blockers were synthesized and modified as fully phosphorothiolated locked nucleic acids (LNA) in order to increase their affinity and selectivity to the target. For the validation of miR-155- 5p on HuR mRNA, the following blocker and control were used; 5′-TTAATATATATCTTAAAGGAAAT-3′ and TSB2 of HuR; 5′-TTAATGGTCTTAAATGCAAAAGT-3′ and TSB1 Ctrl of HuR 5′-TAACACGTCTATACGCCCA-3′. For the validation of miR-340- 5p on Rho mRNA, the following blocker was used: TSB_RhoA_miR-340-5p; 5′-TTATAAAGTAGTTACAGCCT-3′. For the validation of miR-340- 5p on FHL2 mRNA, the following blocker was used: TSB_FHL-2_miR-340-5p; 5′-TTATAAAGTAGTTACAGCCT-3′

8- RIP assay

The RIP allows the identification of subsets within the RNAs, including those of microRNAs that are connected with RNA-binding proteins and also provide information the composition of miRNA-ribonucleoproteins (miRNPSs) (115)**.** The RIP assay was performed through the use of the EZ-Magna RIP kit, according to the protocol of the manufacturer. After experiments,

cells were lysed through complete RIP lysis buffer containing protease inhibitor cocktail. Afterwards, the 100 µl of whole cell extracts were incubated with RIP buffer containing magnetic beads conjugated with an anti-Ago2 antibody, or Ctrl-IgG antibody. Samples were then rotated for 3 hours at 4ºC. After several washing samples were incubated with Proteinase K to digest out proteins at a temperature of 55ºC. The co-immunoprecipitated (co-IP) RNA, including microRNA: mRNA complexes, were then analyzed by using qRT-PCR, and measured according to the relative enrichment of miR-155-5p.

9- Confocal microscopy

In this thesis, we have used confocal microscopy to detect and image FHL-2, E-cadherin and ki67. The colon cancer cells were grown to 60-70% confluence and then cells were transfected with either mimic-miR-340-5p (50 nM) alone or mimic-ctrl (50 nM) alone or miR-340-5p (50 nM) in the presence of TSB or TSB-Ctrl for 24 h in Opti-MEM serum reduced media on glass coverslips as described above. Next day, cells were exposed to 10% BSA for 30 min. Cells were washed and fixed with 2% formaldehyde and then permeabilized with 0.2% Triton X-100 for 20 min. After fixation and permeabilization, cells washed two times with PBS containing 2% fetal bovine serum. Samples were then incubated with primary antibodies: fluorescein isothiocyanate (FITC) conjugated anti-ki67 antibody (ab206633; Abcam) and rabbit anti-human FHL-2 antibody (ab12327, Abcam, Cambridge, MA) in PBS containing (2% BSA) serum overnight. In a separate experiment for E-cad staining, the samples were first incubated with rabbit anti-human E-cad (ab40772; Abcam) primary antibody in PBS containing 2% BSA serum overnight. The samples are washed two times, and then incubated with rat anti-rabbit allophycocyanin (APC) conjugated secondary antibody (A-21038, Thermo Scientific, Rockford, IL, USA) for 20 min. After immunostaining, coverslips were collected and rinsed with PBS twice and then stained with Hoechst 33258 (Thermo Scientific) for 10 min. ProLong Diamond Antifade Mountant (Thermo Scientific) was added to preserver fluorescence intensity. LSM 800 confocal (Carl Zeiss, Jena, Germany) was used for imaging and orthogonal projection images were created by using all slices for a total height of 10 µm. Images were taken by using ×63 oil immersion objective (numeric aperture = 1.25) and processed later using ZEN2012 (Carl Zeiss, Germany) software.

10- Statistical analysis

Statistical analyses for in vitro experiments were performed using GraphPad Prism 8 software. In study I and II, for multiple comparisons we used Kruskal-Wallis One Way Analysis of variance (ANOVA) on ranks followed by the Dunn's post hoc test. Mann Whitney rank sum test was used for comparison between two groups. P-value < 0.05 was considered significant. In study III, we used One-Way Analysis of variance (ANOVA) followed by the Tukey's post hoc test for multiple comparison of microarray and experimental data. For comparison between two groups, we used to two-tail t-test. P-value < 0.05 was considered significant.

Chapter 6

Results and discussion

Contents

1- Paper I

The discovery of microRNAs was a revolutionary event in relation to the study of cell biology, as well as different cellular functions not only in relation to personal health but also to many diseases. In spite of all efforts while treating colon cancer, the predicted five-year survival rate is still remained low when the disease is spread to other distant organs (41). According to recent studies, miR-155-5p has been shown to increase in colon cancer, and it also plays key role in colon cancer cells migration (70, 71). Herein, for the first time we have reported that miR-155-5p- induced tumor cell migration is mediated by HuR mRNA through direct positive regulation in serum starved HT-29 colon cancer cell lines.

HuR (ELAVL1), a nuclear RNA binding protein (RBP), is demonstrated to play a fundamental role in the process of tumorigenesis. For example, HuR is shown to promote proliferation by stabilizing important proteins those control cell growth, such as cyclin A, cyclin B1, c-fos and COX-2 (116, 117). Therefore, in study I, we decided to investigate the role of miR-155-5p on HuR expression and colon cancer cells migration. First, we analyzed the role of miR-155-5p in regulating HuR expression in stressed colon cancer cells. We knocked down miR-155-5p in serum-starved HT-29 colon cells using AntagomiR-155-5p. Transfection with AntagomiR-155-5p decreased HuR mRNA expression in colon cancer cells while transfection with miR-155 mimic increased HuR expression (**Figure 6A**). Transfection with AntagomiR-155-5p in serum-grown HT-29 cells increased HuR mRNA expression in colon cancer cells while transfection with miR-155 mimic decreased HuR expression **(Figure 6B)**.

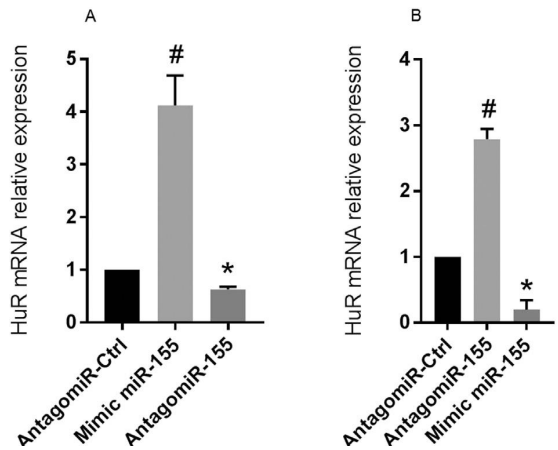

Figure 6. AntagomiR-155-5p reduces HuR expression in colon cancer cells. HuR mRNA expression in **A)** serum-starved and **B)** serum-grown HT-29 colon cancer cells transfected by antagomiR-Ctrl, AntagomiR-155-5p (25 nM) and miR-155 mimic. Data represents mean ± SEM and (n =4). $^{\#}P < 0.05$ versus antagomiR-ctrl and $*P < 0.05$ versus mimic or antagomiRCtrl**.**

Although in most cases miRNAs regulate cellular functions by inhibiting mRNA translation, accumulating evidence suggest miRNAs can enhance RNA translation in cells under stress conditions, such as nutrient deprivation (serum starvation) (77, 118, 119). This is also supported by our observation that miR-155 increases RhoA levels and activity in serum-starved condition, thus regulates colon cancer cells migration (120), suggesting that depending on the conditions miRNAs can either positively or negatively regulate cancer cell functions. In addition, it has been reported that different cell types respond differently to same miRNA. For example, miR-21 was found to downregulate Bcl-2 in breast and glioblastoma cancer cells, on contrary, upregulate Bcl-2 expression in pancreatic carcinoma (80). Taken together, our results support the idea that miR-155-5p works as pro-oncogenic miRNA for serum-starved colon cancer cells by increasing HuR expression. We found that miR-155-5p is a positive regulator of HuR expression in HT-29 colon cancer cells in stress condition. We know that positive regulation of mRNA translation by miRNAs is related to miRNA binding to AU rich elements at 3′-UTR target sites of miRNAs during cell cycle arrest (118)**.** Next, we examined whether HuR is a direct target of miR-155- 5p. Bioinformatics analysis using RNAhybrid revealed that 3′-UTR of HuR mRNA has several potential binding sites for miR-155. We found two interesting regions, containing the ARE motifs AUUA and AUUU and these regions are complementary to the seeding region of miR-155-5p.

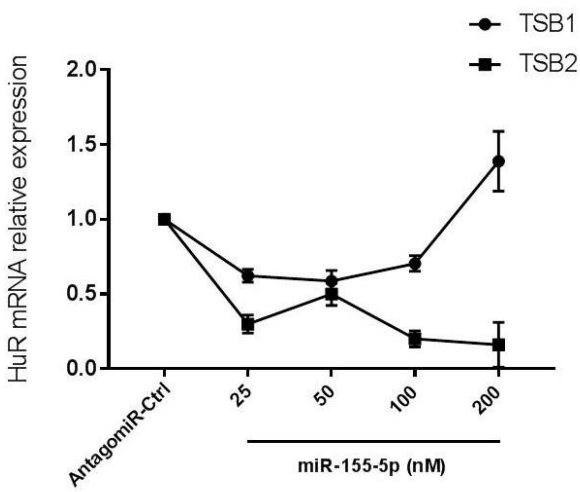

Figure 7. HuR is a direct target of miR-155-5p. Antagomir-155-5p mediated reduction of HuR mRNA expression in serum starved HT-29 colon cancer cells was dose-dependently increased by TSB1. Data represent mean ± SEM and (n = 5).

We then designed two competitive blockers for these regions. Interestingly, co-transfection with one blocker targeting AUUA and AUUU regions, dose-dependently increased HuR mRNA expression that was inhibited by antagomiR-155-5p **(Figure 7)**. Our finding revealed a new target site for translational activation of HuR by miR-155-5p at ARE region of 3′-UTR of HuR mRNA.

