Table of contents

List of figures and tables

List of abbreviations

3’UTR	*3’untranslated region*
ADAR	*Adenosine deaminase, RNA-specific*
Ago	*Argonaute*
Akt	*Protein kinase B*
AL	*Annulate lamellae*
APE1	*apurinic/apyrimidinic endonuclease 1*
ATP	*Adenosine triphosphate*
cDNA	*complementary DNA*
C. elegans	*Caenorhabditis elegans*
Co-IP	*Co-Immunoprecipitation*
c-Src	*c-src tyrosine kinase*
DDX17	*DEAD-box RNA helicase p72*
DGCR8	*DiGeorge syndrome Critical Region 8*
DUSPs	*Dual specificity phosphatases*
EGFR	*Epidermal growth factor receptor*
ERK	*Extracellular signal-regulated kinases*
XPO	*Exportin*
GLS2	*Tumor suppressor glutaminase 2*
GSK3β	*Glycogen synthase kinase 3β*
GTP	*Guanosine-5'-triphosphate*

GW182	*Trinucleotide repeat containing 6A*
HCC	*Human hepatocellular carcinoma*
HDAC5	*Histone deacetylase 5*
HEN1	*Hua ENhancer 1*
HIF	*Hypoxia inducible factor*
HNRNPA1	*Heterogeneous nuclear ribonucleoprotein A1*
HSP27	*Heat shock protein 27*
HSP90	*Heat shock cognate 70 (HSC70)-heat shock protein 90*
ICAM-1	*Intercellular adhesion molecule-1*
IGF	*Insulin-like growth factor*
IRAK	*Interleukin-1 receptor-associated kinase*
IL-1β	*Interleukin-1β*
JAK1	*Janus kinase 1*
JNK	*c-jun NH2-terminal kinases*
kDa	*kiloDalton*
KLF	*Krüppel-like factor*
KSRP	*KH-type splicing regulatory protein*
MAKAPK2	*MAP kinase-activated protein kinase-2*
MAPK	*Mitogen-activated protein kinase*
MCPIP1	*MCP-induced protein 1*
MEK	*Mitogen-activated protein kinase kinase*
miRISC	*microRNA induced silencing complex*
miRNA	*microRNA*

mRNA	*messenger RNA*
MVB	*Multivesicular bodies*
MKK	*MAP kinase kinase*
MLC	*Myosin light chain*
NEAT1	*Nuclear enriched abundant transcript 1*
PARN	*poly(A)-specific ribonuclease*
P-bodies	*Processing bodies*
PDCD4	*Programmed cell death 4*
PI3K	*Phosphoinositide-3-kinase*
PIWI	*P-element induced wimpy testis*
Poly(A) tail	*Poly adenylate tail*
Pre-miRNA	*Precursor microRNA*
Pri-miRNA	*Primary microRNA*
PTEN	*Phosphatase and tensin homolog*
RISC	*RNA-induced silencing complex*
RBP	*RNA binding protein*
RdRP	*RNA-dependent RNA polymerase*
RNAi	*RNA interference*
RNases	*Ribonucléases*
Ser	*Serine*
SGs	*Stress granule*
siRNA	*Small interfering RNA*
snoARNs	*Small nucleolar RNAs*

STAT3	*Signal transducer and activator of transcription 3*
SIM	*SUMO-interacting motif*
TDP43	*TAR DNA-binding protein 43*
TGFβ	*Tumor growth factor β*
Thr	*Threonine*
TMG	*Trimethylguanosine*
TNRC6A	*Trinucleotide repeat-containing gene 6A*
TNFα	*Tumor necrosis factor α*
TRAF6	*TNF receptor associated factor 6*
TRBP	*Transactivation-responsive (TAR) RNA binding protein*
TUTase/TUT	*terminal nucleotidyl transferase*
Tyr	*Tyrosine*
VCAM-1	*Vascular cell adhesion molecule 1*
VE-Cadhérine	*Vascular endothelial-cadherin*
VEGF	*Vascular endothelial growth factor*
VEGFR	*Vascular endothelial growth factor receptor*

Acknowledgement

I would like to acknowledge my supervisors Prof. Jacques Huot and Prof. Martin Simard, for giving me the opportunity to join their laboratories in which I completed my graduate studies. Throughout the lows and highs of these years, their advice, kindness and determination have been essential to what I have been able to contribute to science. I would also like to acknowledge present and past lab members, Bryan, Francois, Maëva, Isabelle, Guillaume, Sandra, Pascale, Lucile, Alexandra, Pavan and Miguel who contributed in some way what I have achieved and for the nice moments we shared together. I owe special gratitude to Prof. Steve Bilodeau for his friendly advice in bioinformatics.

I would like to express my deepest gratitude to my fiancée, Nathalie Hu, for being my fiancée and for being there for me.

Chapter 1. Introduction and reviews of the literature

Foreword

The present chapter covers two introductory aspects relevant for the understanding and presentation of the subsequent chapters, namely cancer, including one subdivision: endothelial cell adhesion molecule-mediated cancer metastasis; and microRNA, containing two subdivisions: pro-inflammatory pathways mediating microRNA pathway and microRNAs modulating endothelial cell adhesion molecules.

Sub-subdivision 1.1.5 *Endothelial cell adhesion molecules and cancer metastasis* contains information reported in a book chapter published in Encyclopedia of Cancer: E-Selectin-Mediated Adhesion and Extravasation in Cancer (Zhong et al., 2017). Subdivision 1.2.3 *microRNAs mediating endothelial cell adhesion molecules* contains information reported in a review article published in the journal of Federation of American Societies for Experimental Biology (FASEB J): Endothelial microRNAs regulating the NF-κB pathway and cell adhesion molecules during inflammation (Zhong et al., 2018).