Next, the association between miR-155-5p and HuR mRNA in HT-29 cells was confirmed by RIP assay using anti-Ago-2 antibody beads. Ago2, core catalytic component of the RISC complex, is known to silence mRNA targets (69). RIP assays were performed to determine the association between miR-155-5p and HuR mRNA on HT-29 cells transfected with antagomiR-155-5p. Immunoprecipitates (IPs) containing Ago-2 were used to detect HuR mRNA by quantitative RT-PCR. We found reduced miR-155-5p in Ago-2 IPs when transfected with antagomiR-155-5p in HT-29 carcinoma cells **(Figure 8A)**. Interestingly, knocking down of miR-155-5p significantly reduced HuR mRNA in Ago-2 immunoprecipitates compared to antagomiR-Ctrl **(Figure 8B)**, indicating that miR-155-5p is associated with HuR mRNA in HT-29 cells when facing serum starvation.

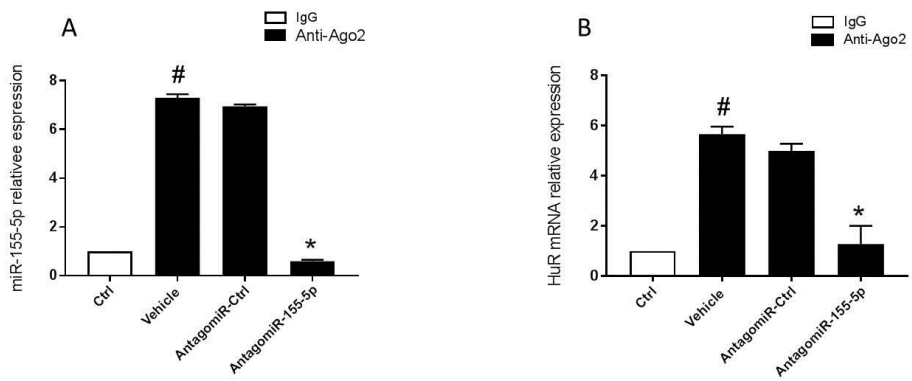

Figure 8. MiR-155-5p is associated with HuR mRNA. After immunoprecipitation, miR-155-5p and HuR mRNA was measured by qRT-PCR. Transfecction with antagomiR-155-5p reduced relative enrichment of **(A)** miR-155-5p and **(B)** HuR mRNA in Ago2 immunoprecipitates. Data are expressed as mean ± SEM and n = 5 and represented as fold change compared to anti-IgG ctrl and U6 was used as a housekeeping gene. $^{\#}P < 0.05$ versus IgG-IP and $^{*}P < 0.05$ versus anti-

Knowing that cancer cell migration is a prerequisite of metastasis (121) and HuR regulates migration of cancer cells (122-124), we next examined miR-155 role in colon cancer cells migration via HuR. We performed transwell migration assays with serum-starved HT-29 colon cancer cells using 10% FBS as a chemoattractant. Migration assay confirmed that miR-155-5p regulates serum-induced colon cancer cell migration and miR-155-5p antagomir reduced serum-induce migration (**Figure 9**). Co-transfection with a TSB reversed the effect of colon cancer cell migration, suggesting that miR-155-5p regulates colon cancer cell migration via this specific and functional binding site on HuR mRNA (**Figure 9**).

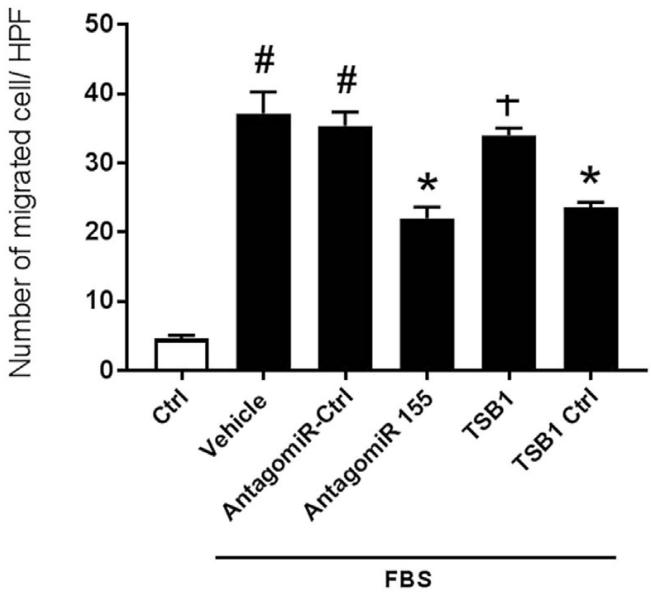

Figure 9. MiR-155-5p regulates colon cancer cell migration. Chemotaxis of HT-29 colon cancer cells was stimulated by use of 10% FBS. Cells were starved 24h and then transfected with antagomiR-Ctrl, AntagomiR-155-5p (25 nM), TSB1, and TSB1 Ctrl. Cells were counted microscopically using 100x High Power Fields (HPF) in five different fields. Data are expressed as mean ± SEM and n = 5. $^{\#}P < 0.05$ versus negative ctrl, $^{*}P < 0.05$ versus 10% FBS + antagomiR-Ctrl, and $^{+}P < 0.05$ versus FBS + AntagomiR-155-5p.

Cytoplasmic localization of HuR is shown in colorectal adenomas and colorectal adenocarcinomas (125). Two studies also found correlation between higher HuR expression and cytoplasmic localization in tumors with poor disease prognosis (126, 127). Thus, we examined cytoplasmic accumulation of HuR protein in colon cancer cells by use of western blot. We found that transfection of HT-29 cancer cells with antagomiR-155-5p decreased cytoplasmic HuR protein (**Figure 10**). Interestingly, blocking by TSB reversed the inhibitory effect of antagomiR-155-5p on HuR, indicating that cytoplasmic levels of HuR protein in HT-29 cancer cells is regulated by miR-155-5p (**Figure 10**).

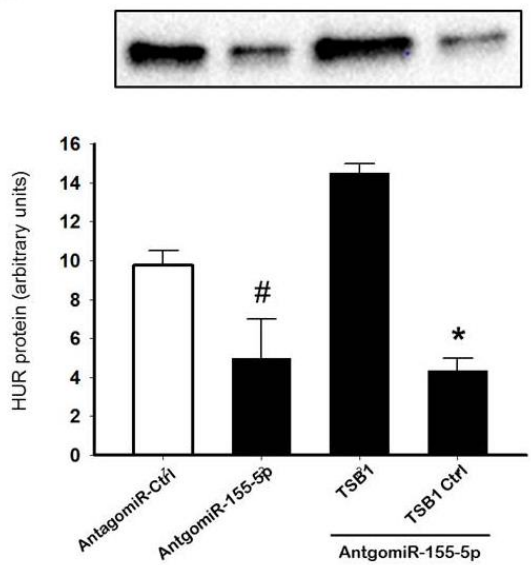

Figure 10. **Cytoplasmic expression of HuR protein determined by western blot.** The protein fractions were normalized to the total protein and analyzed by Image Lab™ software. Quantitative assessment of HuR protein in each fraction is illustrated as a histogram in arbitrary unit. Data are expressed as mean ± SEM and n = 4. #P < 0.05 versus antagomiR-ctrl and *P < 0.05 versus TSB1.

This finding is similar to our recent finding where we have shown that miR-155-5p positively regulates migration of colon cancer cells via RhoA (120). Our observations suggest that miR-155-5p can control colon cancer cell migration via multiple mediators, such as, HuR and RhoA. Our observation is also in line with several previous findings where studies have shown that miRNAs can act on multiple target genes (128-130). Furthermore, miR-155 is shown to associate with higher number of distant metastases in patients with colorectal cancer (71). We conclude that that miR-155-dependent tumor cell migration to distant organs is one of the many mechanisms in patients with high expression of miR-155. Thus, targeting miR155-5p or HuR could be new pathways to antagonize colon cancer cell metastasis to distant organs.

2- Paper II

The most predominant cause of morbidity and mortality in colon cancer patients is the metastatic spread of tumor cells (131). Cytoskeletal proteins, such as, RhoA-C, Rac1/2 and Cdc42 (132) is known to play an important function as a molecular switch during the process of cell migration (133). In fact, the activation of Rho protein is observed during the cancer development and overexpression of RhoA has been reported to be related to worse prognosis of patients with CRC (134).

In the study II, we investigated the expressions of RhoA mRNA and miR-340-5p in colon cancer cells. We found that the levels of miR-340-5p was downregulated in serum-grown cells compared to serum-free cells. In addition, RhoA mRNA expression was also shown to be much higher in serum-grown cells compared to the serum-free cells (**Figure 11A and 11B**). Therefore, it could be stated that miR-340-5p and RhoA is inversely related to each other in colon cancer. One of the most significant steps in the metastatic process is tumor cell migration. The migration response of cancer cells is dominated by complex interactions as well as crosstalk between different types of cell cytoskeleton proteins which are including the family of Rho that is necessary for a wide range of cellular functions, such as cytoskeletal organization, growth, apoptosis, membrane trafficking, cell-cell and cell-matrix adhesion, as well as differentiation (88).