1.1 Cancer

Cancer is not a single disease but a collection of disorders involving abnormal cell growth. In normal conditions, cell division is a highly controlled process in that an abnormality such as un-repaired genetic mutation will be detected during the cell cycle, leading to programmed cell death or apoptosis. When cancer cells grow out of control they usually form a mass, called a tumor. Some tumors not only enlarge locally, but also have the potential to invade or spread to other parts of the body to form secondary tumors, or metastases. These tumors are called malignant tumors in contrast to benign tumors, which do not spread to other parts of the body. Increasing exposition to mutagens such as radiations and DNA reactive chemicals, combined with the aging population in developed countries, significantly augmented the chance of getting cancer in an individual's lifetime. One in two Canadians will develop cancer in their lifetime and one in four will die of it. In 2017 in Canada, an estimated 206,200 new cases of cancer and 80,800 cancer-associated deaths will occur (Canadian Cancer Statistics - 2017). Intriguingly, a wild life study of the beluga population in St. Lawrence river in Quebec showed that the annual cancer rate of all cancer types (163/100,000 belugas) for this long-lived mammal is much higher than that reported for any other population of cetacean and is similar to that of humans inhabiting along the river, strongly suggesting the involvement of environmental contaminants (*Figure 1-1*). It is both worth noticing and frustrating that the similar cancer-associated death rate of 27% was observed for St. Lawrence beluga as for humans (Martineau et al., 2002), providing us with a glance of how much remains to be done to fight this threat to our health.

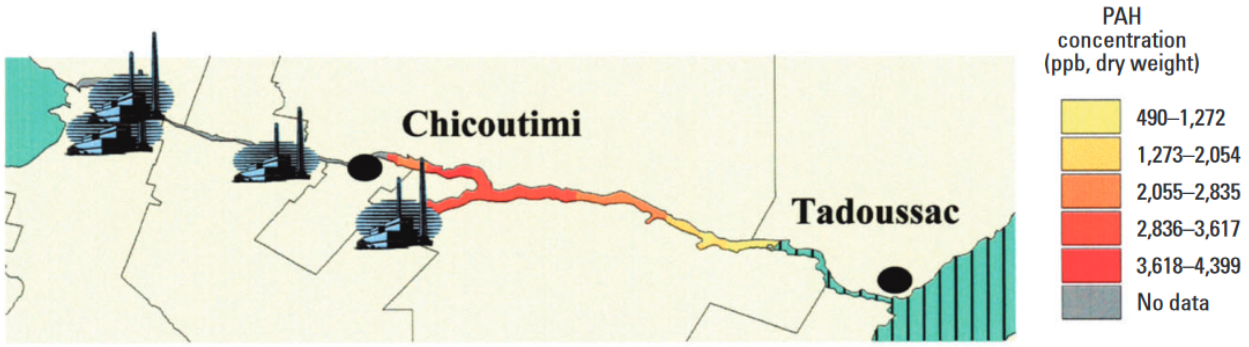


Figure 1-1: Polycyclic aromatic hydrocarbon (PAH) concentration in sediments in Saguenay River, Québec, Canada. *Chimney icon: aluminum smelters; PAH: parts per billion. Adapted from (Martineau et al., 2002).*

1.1.1 Causes of cancer

1.1.1.1 Environmental cues

Environmental cues that can give rise to cancer include chemical substances and radiations. Together they are called carcinogens.

Chemicals including polycyclic aromatic hydrocarbons (PAHs), nitroaromatic compounds, aromatic amines, phenols, dihydrodiols and nitroaromatics have been confirmed as carcinogen as early as in 1950 (Boyland and Wolf, 1950). Many of the carcinogenic chemicals are metabolically activated to electrophilic species that bind to DNA or cause DNA damage (Wogan et al., 2004) and extensive investigations were performed to establish how each carcinogenic agent damage DNA or form DNA adducts (Wogan et al., 2004). Another important environmental cue acting as carcinogen is radiation. For such instance, UV light can either directly damage DNA, or damage DNA indirectly via reactive oxygen species (ROS). UV light is responsible for most types of skin cancers. In duplex DNA, UVB (280–320 nm) and UVC (240–280 nm) lead to the photolesion pyrimidine(6-4)pyrimidone photoproduct, which is

chemically stable but may undergo conversion to its more toxic Dewar isomer by UVA or UVB light (Figure 1-2) (Perdiz et al., 2000).

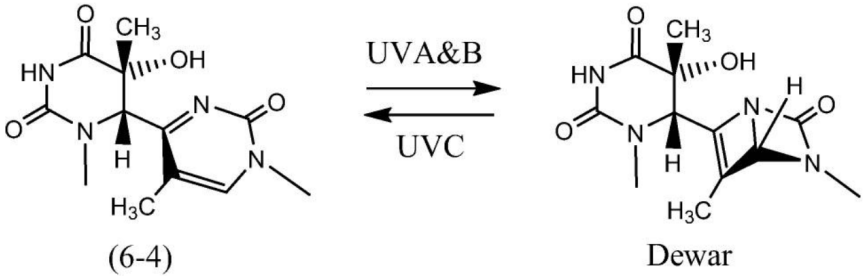


Figure 1-2: UV light induced photoproducts (Basu, 2018).

1.1.1.2 DNA damage and cancer

Most types of cancers are the result of at least a few mutations in critical genes (Greenman et al., 2007). The currently most accepted model for carcinogenesis is the somatic mutation theory (SMT) proposed by Theodor Boveri, and according to which cancer is caused by nonlethal mutation(s) associated with increased proliferation and survival in body cells instead of germ cells (Boveri, 2008). Based on his observations of retinoblastoma cases, Alfred Knudson modified Theodor's proposal, proposing that cancer is the result of accumulated mutations which could be as little as two hits. The two-hit model proposes that dominantly inherited predisposition to cancer requires a germline mutation, while tumorigenesis necessitates at least a second somatic mutation (Knudson, 1996). In our case, colon cancer necessitates five gene mutations for its malignant development (Fearon and Vogelstein, 1990). These mutations have been referred to as "driver" mutations conferring growth advantage to the cells (Fearon and Vogelstein, 1990).

Genes involved in carcinogenesis include oncogenes and tumor suppressor genes. Oncogenes drive a normal healthy cell into a cancerous cell. For such instance, *Ras* is engaged in cell communication and cell growth and its activating mutations are of importance in colon cancer. By contrast, tumor suppressor genes protect a cell from becoming cancerous. The tumor suppressor proteins control cell growth by monitoring cell division, repairing base mismatches in DNA, and controlling apoptosis. Famous tumor suppressor genes include *p53*, *BRCA1*, and *BRCA2*. p53 is a gatekeeper of DNA damage responses. Impressively over 50% of human cancers are characterized by mutations in *p53* (Greenblatt et al., 1994).

1.1.1.3 The cells' toolbox for DNA repair

Cancer arises from the instability of our genomic sequence, and the failure to repair it.