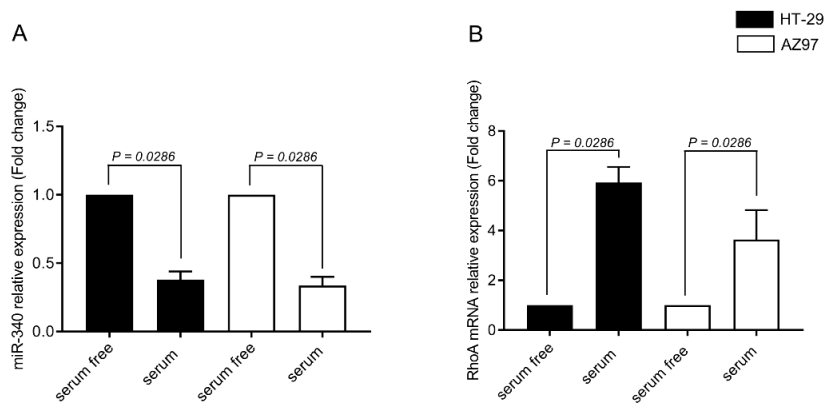

Figure 11. MiR-340-5p and RhoA expression in colon cancer cells lines. Expression of **A)** miR-340-5p and **B)** RhoA mRNAs relative to housekeeping gene were determined using qRT-PCR in serum-free and serum-grown HT-29 cells. Relative expressions were calculated using qRT-PCR where U6 was used as a housekeeping gene for miR-340-5p and beta-actin was used as a housekeeping gene for RhoA mRNA. Relative expressions were determined by using $2^{-\Delta\Delta CT}$ method. Data represent mean ± SEM and $n = 4$. $P < 0.05$ versus serum-free**.**

Next, the cells were transfected with mimic-miR-340-5p and mimic-control. We observed that transfection with the mimic-miR-340-5p for 24h resulted in higher levels of miR-340 compared to mimic-control (**Figure 12A**). Consequently, the transfection with mimic miR-340-5p dose-dependently decreased RhoA mRNA levels in colon cancer cells (**Figure 12B**).

Interestingly, it was revealed that miR-340-5p is downregulated in various different types of cancers such as breast, lung, glioblastoma, skin, gastric, and prostate cancers as well as colorectal cancer cells (82-87, 135). In addition, it has been revealed that miR-340-5p can regulate various cellular functions in cancer cells. For instance, miR-340-5p can suppress prostate cancer cell growth by targeting high mobility group nucleosome binding domains, while in breast cancer it can inhibit cell invasion and migration by targeting myosin X and ROCK1 (84, 136). Furthermore, the miR-340-was shown to lower glioblastoma cell proliferation by targeting CDK6, cyclin D1 and D2 directly (137), and it can also inhibit the tumorigenic phenotype of melanoma by regulating RAS-RAF-MAPK signaling pathways (138).

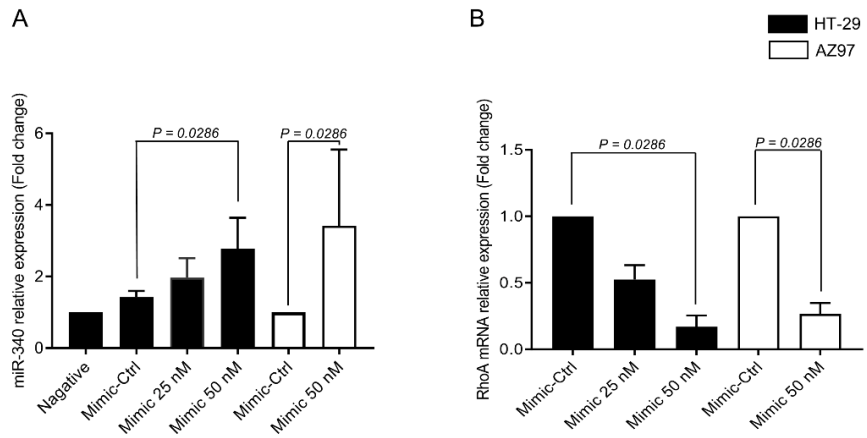

Figure 12. Mir-340-5p-mediated regulation of RhoA mRNA expression in HT-29 and AZ97 colon cancer cells. Cells were transfected with Mimic-Ctrl (50 nM) or miR-40-5p mimic (25 nM and 50 nM). **A**) After transfection, upregulation of miR-340-5p and **B)** downregulation of RhoA mRNA expression in colon cancer cells. Relative expressions were calculated by using qRT-PCR where U6 was used as a housekeeping gene for miR-340-5p and beta-actin was used as a housekeeping gene for RhoA mRNA. Relative expressions were determined by using $2^{-\Delta\Delta CT}$ method. Data represents mean ± SEM and (n = 4). $P < 0.05$ versus mimic control.

In the next stage, we investigated whether miR-340-5p could be a target of RhoA mRNA in colon cancer cells. By using bioinformatics tool, we found that the seeding region of miR-340-5p has one potential binding site on RhoA mRNA 3'-UTR (**Figure 13A**). The binding of miR-340-5p on mRNA of RhoA was confirmed and validated by using a specific target site blocker (known as TSB). Our study showed that the level of RhoA has significantly decreased after transfecting the cells with mimic-miR-340-5p. Meanwhile, co-transfection of cancer cells with TSB has reversed the inhibitory effect of mimic-miR-340-5p on RhoA mRNA. In contrast to this, co-transfection with a control TSB had no effect on the expression of mRNA of RhoA in colon cancer cells transfected with mimic-miR-340-5p (**Figure 13B**).

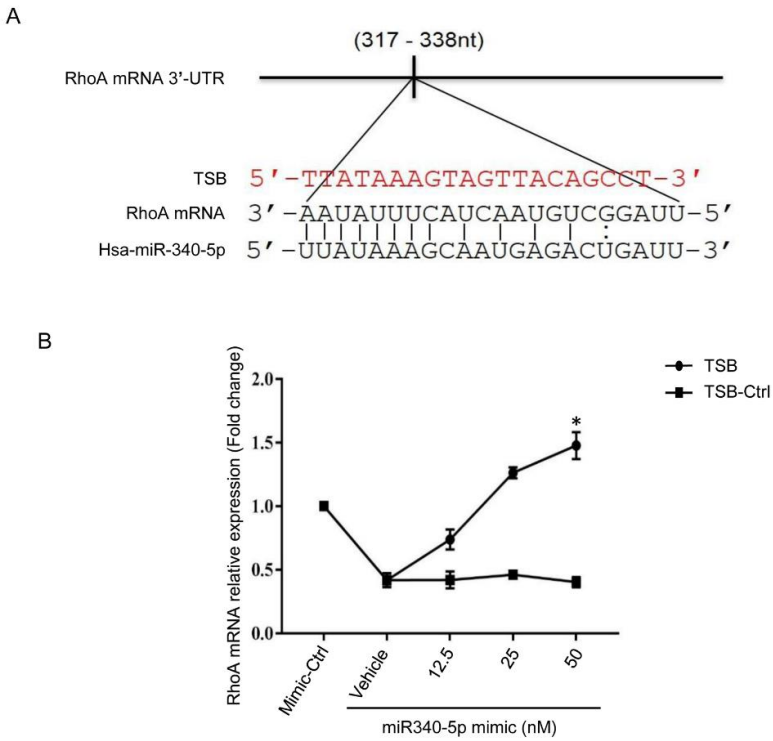

Figure 13. miR-340-5p directly targets RhoA mRNA. A) Bioinformatics analysis predicted a target site of miR-340-5p containing an (AAUAUUUC) motif in RhoA mRNA at 3′-UTR sequence. TSB (red sequence) was designed to block the seeding region of miR-340-5p complementary to (UUAUAAAG) of RhoA mRNA. **B)** The inhibitory effect of miR-340-5p on RhoA mRNA expression was TSB dose-dependently increased in HT-29 colon cancer cells. Data represent mean ± SEM and (n = 4). *P < 0.05 versus TSB.

Short-term serum stimulation increased RhoA activity in colon cancer cells and transfection of miR-340-5p in serum-stimulated cells resulted in lower RhoA activity (**Figure 14**). Next, we performed migration and invasion assays where we used 10% serum as a chemoattractant. We found that colon cancer cell migration and invasion have increased significantly (**Figure 15**). Transfection of colon cancer cells with miR-340-5p mimic reduced cancer cell migration and invasion (**Figure 16**). The activation of Rho proteins becomes one of the most well-known and frequently reported oncogenic signals. Moreover, role of RhoA is the well-documented in the progression of different types of cancers (89). In addition, it was demonstrated that patient who have high levels of RhoA, had lower survival rate (139). Dysregulated miRNAs are implicated in the pathogenesis of various types of cancers (140).

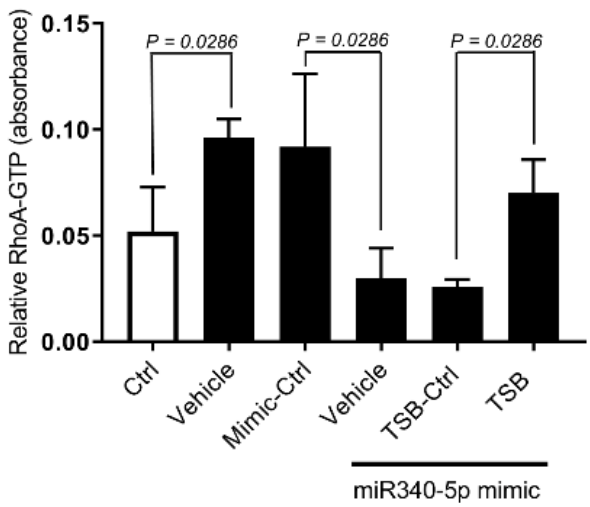

Figure 14. Activation of RhoA in HT-29 colon cancer cells. Relative RhoA-GTP activation was measured by using commercially available G-LISA activation assay kit. HT-29 cells were transfected with miR-340-5p mimic, mimic control, TSB control and TSB. Data are presented as mean ± SEM and $n = 4$. $P < 0.05$ versus Control, $P < 0.05$ versus mimic control and $P < 0.05$ versus TSB control.