Upon its discovery, DNA was believed to be extremely stable, but Tomas Lindahl demonstrated that DNA decays at a rate that would have made the life impossible (Lindahl, 1993). He further discovered the mechanism counteracting the collapse of DNA: base excision repair (Barnes and Lindahl, 2004; Lindahl and Wood, 1999), which is the first discovered molecular machinery for DNA repair. Nucleotide excision repair is another mechanism repairing DNA damage caused by mutagenic substances, especially UV light. People born with defects in this repair system will develop skin cancer if they are exposed to sunlight (Hu et al., 2017; Li et al., 2018). DNA repair mechanism also repairs innate damage. Mismatch repair corrects errors occurring during DNA replication. Congenital defects in mismatch repair are known to cause at least one hereditary

variant of colon cancer (Iyer et al., 2006). These DNA repair mechanisms safeguard genetic information and have been applied for the development of new cancer treatments.

1.1.2 Multi-stepped carcinogenesis

The process of carcinogenesis was at first divided into two distinct steps. Mottram and colleagues showed that a single application of a carcinogen, such as B[*a*]P, followed by multiple applications of an "irritant", such as croton oil, induce tumors in mouse, while croton oil alone has no effect. This led to the model of "initiation" followed by "promotion" (Berenblum and Shubik, 1949). Now carcinogenesis is known as a complex process which can be divided into three distinct stages, which are: initiation, promotion and malignant progression. During initiation and promotion, apoptosis and cell proliferation can remain balanced. During progression, this balance is disturbed and from there malignancy arises. Changes in the genome's structure occur across the three stages of carcinogenesis (*Figure 1-3*) (Basu, 2018; Trosko, 2001).

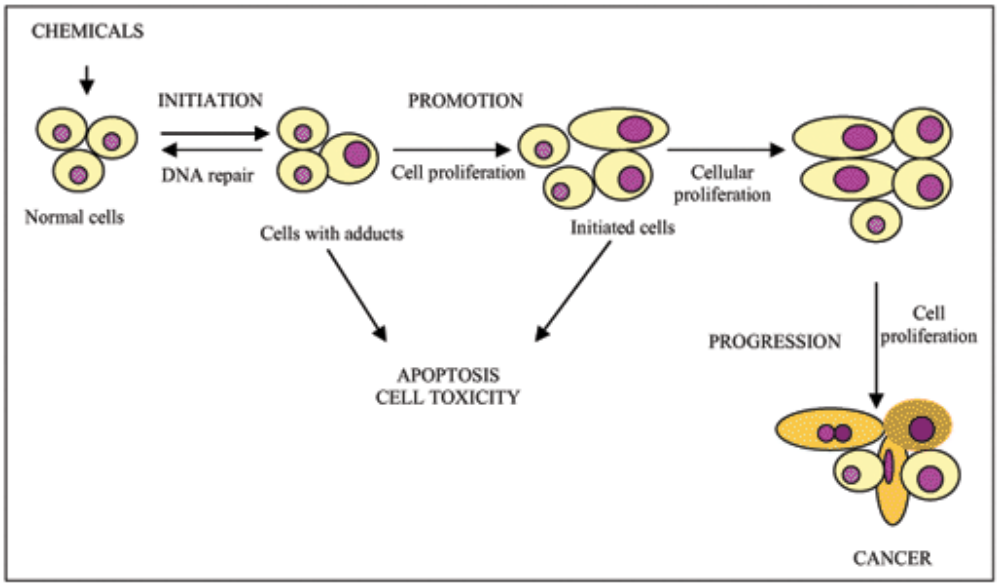


Figure 1-3: Chemical carcinogenesis. *By inducing irreversible genetic changes, carcinogenic chemicals initiate the transformation of normal cells, predisposing susceptible normal cells to malign evolution and potential immortality; after initiation, promotion mediated by chemical substances with low carcinogenic activity may occur; the multiplying cells can undergo a genetic event that confers a permanent genetic growth advantage known as progression, which may eventually lead to malignant conversion. Adapted from (Oliveira et al., 2007)*

1.1.2.1 Initiation

Initiation, induced by a single exposure to a genotoxic carcinogen, can result from a mutation in a single critical gene or genes in only a few cells. Cell culture studies of initiated epidermal cell lines have indicated that critical mutations alter cells' ability to respond to signals that induce terminal differentiation (Abel et al., 2009). A single mutation may accomplish initiation, as indicated by initiation by introduction of a virus containing an activated ras^{Ha} gene in a mouse

multistage epidermal carcinogenesis, and the gene(s) that regulate the terminal differentiation must be mutated (Brown et al., 1986).

The change is irreversible. DNA damage has been established as the event that kick-starts chemical carcinogenesis (Santella et al., 2005). Cell proliferation plays a role in initiation either before or soon after exposure to the initiator, perhaps in fixing the mutation (Hennings et al., 1973). Normal somatic cells can repair DNA damages by removing damages such as adducts, but proliferating cells have less time to do so (Frowein, 2000; Okafor et al., 2018). In other words, if cellular division occurs before DNA repair then the injury becomes permanent and irreversible, even when the carcinogenic substance is removed (Farber, 1984; Trosko, 2001).

The irreversible genetic changes predispose susceptible normal cells to malign evolution and potential immortality (Berenblum and Shubik, 1947; Shacter and Weitzman, 2002). An initiator is a complete carcinogen. Its effect is irreversible and additive, in contrast to the reversible action of a promoter at early stages. It is worth mentioning that an initiated cell is not yet a neoplastic cell but has taken its first step towards this state. The initiated cell is phenotypically similar to a "normal" cell but has undergone mutations inducing proliferation without differentiation (Trosko, 2001).

Initiation can also begin with spontaneous mutations such as DNA depurination and deamination. In addition, errors in DNA replication are also associated with initiation (Gomes-Carneiro et al., 1997).

1.1.2.2 Promotion

Promotion, accomplished by repeated treatments with chemicals at the proper dosage and frequency, is characterized by selection for the growth of initiated cells resulting in their clonal expansion into benign tumors (Hennings et al., 1993).

The concept of promotion was introduced when non-mutagenic chemical substances with low carcinogenic activity were discovered. These substances do not interact directly with DNA and unchain biological effects without being metabolically activated (Weisburger, 1998). In most cases, these compounds increase cell proliferation in susceptible tissues and enhance alterations in genome by causing errors in gene repairing. Some promoters may indirectly damage DNA by oxidation (Pisoschi and Pop, 2015). The promoter must be present for weeks, months or even years in order to be effective and the process is **reversible** -- the disappearance of the promoter results in the disappearance of proliferating initiated cells, probably by apoptosis. Additionally, benign tumors do not develop after insufficient exposure of initiated cells to promoters or when the interval between individual promoter applications is increased sufficiently. The reversibility of promotion suggests an epigenetic mechanism. Promoter treatment provides an environment that allows the selective clonal expansion of foci of initiated cells (Butterworth and Bisset, 1992; Wattenberg, 2007).

Known promoters include phenobarbital, benzene, asbestos and arsenic. The most frequently studied tumor promoter, TPA, is isolated from croton oil and is not directly mutagenic. Prolonged exposure to high doses of them can induce neoplastic development independent of initiating agents (Heidelberger, 1977; Pitot and Dragan, 1991). Alterations in gene expression, induction of inflammation, proliferation, and terminal differentiation result from exposure to

promoters, but no single critical characteristic has been defined for promotion (Hennings et al., 1993).

1.1.2.3 Progression

The lesions identified between initiation and promotion are designated as pre-neoplastic lesions, also called benign neoplasia. Their transformation into malign neoplasia is the last and the most extended stage of carcinogenesis – progression, which is a genetic event that confers a permanent growth advantage, and thus benign neoplasms become malignant and invasive lesions, eventually reach the ten hallmarks of cancer (Klaunig et al., 2000) (see below). Progression may eventually lead to malignant conversion. The neoplastic phenotype is acquired through genetic and epigenetic mechanisms (Shacter and Weitzman, 2002; Szigeti et al., 2018). Unlike initiation and promotion, progression does not require the presence of a stimulus. It occurs spontaneously at a low frequency (Lutz, 2000).

Progression of benign tumor into a malignant tumor is characterized by an increased autonomy from both the environment and the host. This stage of conversion to malignancy, associated with an increased frequency of genetic changes, is **irreversible** (Brown et al., 1990). The rate of progression to malignancy can be significantly increased by treating benign tumors with certain genotoxic agents such as ethylnitrosourea and benzoyl peroxide. These progressor agents or converting agents are likely to act via a second genetic change in benign tumors already bearing the initiating mutation. However, this second genetic change can also be due to spontaneous mutation, or instabilities introduced by the initiating mutation. (Moolgavkar and Knudson, 1981).

1.1.3 Hallmarks of cancer

Hanahan and Weinberg proposed ten hallmarks of cancer to characterize the progression of this neoplastic disease, which are: 1. sustaining proliferative signaling, 2. evading growth suppressors, 3. resisting cell death, 4. enabling replicative immortality, 5. inducing angiogenesis, 6. **activating invasion and metastasis**, 7. genome instability, 8. **inflammation**, 9. reprogramming of energy metabolism, 10. evading immune destruction. In addition to these hallmarks of cancer cells, and 11. creating the tumor microenvironment (Hanahan and Weinberg, 2011). Flavahan et al. have expanded the model by introducing 12. **epigenetic plasticity** as an additional hallmark.

These hallmarks must be acquired for development of a human cancer, and new treatments of cancer can be developed by tackling one of these eleven hallmarks (*Figure 1-4*). This thesis tackles two hallmarks of cancer, namely metastasis and inflammation using microRNA as the weapon. Given the recent addition of a 12th hallmark: **epigenetic plasticity** in non-coding RNA biology, it is also elaborated below.

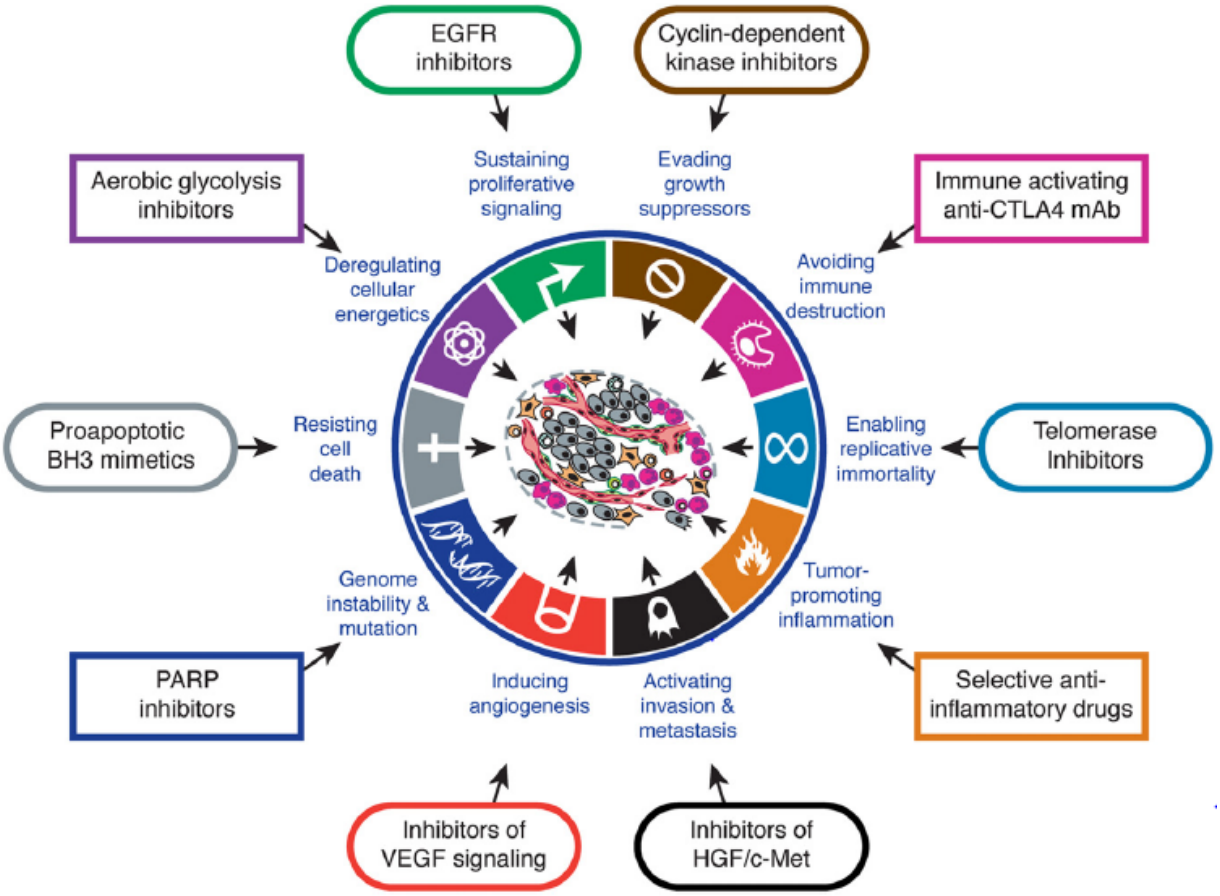


Figure 1-4. Ten hallmarks of cancer and some therapeutics targeting them. Adapted from (Hanahan and Weinberg, 2011)