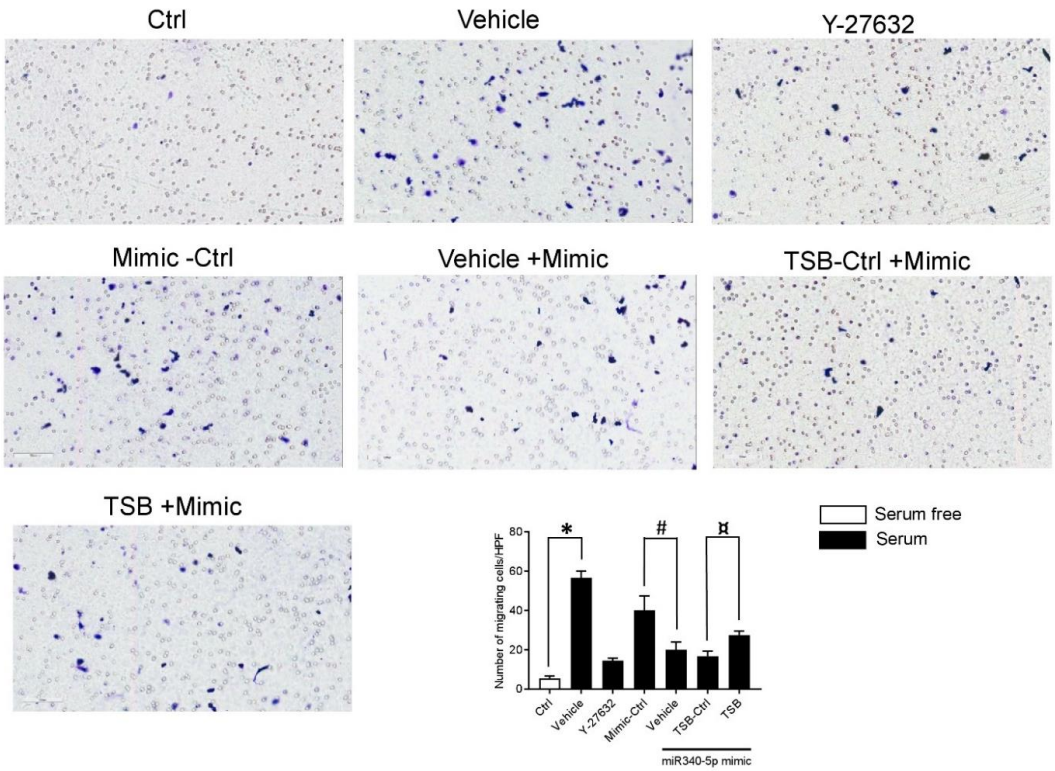

Figure 15. Migration of colon cancer cells were stimulated by use of 10% FBS. Cells were counted microscopically by using high power fields in five different fields. Data represent mean ± SEM and $n = 4$. $^{\#}P < 0.05$ versus Control, $^{*}P < 0.05$ versus mimic control and $^{¤}P < 0.05$ versus TSB control. Ctrl.

The results of this study is in accordance with the previous studies showing that migration of cancer cells is regulated by miR-340-5p in lung, breast and squamous cancers (84, 135, 141). Furthermore, other studies showed that miR-340-5p could regulate cancer cell growth (142), apoptosis (143) and chemo-sensitivity (87). Together with other studies our finding suggests that miR-340-5p can regulate the progression of colon cancer at multiple levels.

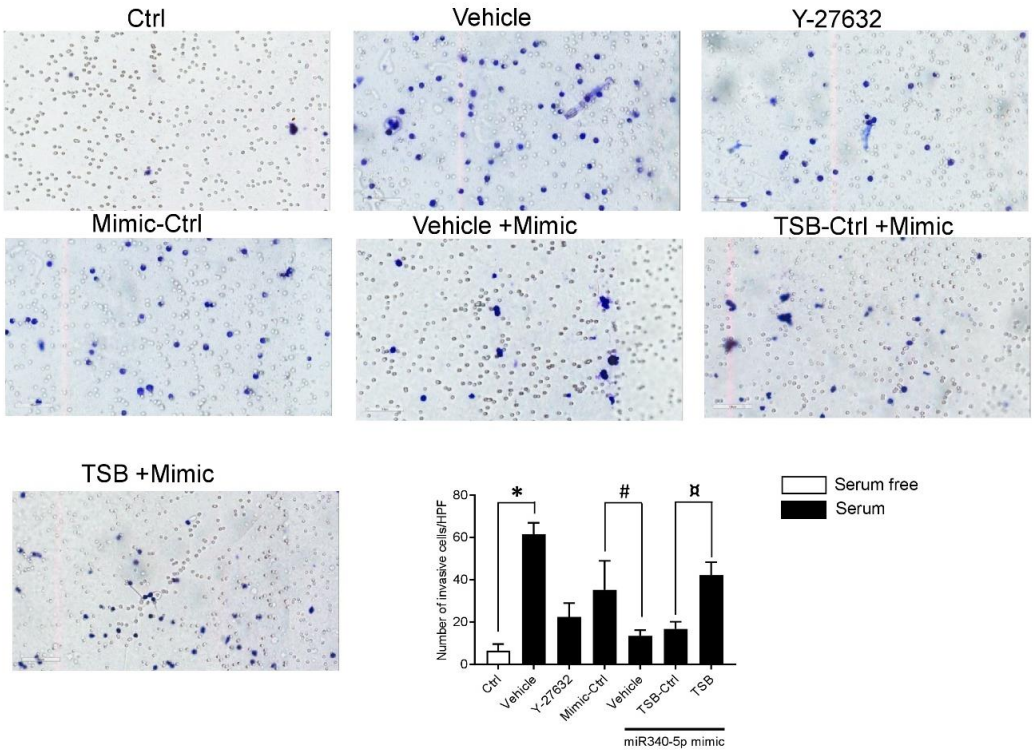

Figure 16. Invasion of colon cancer cells was stimulated by use of 10% FBS. Cells were counted microscopically by using high power fields in five different fields. Data represent mean ± SEM and $n = 4$. $^{\#}P < 0.05$ versus Control, $^{*}P < 0.05$ versus mimic control and $^{¤}P < 0.05$ versus TSB control**.**

In conclusion, our study revealed that miR-340-5p inversely regulates colon cancer cell by activating RhoA. The results not only show how miR-340-5p-RhoA axis mediate colon cancer cells migration, but also provide a possibility of developing new strategies that can be used against colon cancer cell metastasis.

3- Paper III

Metastasis of colon cancer represents a leading cause of deaths in all over the world. Accumulating data suggest that FHL2 oncogene plays a significant role in cancer cell migration and invasion. The short non-coding RNAs (miRNAs) are shown to regulate various types of cancer progression as well as metastasis (90, 144, 145). The role of FHL2 with regard to miRNA in colon cancer migration and invasion is not demonstrated yet. Thus, in this study we demonstrate for the first time that FHL2-miR-340 axis regulates colon cancer migration and invasion under stress condition.

We first analyzed multiple microarray datasets to compare FHL2 expression between normal colon tissues and colon cancer. Datasets GDS4382 and GSE115313 revealed that colon cancer tissue had significantly lower levels of FHL2 expression than matched normal colonic tissue (**Figure 17A and 17B**).

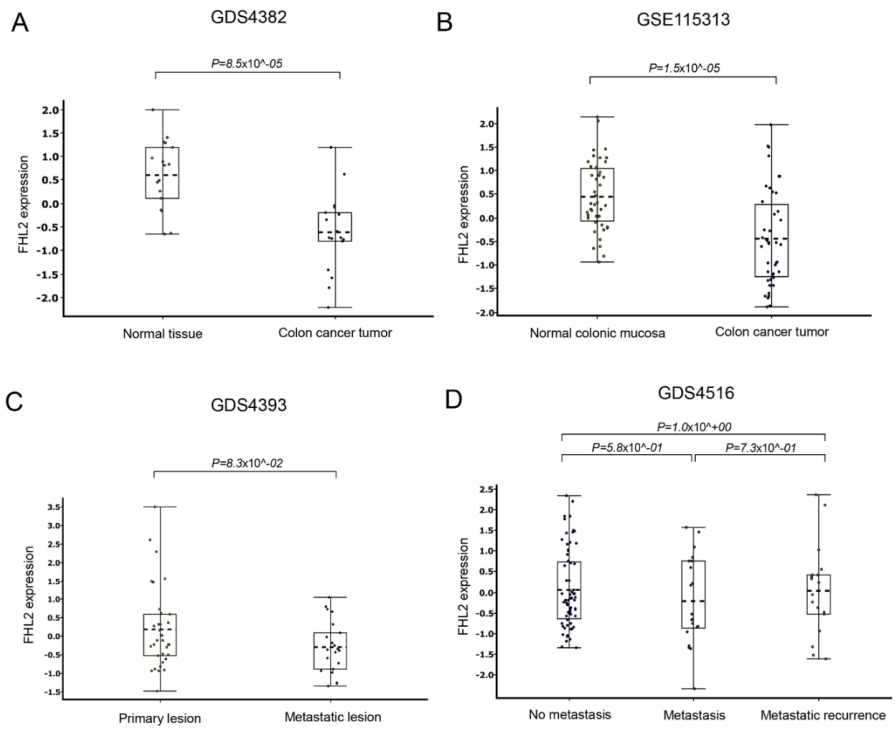

Figure 17. Expression of FHL2 in colon cancer from different microarray datasets. Comparison of FHL2 expression between normal colon mucosa and CRC tissue in (**A**) GDS4382 and (**B**) GSE115313 datasets. Comparison of FHL2 expression between primary colon cancer and metastatic colon cancer or recurrence metastasis in **(C)** GDS4393 and **(D)** GDS4516 datasets. Box plots created using Qlucore program represent the median (25-75 percentile) and the whiskers extend from the minimum to the maximum levels and dots represent sample values.

However, we observed that primary colon cancer tissue had no significant difference with metastatic colon cancer tissue in terms of FHL2 expression. Analysis of microarray datasets containing different types of colon cancer cell lines revealed that there might be two different levels of FHL2 mRNA expression in colon cancer depending on the cell line types (**Figure 17**). Such observation is similar to those of Amann et al (146), where they have shown that HT-29 cell lines have the lowest levels of FHL2 expression in compared to CaCo, HCT116, Lovo, SW48, and SW480 colon cancer cell lines.