1.1.3.1 Metastasis

Metastases are tumors that develop at a distance from their primary origin and are responsible for the death of 90% of cancer patients (Sporn, 1996). For over a century the notion of seed and soil, where cancer cells being the "seeds" and the specific organ microenvironments being the "soil", and the interaction between the "seeds" and the "soil" determines the formation of a secondary tumor, provided an accurate account of the metastatic preference of cancer cells for specific organs (Langley and Fidler, 2011).

Metastasis consists of sequential interrelated steps, all of which must be successfully completed to give rise to a secondary tumor. These include (A) shedding of cancer cells from the primary tumor and local invasion of detached cells, (B) intravasation into the blood or lymphatic circulation, (C) aggregation with leukocytes/platelets/tumor cells and survival in the circulation, (D) arrest in the capillary and extravasation, and (E) proliferation in the new tissue and formation of the secondary neoplasm (*Figure 1-5*). Endothelial cell adhesion molecules determine the step (D) arrest in the capillary and extravasation (Gout et al., 2008) (see 1.1.4 Endothelial cell adhesion molecules and cancer metastasis).

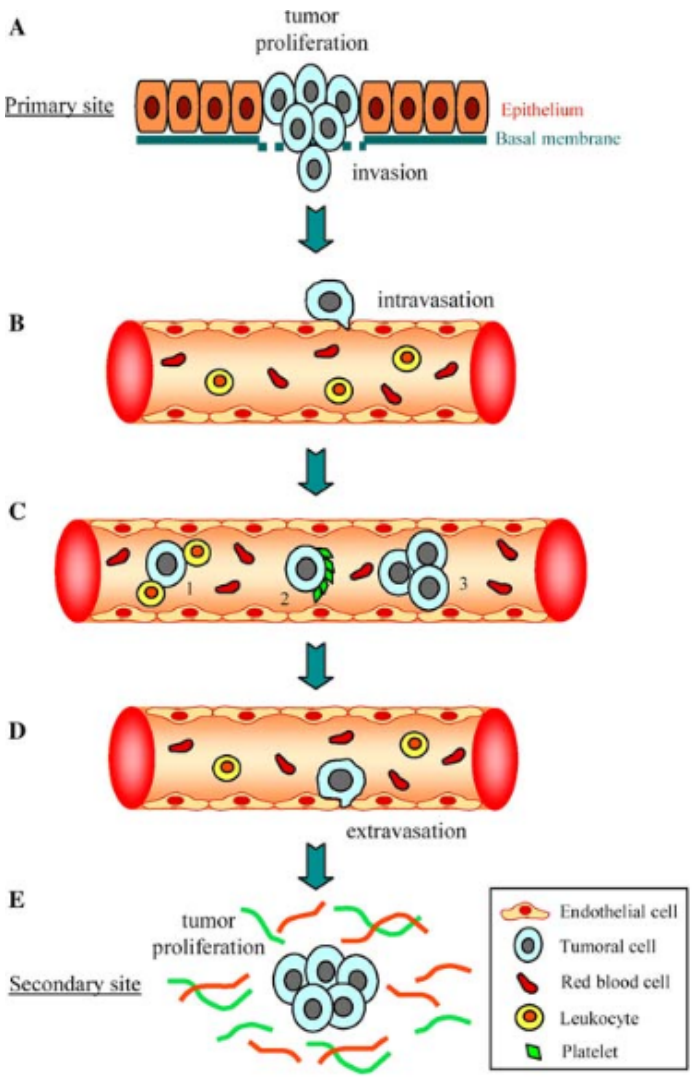


Figure 1-5. Metastasis formation. *(A) shedding of cancer cells from the primary tumor and local invasion of detached cells, (B) intravasation into the blood circulation, (C) aggregation with leukocytes/platelets/tumor cells and survival in the circulation, (D) arrest in the capillary and extravasation, and (E) proliferation in the new tissue and formation of the secondary neoplasm. Adapted from (Gout et al., 2008).*

1.1.3.2 Inflammation and cancer

Some tumors are densely infiltrated by cells of the immune system, resembling inflammatory conditions in non-neoplastic tissues. Dvorak thereby called tumors “wounds that do not heal” (Dvorak, 1986). Virtually all neoplastic lesions contain some immune cells (Pagès et al., 2010). Such phenomena were thought to reflect an attempt by the immune system to eradicate tumors. However, the tumor-associated inflammatory response was found to have an unanticipated, paradoxical effect of enhancing tumorigenesis and progression (Qian and Pollard, 2010). Inflammation is capable of fostering the development of incipient neoplasia into full-blown cancers (de Visser et al., 2006). Up to 20% of all human cancers result from chronic inflammation and persistent infections (Wang and Karin, 2015).

Proinflammatory cytokines and tumor-infiltrating immune cells play critical roles in almost every developmental stage of inflammation-induced cancers, from initiation, promotion, and progression to malignant metastases. Immune cells can release chemicals, notably reactive oxygen species, that are actively mutagenic for nearby cancer cells, accelerating their genetic evolution toward states of heightened malignancy (Grivennikov et al., 2010). Inflammation especially chronic inflammation triggers cellular events that can promote malignant transformation of cells and carcinogenesis. Several inflammatory mediators produced by immune cells, such as TNF-α, IL-6, TGF-β, and IL-10, have been shown to participate in both the initiation and progression of cancer, partially by mediating the generation of reactive oxygen species and nitrogen species, by their direct mutagenic effect, and by regulating epithelial mesenchymal transition (EMT), angiogenesis, and metastasis (Landskron et al., 2014).