In this study, we have used two different cell lines (HT-29 and AZ-97) which represent both low and relatively high FHL2 expressing cell lines (**Figure 18A, 18B**). According to two recent studies, the colon cancer tissue has significantly lower levels of miR-340-5p than normal colon tissue, such decreased expression of miR-340-5p correlates with an increased incidence of metastasis in the liver (147) and the lymph node (148). Moreover, we have shown that miR-340-5p can reduce colon cancer migration and invasion through the control of RhoA activity (145), thus indicating that miR-340-5p can regulate the pathogenesis of colon cancer.

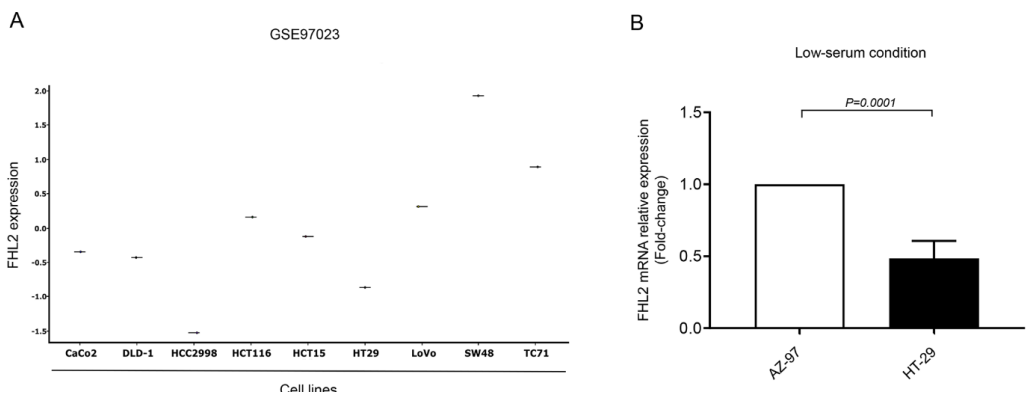

Figure 18. FHL2 expression in colon cancer cell lines. (**A**) Expression of FHL2 in different types of colon cancer cell lines. (**B)** FHL2 mRNA expression in AZ-97 and HT-29 cells grown in low-serum conditions. Data represent mean ± SEM and $\boldsymbol{n} = 4$. $^{\#}P < 0.05$ was considered significant.

In order to investigate the transient effect of serum in stressed cancer cells, we have cultured cells in low serum condition and after that exposed to serum for 30 minutes. We have shown that short serum exposure leads to an increased expression of FHL2 in colon cancer cells (**Figure 19**) as well as transfection the cells with mimic-miR-340-5p, significantly reduced short serum-induced FHL2 expression (**Figure 20**).

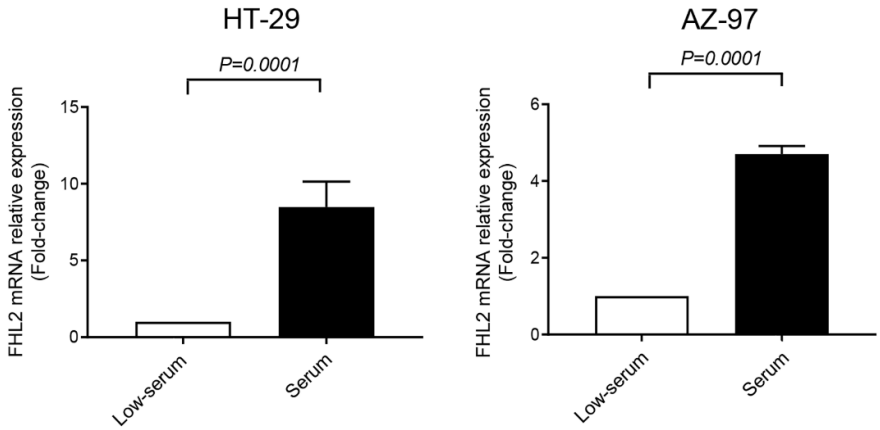

Figure 19. The expression of FHL2 in HT-29 and AZ-97 colon cancer cell lines. Cells were grown at different conditions such as low-serum and serum-rich. Relative expressions were quantified by using qRT-PCR where beta-actin was used as a housekeeping gene for FHL2 mRNA.

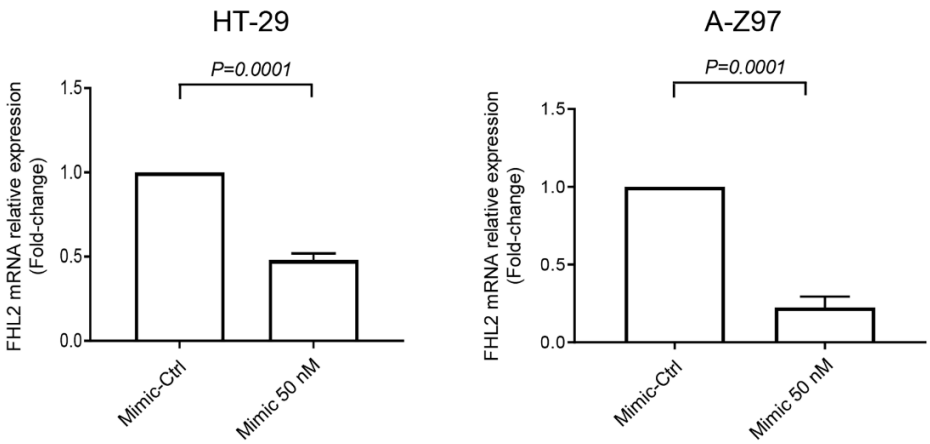

Figure 20. The expression of FHL2 in HT-29 and AZ-97 colon cancer cell lines. Transfection of HT-29 and AZ-97 cells with mimic-miR-340-5p reduced FHL2 mRNA expression. Relative expressions were quantified by using qRT-PCR where beta-actin was used as a housekeeping gene for FHL2 mRNA.

In addition, we have revealed that transfection of HT-29 cells with mimic-miR-340-5p reduced cells proliferation in the terms of ki67 positive cells (**Figure 21**). Given the fact that FHL2 can regulate cancer cell proliferation and migration (144, 149), such findings have shown that miR-340-5p can regulate the progression of colon cancer, alongside an inverse relationship with FHL2 expression as well as cell proliferation.

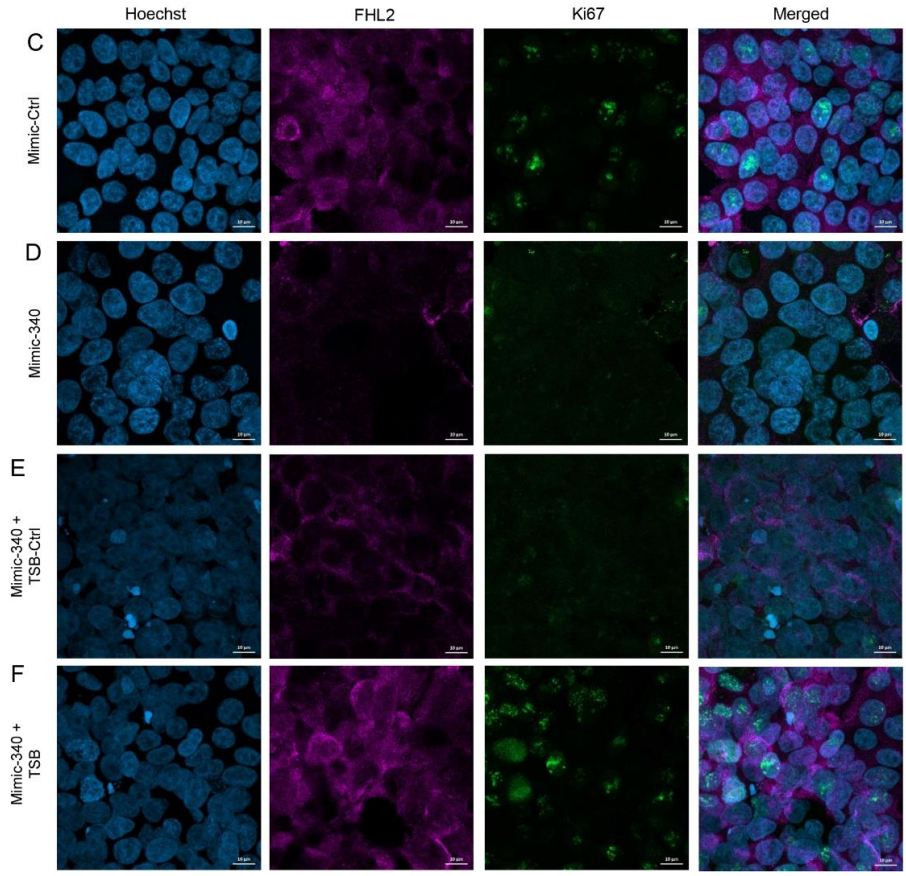

Figure 21. Transfection of HT-29 cells with mimic-miR-340-5p reduced cells proliferation and down-regulates FHL2 expression in colon cancer cells. FHL2 expression was evaluated by using confocal microscopy (C, D, E, F). Cells were transfected with miR-340-5p mimic, mimic control, TSB control and TSB for 24 hours in low-serum condition. Colon cancer cells were then stimulated using 10% FBS for 30 min.

Next, by using the bioinformatics tools we have checked if the FHL2 has any binding sites for miR-340-5p, we have found that miR-340-5p has one a binding site at 3'-UTR of FHL-2 mRNA. Next, we have checked the functionality of this binding site by using a target site blocker (TSB) that specifically compete with miR-340-5p as shown in the (**Figure 22**).