Inflammation can contribute to multiple hallmark capabilities by supplying bioactive molecules to the tumor microenvironment, including growth factors that sustain proliferative signaling,

survival factors that limit cell death, proangiogenic factors, extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis, and inductive signals that lead to activation of EMT and other hallmark-facilitating programs (DeNardo et al., 2010; Karnoub and Weinberg, 2006).

Colon cancer is deeply attributable to chronic inflammatory disease (Landskron et al., 2014). In this sense, the remedy that can attenuate the extravasation and the infiltration of immune cells by targeting endothelial cell adhesion molecules might provide a path to reduce the metastases of colon cancer and cancerogenic inflammation at the same time.

1.1.3.3 Epigenetics and cancer

While each cell in the body is packed with the same genetic information, epigenetic instructions determine the access to the information by directing the way DNA is packaged into chromatin. Epigenetic patterns are transferred into subsequent generations of cells. The most remarkable epigenetic pattern is the inactivation of one X chromosome in human females, where one whole allele is silenced by epigenetic modifications (Egger et al., 2004).

In the eukaryotic nucleus, DNA is compacted into nucleosomes, in which histone octamer composed of H3, H4, H2A and H2B (two of each) is surrounded by 147 bases (Luger et al., 1997). This packaging of DNA represents a potential barrier to transcription. DNA methylation and Covalent histone modification (acetylation, methylation, phosphorylation, ubiquitination and substitutions) are two major players in epigenetic organization (Bostick et al., 2007). They jointly constitute the ‘Epigenetic code’ to modulate the expression of the mammalian genome in different cell types and in diverse diseases including cancer (Rijnkels et al., 2010).

1.1.3.3.1 DNA methylation

DNA methylation is a widespread modification in bacteria, plants and mammals, and this covalent molecular transformation is a natural modification of DNA, which takes place at position 5 of cytosine, and especially of cytosines preceding guanine (CpG) in human cells (Illingworth et al., 2008). DNA methylation produced during DNA replication is considered as a stable gene-silencing mechanism. DNA methylation is established and maintained by specific DNA methyltransferases (DNMT1, 3A and 3B) with S-adenosyl-methionine as methyl donor (Bostick et al., 2007). DNMT3A and 3B are required for initial DNA methylation, and DNMT1 maintains the methylation status (Bernstein et al., 2007). DNA methylation inhibits transcription either "passively" by blocking the access of transcription factors to their binding sites, or "actively" through recruiting methylated binding domain proteins that inhibits gene expression (Nan et al., 1998).

CpG-rich DNA fragments are called 'CpG islands', which are not methylated in dividing and differentiating tissues. However, in normal body cells, most of these CpG islands are methylated in a tissue-specific manner and certain subsets of methylated CpG islands at the promoter can lead to long-term silencing of transcription. In this sense, DNA methylation is formed during differentiation and can cause cells to partially or completely lose the ability to divide (Weber et al., 2007). CpG island-containing gene promoters are usually unmethylated in normal cells to maintain their euchromatic structure. Nevertheless, during cancer development, many of these CpG island-containing gene promoters are hypermethylated, changing open euchromatic structure to compact heterochromatic structure (Illingworth et al., 2008).

On the other hand, 5-methylcytosine is prone to spontaneous deamination and point mutation to thymine and represents potential oncogenic hazard (Pfeifer, 2006).

1.1.3.3.2 Histone modification

Histone octamer is the basic structure of nucleosome components (Luger et al., 1997). The N-terminals of histones protrude out of the nucleosome core, and amino acids bearing these N-terminals easily undergo covalent modifications, including both activation modifications (acetylation, phosphorylation, H3K4 methylation, etc.) and repressive modifications (H3K9 methylation, H3K27 methylation, etc.), to shape chromatin into open or tightly packed structures, respectively (Biswas and Rao, 2017; Sharma et al., 2010). Mechanistically, these modifications either directly alter nucleosome interactions with chromatin, or indirectly by recruiting auxiliary effector proteins, and the specificity of these epigenetic codes stems from the substrate specificity of the enzymes that catalyze the covalent modifications as well as the enzymes that remove them (Chen et al., 2014).

1.1.3.3.3 Epigenetics and microRNAs in cancer development

Since epigenetic mechanisms are required to maintain normal growth and differentiation, abnormal epigenetic regulation may alter gene expression and function which may lead to diseases such as cancer (Barber and Rastegar, 2010). Non-coding RNAs have an important role in the molecular mechanisms that sustain epigenetics, and alterations of non-coding RNAs, especially microRNAs (miRNAs), can cause abnormal epigenetic patterns at canonical promoter regions or distant regulatory elements, which may contribute to deregulate critical genes

involved in proliferation, programmed cell death, and cell differentiation (Bernstein and Allis, 2005; Murtha and Esteller, 2016). Compelling evidence demonstrated that miRNAs can also be deregulated in cancer by abnormal CpGs methylation and/or histone modifications (Suzuki et al., 2012). On the other hand, several miRNAs are not only regulated by epigenetic mechanisms, but themselves have an active role on the epigenetic machinery. These miRNAs, called "epi-miRNAs", are often deregulated in human cancer and target specific epigenetic regulators, such as components of the polycomb repressive complexes 1 and 2 (PRC1 and PRC2), DNA methyl-transferases (DNMTs) and histone deacetylases (HDACs) enzymes, and the Retinoblastoma-Like protein 2 (RBL2) (Ramassone et al., 2018). In this regard, the deregulation of miRNAs may be both the cause and consequence of a cancer-related epigenetic alteration, which is further discussed in section 1.2.4.

1.1.4 Endothelial cell adhesion molecules and cancer metastasis

Extravasation of circulating cancer cells is a multi-step process. Firstly, two endothelial cell adhesion molecules E-selectin and P-selectin initiate the selectin-mediated transient adhesion of cancer cells to endothelium, which is associated with the rolling of circulating cancer cells. Rolling cancer cells then become activated by locally released chemokines, which triggers the activation of integrins on cancer cells, allowing their firm adhesion to immunoglobulin cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), leading to the trans-endothelial migration (TEM) of circulating cancer cells (Walzog and Gaehtgens, 2000) (*Figure 1-6*).

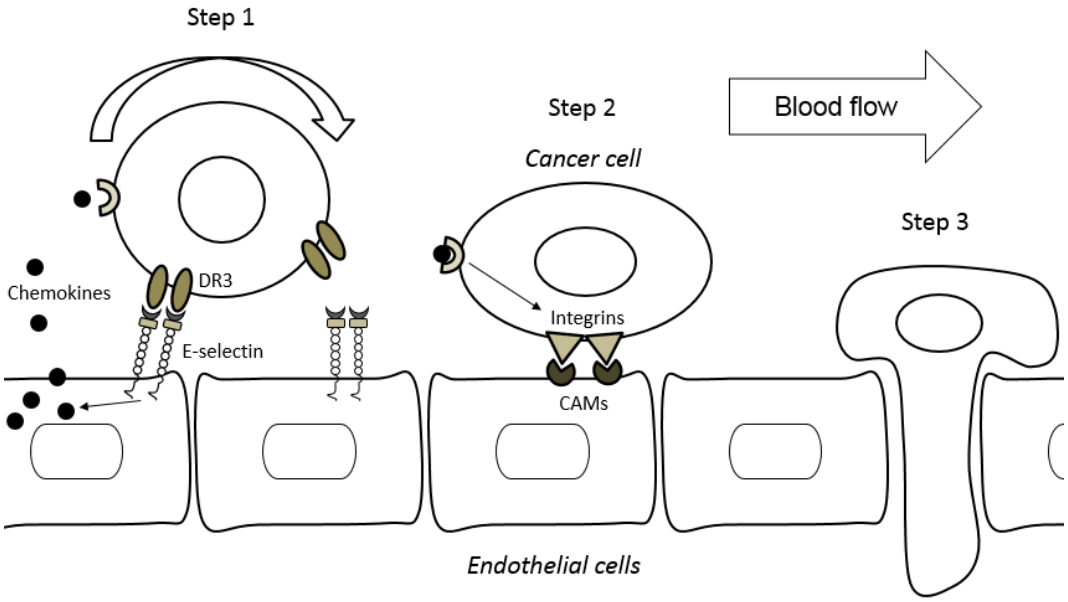


Figure 1-6. E-Selectin-Mediated Adhesion and Trans-endothelial Migration of Cancer Cells. *Extravasation of cancer cells is a multi-step process. The first step involves the transient adhesion of cancer cells to the endothelium. It requires endothelial E- and P-selectins, and their counter-receptors (such as DR3 and CD44 for E-selectin) on cancer cells. This step is associated with the rolling of cancer cells on the endothelium. The second step involves a firmer adhesion of cancer cells to the endothelium, which is mediated by cell adhesion molecules (CAMs) on the endothelium and integrins on cancer cells.*

The third step is the extravasation of cancer cells through endothelial cell-cell junctions. (Zhong et al., 2017)

1.1.4.1 E-selectin and cancer metastasis

1.1.4.1.1 Characteristics of E-selectin

Structure

E-selectin (64 kDa) is a transmembrane receptor of the selectin family that also contains L- and P-selectins. Two glycosylated forms of E-selectin are detected at 100 and 115 kDa. The extracellular part of selectin is constituted of three domains: an N-terminal C-type lectin domain, which is calcium-dependent and mediates ligand interaction; an epidermal growth factor (EGF) domain, which also regulates ligand interaction; and consensus complement regulatory protein (CRP) repeats of ~60 amino acids each, which serve as spacers to hold the other two domains away from cell surface, and mediate the rolling of adhering cells (see below). The number of CRP repeats distinguishes the extracellular domain of different selectins. E-selectin has six CRP repeats. Selectins are anchored in the membrane through a single helicoidal transmembrane domain followed by a short cytoplasmic tail (*Figure 1-7*). The cytoplasmic tail of E-selectin can trigger signaling in the endothelial cell and is connected to the actin cytoskeleton via actin-binding proteins, which are important mediators of extravasation.

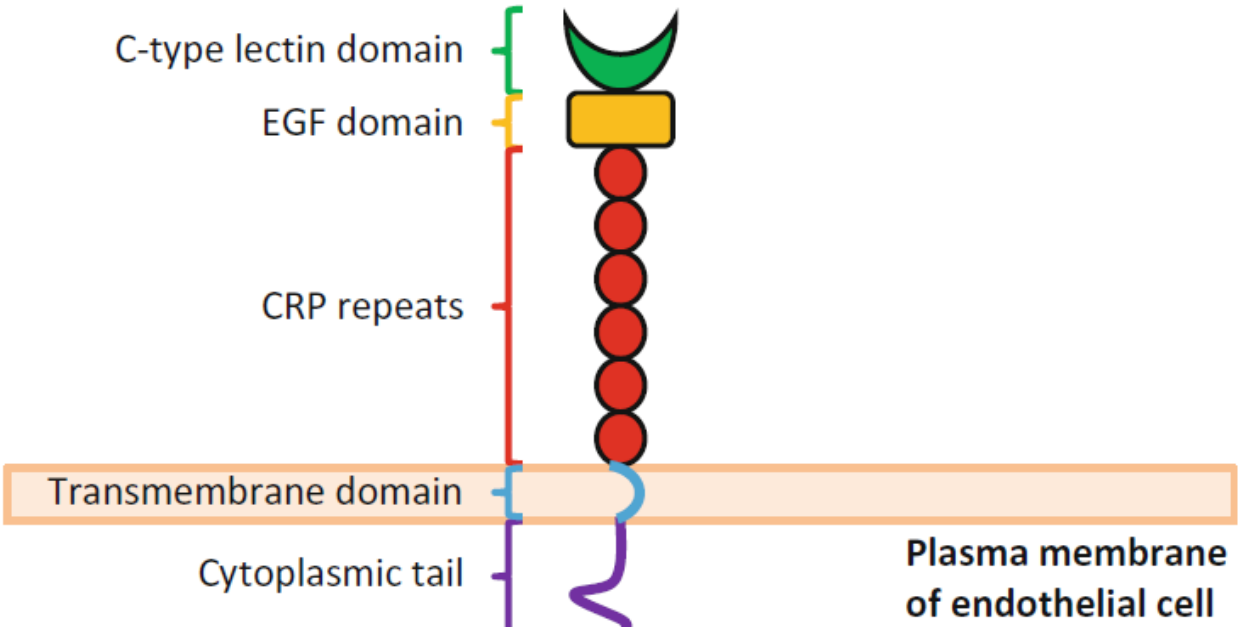


Figure 1-7. Structure of E-selectin. *The extracellular part of E-selectin is divided into three domains: an N-terminal C-type lectin domain, an epidermal growth factor (EGF) domain, and consensus complement regulatory protein (CRP) repeats. E-selectin is anchored in the membrane through a single helicoidal transmembrane domain, followed by a short cytoplasmic tail.*