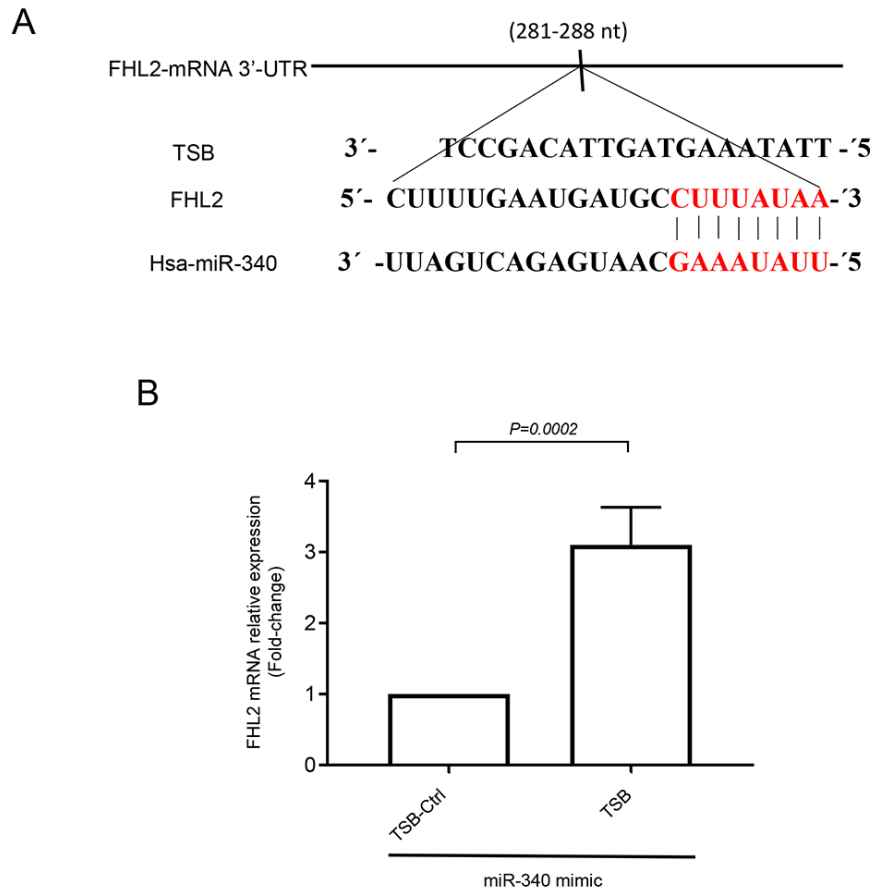

Figure 22. MiR-340-5p directly targets FHL2. A) Bioinformatics analysis predicted a target site of miR-340-5p containing an (CUUUAUAA) motif in FHL2 mRNA at 3′-UTR sequence. TSB (red sequence) was designed to block the seeding region of miR-340-5p complementary to (GAAAUAUU) of FHL2 mRNA. **B)** The inhibitory effect of miR-340-5p on FHL2 mRNA expression in HT-29 colon cancer cells was dose-dependently reversed by TSB. Data represent mean ± SEM and n=4.

We demonstrated that miR-340-5p had a functional binding site in FHL2 mRNA, and targeting of FHL2 by miR-340-5p mimic reduced cancer cells migration and invasion (**Figure 23 and 24**). Therefore, targeting FHL2 through miR-340-5p could be a useful strategy to inhibit colon cancer metastasis.

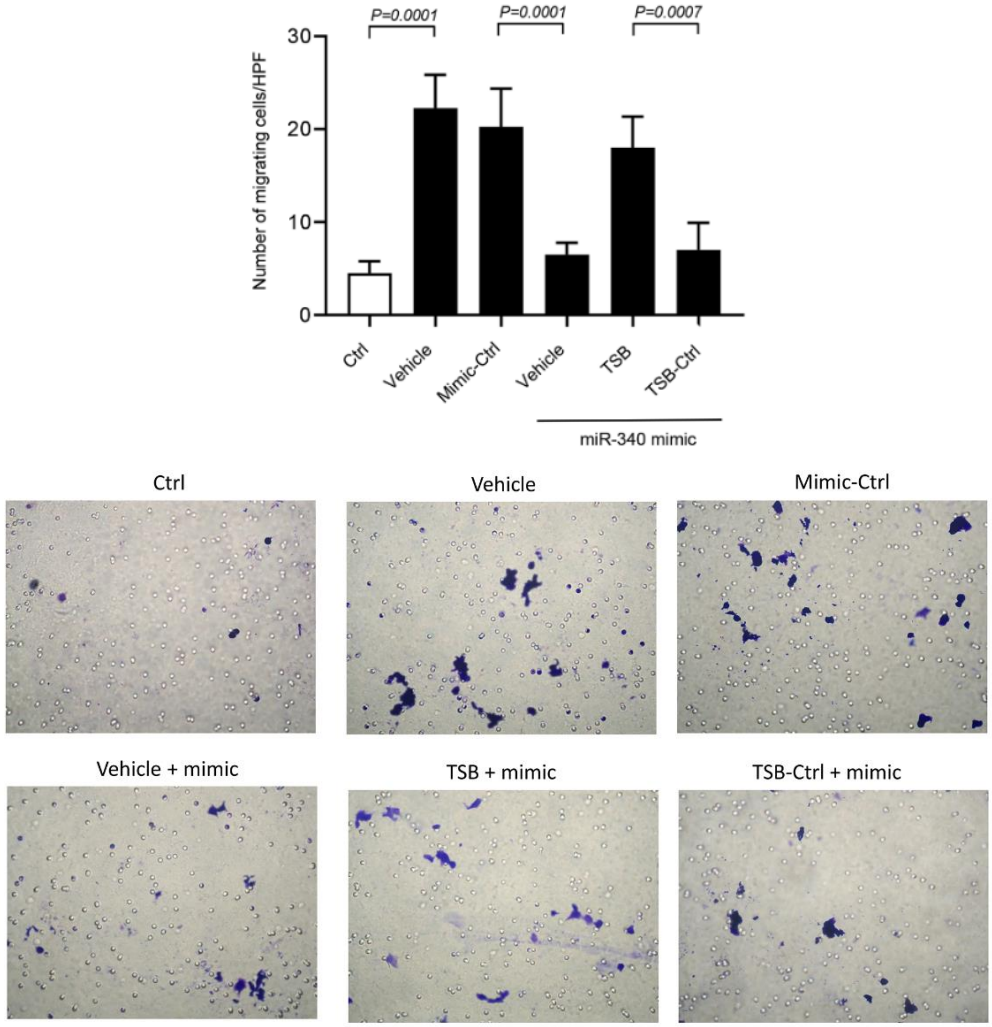

Figure 23. Migration of colon cancer cells toward serum. HT-29 cells were transfected with miR-340-5p mimic, mimic control, TSB control and TSB in low-serum condition for 24 hours. Serum-stressed transfected cells were then loaded in the upper chamber of the transwells and lower chamber were filled with 10% BSA. A). cells were counted by microscope in five different fields. Data represent mean ± SEM and n=4.

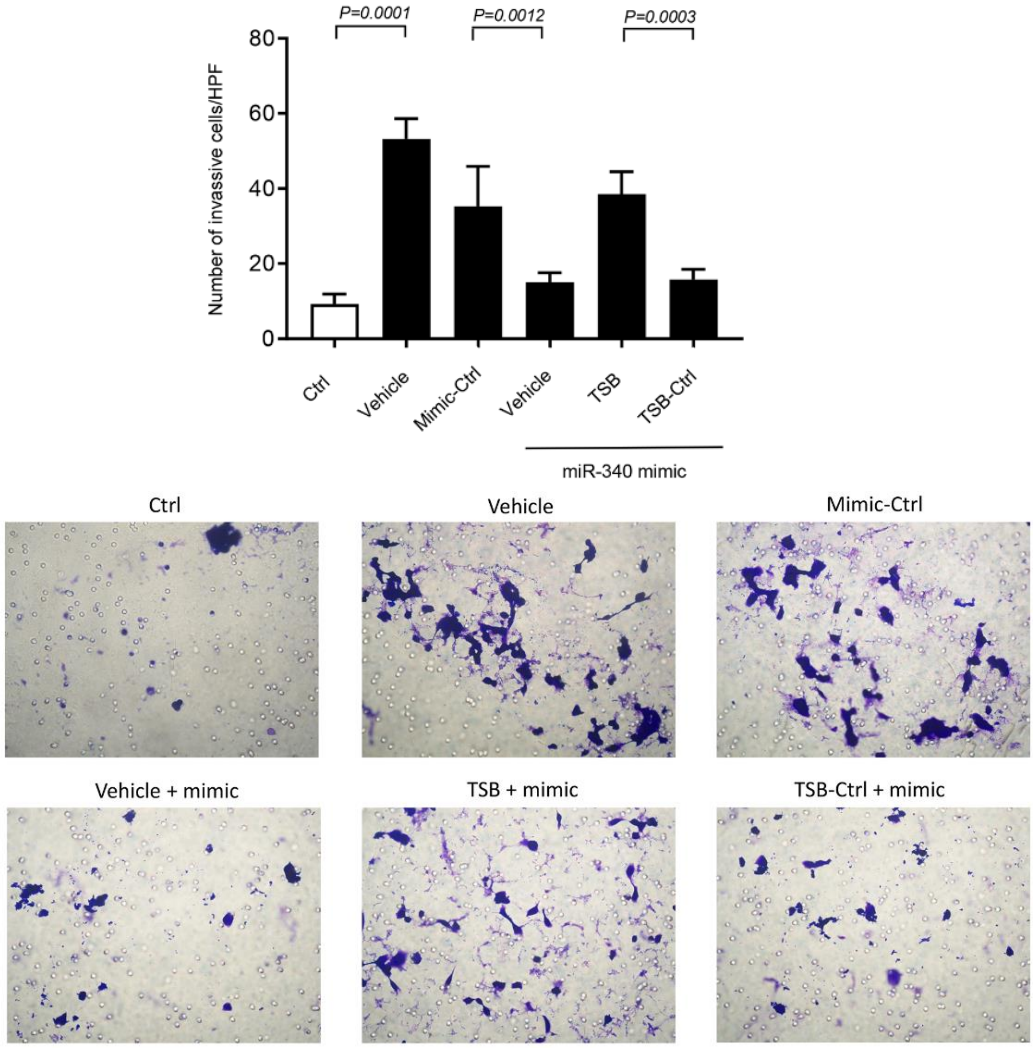

Figure 24. Invasion of colon cancer cells toward serum. HT-29 cells were transfected with miR-340-5p mimic, mimic control, TSB control and TSB in low-serum condition for 24 hours. Serum-stressed transfected cells were then loaded in the upper chamber of the transwells and lower chamber were filled with 10% BSA. A). Cells were counted by microscope in five different fields. Data represent mean ± SEM and n=4.

To examine downstream target protein of FHL2, we examined E-cad expression. QRT-PCR revealed that short-term serum stimulation significantly decreased E-cad expression in HT-29 cells (**Figure 25A-B, C-F**).

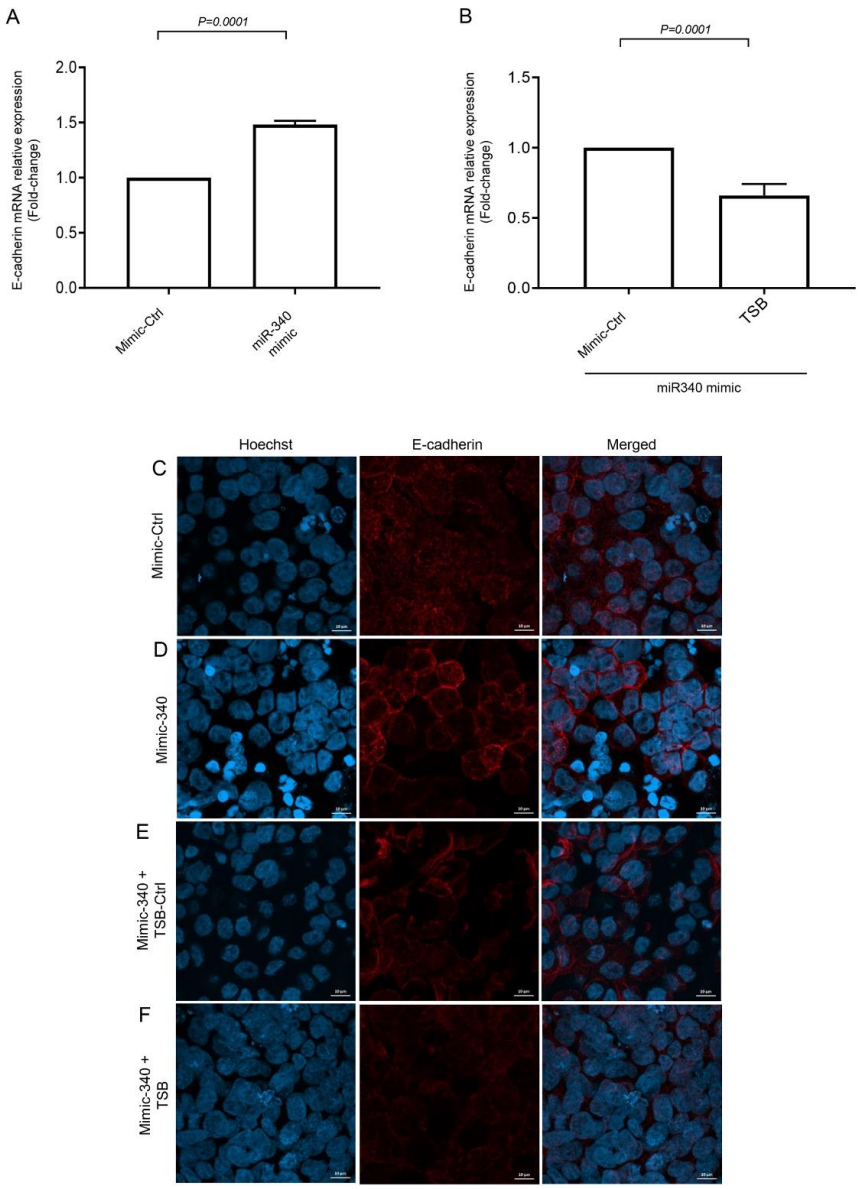

Figure 25. miR-340-5p up-regulates E-cad expression in colon cancer cells. E-cad expression was evaluated by using **A-B)** RT-qPCR and **C-F)** confocal microscopy. Cells were transfected with miR-340-5p mimic, mimic control, TSB control and TSB for 24 hours in low-serum condition. Cells were then stimulated by using 10% FBS for 30 min. Relative expressions of E-cad mRNA was quantified by using qRT-PCR where beta-actin was used as a housekeeping gene and expressions were determined by using 2−ΔΔCT method. Data represent mean ± SEM and n=4.

Interestingly, short serum-induced downregulation of E-cad in cancer cell was reversed by miR-340-5p and TSB could neutralize the effects of miR-340-5p. Thus, we concluded the inhibition of colon cancer cell invasion and migration by miR-340-5p might be through the regulation of E-cad expression. Our finding is also supported by other studies showing that deregulation of E-cad is associated with metastasis of breast (150), colon (151), and prostate cancer (152). In addition, Zhang et al (153) have shown that FHL2 regulates the E-cad inversely through the process of transcription repression.

In the current study, we have shown that the transfection of colon cancer cells by mimic-miR-340-5p reduced colon cancer cell invasion and migration compared to mimic-control (**Figure 23**). This observation is in line with other studies showing that increase of miR-340-5p in many cancers such as breast (154), cervical (155), lung (135) and ovarian (144) cancers, reduced metastasis. Thus, our findings indicate that miR-340-5p regulate colon cancer cell migration and invasion by regulating FHL2-E-cad axis.

We also observed that specific TSB reversed miR-340-5p mimic-induced FHL2 expression in colon cancer. This suggests that the binding site for miR-340-5p on the FHL2 mRNA represents a functional binding site. It should be pointed out that FHL2 expression is in fact documented as promoter of colon cancer cells invasiveness by the transformation of epithelial cells into mesenchymal cells (156). Furthermore, FHL2 is shown to down-regulate multiple oncogenes in the gastrointestinal cancer cells (157).

Taken together, we could conclude that the expression of FHL2 on the stressed cancer cells play a significant role in colon cancer cell invasion and migration. Furthermore, inhibition of FHL2 expression by the use of mimic miR-340-5p could reduce cancer cells proliferation and increase E-cad expression in colon cancer cells. Thus, it could be suggested that targeting of FHL2 via miR-340-5p might be a useful approach to reduce colon cancer cell metastasis and invasion.

Chapter 7

General Discussion and Future Perspective

Colorectal cancer, also commonly known as CRC, this can be noticed through the abnormal growth of various parts of the large intestine. During the early stages, one can observe the appearance of polyps, which is a type of proliferative growth. CRC is most prominently detected in the Western world, where the incidence of diagnosis increasing year by year. To be more specific, the probability of suffering from colorectal cancer is approximately 4% to 5%, and the risk of developing CRC is commonly associated with personal aspects and even daily habits, including the chronic disease history, age, gender, socio-economic circumstances, habits, lifestyle, and many more.

CRC is caused by mutations that target tumor suppressing genes as well as oncogenes and genes which are related to DNA repair mechanisms. Depends on the origin of its mutation, colorectal carcinomas are classified into the following categories and with percentage of its occurrences: inherited (5%), sporadic (70%), and familial (25%). On the other hand, pathogenic mechanisms of CRC are categorized into three types: chromosomal instability (CIN), microsatellite instability (MSI), and CpG island methylator phenotype (CIMP). Moreover, due to the high incidence and mortality rate at a global scale, colorectal cancer has become a global health problem.

CRC starts off and develops depending on a combination of specific factors, which predominantly includes genetic and environmental factors. Such particular study is very important for establishing new strategic methods for preventing CRC, which represents one of the most significant steps towards taking action in order to stop the increase of CRC occurrences. It is very important to conduct preclinical studies, as these help translating the findings and make them applicable for clinical trials.

In the last decade, the major colon cancer genes have been detected and their pathogenic variants have been linked with a high susceptibility to colorectal cancer. In addition, the study of the inheritance patterns was important in order to discover the Hereditary Colorectal Cancer Syndromes, and this has led to the ability to diagnose this disease, as well as developing the ability to provide genetic counselling as well as special risk management programs that that can be applied to patients and their relatives, reducing their risk of suffering from this disease.

On the other hand, the development of colon cancer due to familial lineage can also occur outside the expected syndromes related to the colon cancer family. Therefore, various epidemiological studies are taking place in order to be able to identify underlying susceptibility

to CRC amongst different types of peoples. The results from those studies represent a stepping stone towards new generation of genetic screening tests.

In the current thesis, we have studied miRNA-mediated colon cancer cells migration and analyzed publicly available microarray data to compare specific gene expression between normal and CRC tumor tissue samples. It is commonly believed that such comparisons can provide useful information in regards to the prognosis of the disease, and most suitable methods of treating cancer for each patient.

The MicroRNAs, also commonly referred to as miRNAs, represent noncoding nucleotide RNAs that contain approximately 18 and 25 nucleotides which regulate gene expression through a process of post-transcriptional regulation within human genomes, approximately 30% of the protein-coding genes are tied tightly together according to the regulations of the miRNAs (64, 65). They regulate a wide range of important cellular functions, including cell survival, proliferation, differentiation, apoptosis, and migration (66).

Due to the natural roles of these miRNAs, different types of miRNAs can exert their function upon a single gene, while this single miRNA is also capable of acting on many gene targets (67). Generally, miRNAs are known to regulate their target mRNAs through direct recognition; this often applies through both perfect and even imperfect base pair bindings. These can result into the inhibition of the translation or even the complete degradation of the targets. This process is mediated by argonaute-2 protein (Ago-2), and this represents an RNA-induced silencing complex (RISC), which represents a major catalytic subunit as well as main form of decay in regards to a RISC complex (68, 69).

The mRNA stability is important for controlling gene expression at the posttranscriptional stage, or by influencing the localization, the export, stability and translation efficiency of an mRNA. mRNA contains multiple different sequences which are involving in gene expression, including the AU-rich elements (AREs), microRNA response elements (MREs), and the poly(A) tail. AREs in the 3'-untranslated region (UTR) of the short-lived mRNAs are shown to involve in positive regulation of translation (91). There are many mRNAs which have AU rich elements at 3'-untranslated region (UTR), which includes AUUA and AUUUA. These include the positive regulation in specific conditions, including stress or low serum conditions. On the other hand, the majority of mRNAs that do not have any AU rich elements at 3'-untranslated region (UTR) involve in negative regulations in specific conditions such as stress or even low

serum conditions. However, according to more recent studies and amount of evidence, it has been revealed that the microRNAs, as contrast to their traditional role, could indeed potentially upregulate their own targets, which can be done either through direct or even indirect responses to distinct cofactors, for example, cell types, stress conditions. Because of this, same miRNA performs different regulatory profiles in different types of cancers (75, 76).

In this thesis, we have identified three different genes such as HuR, RhoA and FHL2 which are involved in invasion and migration of colon cancer cells. Suggesting that targeting any of these genes might provide a therapeutic approach to reduce the invasion and migration of colon cancer cells in clinical CRC. As well as we have investigated the role of two different miRNAs, such as, miR-340 and miR-155 in connection with the above mentioned genes. According to previous studies, miR-155 is overexpressed in many different cancers, including colon cancer (70, 71). In addition, there is an evidence that higher expression of miR-155 correlates with poor prognosis of CRC patients (71). As confirmed in various studies, miR-155 plays significant role during the process of tumor cell migration as well as invasion (71-73).

In our first study, we investigated the role of miR-155-5p on HuR expression and colon cancer cells migration. We knocked down miR-155-5p in serum-starved HT-29 colon cells using AntagomiR-155-5p. Knockdown of miR-155-5p decreased HuR mRNA expression in colon cancer cells while knock-in of miR-155 mimic increased HuR expression. We found that miR-155-5p is a positive regulator of HuR expression in HT-29 colon cancer cells in stress condition. Interestingly, our migration assay confirmed that miR-155-5p regulates serum-induced colon cancer cell migration and miR-155-5p antagomir reduced serum-induce migration. We concluded that targeting HuR expression by miR-155-5p or antagonize colon cancer cell metastasis to distant organs.

In study II, we investigated RhoA which is one of the most extensively studied Rho GTPases family, which involves in various types of cellular functions, including the cytoskeletal organization, membrane trafficking, proliferation, and actin stress fiber formation (88). Previous studies suggest that RhoA is overexpressed in various types of cancers including colon cancer (89). miR-340-5p and RhoA is inversely related to each other under certain conditions in colon cancer. We found levels of miR-340-5p was downregulated in serum-grown cells compared to serum-free cells. RhoA mRNA expression was also shown to be much higher in serum-grown cells compared to the serum-free cells. Transfection of cancer cells with mimic miR-340-5p dose-dependently decreased Rho mRNA. In addition, transfection of colon cancer

cells with miR-340-5p mimic reduced cancer cell migration and invasion, suggesting that miR-340-5p inversely regulates colon cancer cell by activating RhoA. Similarly, in study III, we showed that inhibition of FHL2 expression by the use of mimic miR-340-5p could reduce colon cancer cells migration by increasing E-cad expression. In the study II and III, our findings show that miR-340 controls colon cancer cell invasion and migration by two different genes, such as, RhoA and FHL2, which also confirms with the previous studies regarding the migration of cancer cells in lung, breast and squamous cancer cells, which is regulated by miR-340-5p (84, 135, 141).

Targeted therapies such as directly inhibiting cell proliferation, migration or modulating cancer microenvironment using small inhibitors or antibodies, such as inhibition of local blood vessels development and crosstalk between cancer and immune cells, may improve the overall survival rate significantly. Targeted therapy of CRC is getting popular since it has successfully improved overall survival of CRC patients. For example, successful use of anti-EGFR (epidermal growth factor receptor) agent cetuximab and the anti-angiogenesis agent bevacizumab have raised the hope for CRC therapies significantly. In addition, some blocking agents targeting critical pathways and immune checkpoints are emerging as a new hope since significant investigational efforts are going there.

Finally, the finding of this thesis suggest that miRNAs can be used simultaneously as biomarker and therapeutics. Significant numbers of trials have assessed the suitability of ncRNA transcripts, such as, miRNAs, to examine wide range of health conditions such as diabetes, coronary heart disease, breast cancer, lupus, stroke, liver disease. Many miRNA mimics (to overexpress the transcript) and miRNA repressors (to silence the transcript function) are under clinical trial to date. Although no miRNA drug candidates have approved for therapies, several candidates are in phase 1 and phase 2 trails. For example, miR-29 (MRG-201) for the treatment of keloid and scar tissue formation is in phase 1 trial and miR-155 (Cobomarsen; MRG-106) for the treatment of T-cell lymphoma is in phase 2 trial (158). For cancer therapy, direct injections of miRNA mimics or suppressors into tumor can enhance target specificity, efficacy, and minimize side effects. Although some miRNA therapeutics have shown clinical potential, some have failed during the clinical trials. For example, phase 1 trial of a miRNA therapeutics (miRNA-34 mimic, MRX34) targeting melanoma cancer has halted due to serious immune responses. It should be also noted that miRNA-based therapies might lead to unwanted adverse events; identification of potential sensitive patients based on biomarkers might reduce the

adverse events. Thus, extensive preclinical and clinical studies are required to developed miRNA-specific therapies against colon cancer.

Thesis Conclusions

- Stress condition, such as, serum starvation may alter gene expression profile of colon cancer cells.

- MiR-155-5p positively regulates colon cancer cell migration by directly binding to ARE region of HuR mRNA at 3'-UTR in stressed conditions.

- MiR-340-5p negatively regulates colon cancer cell migration by directly binding at 3-UTR of RhoA.

- Inhibition of FHL2 expression by use of mimic miR-340-5p could reduce colon cancer cell migration by increasing E-cad expression and reducing cell proliferation.

- Targeting the mRNA of HuR, RhoA and FHL2 by miR-155-5p and miR-340-5p might be effective approach to antagonize colon cancer migration and invasion.

Chapter 9

Populärvetenskaplig Sammanfattning

Populärvetenskaplig Sammanfattning

Kolorektal cancer (CRC) är den näst vanligaste cancertypen hos kvinnor och den tredje vanligaste cancerformen hos män världen över. Orsaken till majoriteten av dödsfall är relaterad till migrationen av cancerceller till avlägsna organ, som kallas metastasering. Mekanismerna bakom metastasering är inte helt förstådda, men ackumulerande studier tyder på att det kan bero på förbättrad tumörcellmotilitet på grund av överuttryck av metastaserings befämjande proteiner. Man tror att mikroRNA (miRNA) spelar en viktig roll i tumörgenes och metastasering av cancer genom att reglera onkogener. Syftet med denna avhandling är att undersöka mekanismerna bakom miRNA-medierad koloncancercell invasion och migration och möjliga måltavlor för dessa miRNA. Vi fann att knockdown av miR-155-5p med hjälp av antagomiR minskar uttrycket HuR mRNA och migration av koloncancerceller. Våra data visade också att miR-155-5p är inblandad i regleringen av HuR mRNA genom direkt bindning och positiv reglering av HuR-protein under stressförhållanden. Vidare fann vi att miR-340-5p är involverat i koloncancercell invasion och migration genom att reglera RhoA- och FHL2-mRNA-uttryck. Bioinformatikanalys avslöjade att både RhoA-mRNA och FHL2-mRNA har direkta bindningsställen för miR-340-5p, och att miR-340-5p negativt reglerar RhoA- och FHL2-mRNA-expression under stressförhållanden. Våra resultat har visat att hämningen av RhoA- och FHL2-uttryck genom blockering av miR-340-5p, minskade invasion och migration av koloncancerceller. Dessutom fann vi att hämning av FHL2 minskar spridning av cancerceller och ökar E-cadherinuttryck i tjocktarmscancerceller, vilket tyder på att inhibition av FHL2 och RhoA med miR-340-5p kan vara ett användbart tillvägagångssätt för att motverka metastasering av tjocktarmscancer. Resultaten av våra studier visar inte bara hur miRNA kontrollerar migrering av koloncancerceller utan gav också värdefull information om att miRNA kan vara ett viktigt mål för att utveckla nya och effektiva terapeutiska strategier mot metastasering av koloncancerceller. Sammantaget visade våra data nya mekanismer som kan hjälpa till att bättre förstå metastasering av koloncancer och öppnar för en ny potentiell strategi för behandling av patienter med avancerad koloncancer.

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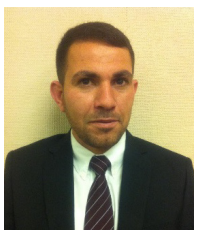
Printed by Media-Tryck, Lund 2021 NORDIC SWAN ECOLABEL 3041 0903

MicroRNA-Mediated Migration of Colon Cancer Cells



Anwar Algaber is a biomedical scientist who obtained his MSc in biomedicine from University of Skövde in the Sweden. He worked as teacher of biology at Ministry of education in the Iraq. Anwar moved to Sweden and started his PhD in clinical medicine and experimental surgery at the faculty of medicine at Clinical Research Center, Lund University. His main research focus is microRNA-mediated colon cancer metastasis.

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Department of Clinical Science, Malmö

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2021:72
ISBN 978-91-8021-078-2
ISSN 1652-8220