Expression

E-selectin is expressed exclusively by endothelial cells. Its constitutive expression has been detected in the skin and parts of bone marrow microvasculature. However, in most vessels, the *de novo* synthesis of E-selectin is induced by pro-inflammatory molecules such as tumor necrosis factor α (TNFα), interleukin 1 (IL-1β), endothelial monocyte activating polypeptide II (EMAPII), and bacterial lipopolysaccharide (LPS). Following stimulation by TNFα, E-selectin relies on PI3K-Akt-NFκB and JNK-c-Jun pathways for its transcription. In physiological conditions such as inflammation, the expression of E-selectin is transient and often reaches its peak 2-6 hours after stimuli. E-selectin is gradually internalized by endocytosis by clathrin-coated pits, and degraded in the lysosomes. In the endothelium areas of chronic inflammation, E-selectin may remain upregulated. Several cancer cells have the ability to induce E-selectin. For

instance, Lewis lung carcinoma cells induce E-selectin in liver sinusoidal endothelium. Moreover, highly metastatic human colorectal and mouse lung carcinoma cells, upon their entry into the hepatic microcirculation, induce TNFα production by resident Kupffer cells, triggering E-selectin expression. Clinically, patients with various cancers including breast, colorectal, lung, bladder, head and neck and melanoma have elevated galectin-3 in their serum. In turn, galectin-3 induces secretion of pro-inflammatory cytokines by blood vascular endothelium, which triggers the expression of E-selectin. A TNFα-inducible microRNA, miR-31, directly targets the mRNA of E-selectin and downregulates its expression, suggesting its involvement in carcinoma dissemination (Suárez et al., 2010).

Function

E-selectin recognizes the sialyl Lewis-a/x tetrasaccharide borne by glycoproteins and glycolipids on the surface of leukocytes and tumor cells. Its glycoprotein ligands include: E-selectin ligand-1 (ESL-1), P-selectin glycoprotein ligand-1 (PSGL-1), β2 integrin, L-selectin, CD43/44, lysosomal-associated membrane protein-1/2 (LAMP-1/2), mucin-16 (MUC16), Mac-2, podocalyxin (PODXL), and death receptor-3 (DR3). Malignant transformation is often associated with abnormal glycosylation such as increased sialyl Lewis-a/x structures. On carcinoma cells, sialyl Lewis-a/x are mostly carried by mucins, making them major E-selectin ligands on carcinoma cells. The physiological role of E-selectin is to mediate the adhesion of leukocytes to the endothelium. In pathological conditions, cancer cells “hijack” the inflammatory system to interact with E-selectin. On the surface of endothelial cell, E-selectin molecules cluster in clathrin-coated pits and lipid rafts. This distribution pattern of E-selectin enhances its ability to mediate rolling in flow conditions (Kannagi et al., 2004).

The sequence of events is as follows: the C-type lectin domain of E-selectin binds its ligand on cancer cells. This primary adhesion is unstable under shear stress, which allows the rolling of cancer cells along the endothelium. In response to the E-selectin-mediated attachment, chemokines are produced and released by endothelial cells, which activate integrins on cancer cells. Integrins are capable of firmly binding to cell adhesion molecules (ICAM-1/2 and VCAM-1) on endothelial cells, allowing the extravasation of cancer cells into tissues (*Figure 1-5*). Breast, bladder, gastric, and pancreatic carcinoma, leukemia and lymphoma form metastasis in an E-selectin-dependent manner in organs as various as liver, bone marrow, skin, and lung.

The interaction between E-selectin and its ligand triggers signals in both endothelial cells bearing E-selectin, and cancer cells bearing the ligand. When E-selectin binds to DR3 on colon carcinoma cells, on one hand, this interaction activates not only the pro-survival ERK MAP kinase and PI3K pathways, but also the pro-migratory p38 MAP kinase pathway in colon carcinoma cells; on the other hand, in endothelial cells, the interaction activates p38 and ERK MAP kinase pathways to increase the permeability of the endothelium (*Figure 1-7*) (Gout et al., 2006; Tremblay et al., 2006). Similar mechanism has also been observed with ESL-1, where E-selectin binds to ESL-1 on the circulating prostate cancer cell and activates the pro-metastatic RAS-ERK-cFos signal cascade in the cancer cell. Moreover, CD44 on melanoma cells can bind to E-selectin on endothelial cells and activate PKCα-p38-SP-1 pathway to up-regulate ICAM-1 on endothelial cells. This bidirectional signal transduction is also revealed for the tethering of leukocytes: E-selectin binding to PSGL-1 on neutrophils activates β2 integrin through the Syk-Src pathway. At the same time, E-selectin transduces signals into endothelial cells through p38 and p42/p44 MAP kinase pathways. Overall, E-selectin-mediated adhesion of cancer cells increases their metastatic potential by inducing bidirectional signaling that enhances their

intrinsic motile and survival abilities, as well as the permeability of the endothelium.

E-selectin is a double-edged sword in cancer therapy, as it also allows lymphocyte infiltration into tumor. Some cancer cells are able to reduce E-selectin to evade immune detection: squamous cell carcinomas can recruit nitric oxide (NO)-producing myeloid-derived suppressor cells, and this local production of NO inhibits vascular E-selectin expression, preventing T cells from entering in squamous cell carcinomas. In this case, lower E-selectin is correlated with lower survival. On the other hand, many types of cancer cells benefit from E-selectin in a variety of ways. The recruitment of leukemia cells by E-selectin sequesters leukemia cells in a quiescent state, rendering them immune to chemotherapy. Given that leukemia cells can stimulate endothelial cells by themselves, they promote their own survival through E-selectin. In addition, proliferating hemangioma endothelial cells from infantile hemangioma constitutively express E-selectin, which enhances hemangioma stem cell adhesion and vasculogenesis.

A new role in stem cell proliferation has been identified recently for E-selectin on bone marrow vascular endothelial cells: it recruits hematopoietic stem cells that express appropriate ligands, and this attachment wakes hematopoietic stem cells up by inducing their proliferation, self-renewal, chemo- and radio-sensitivity.

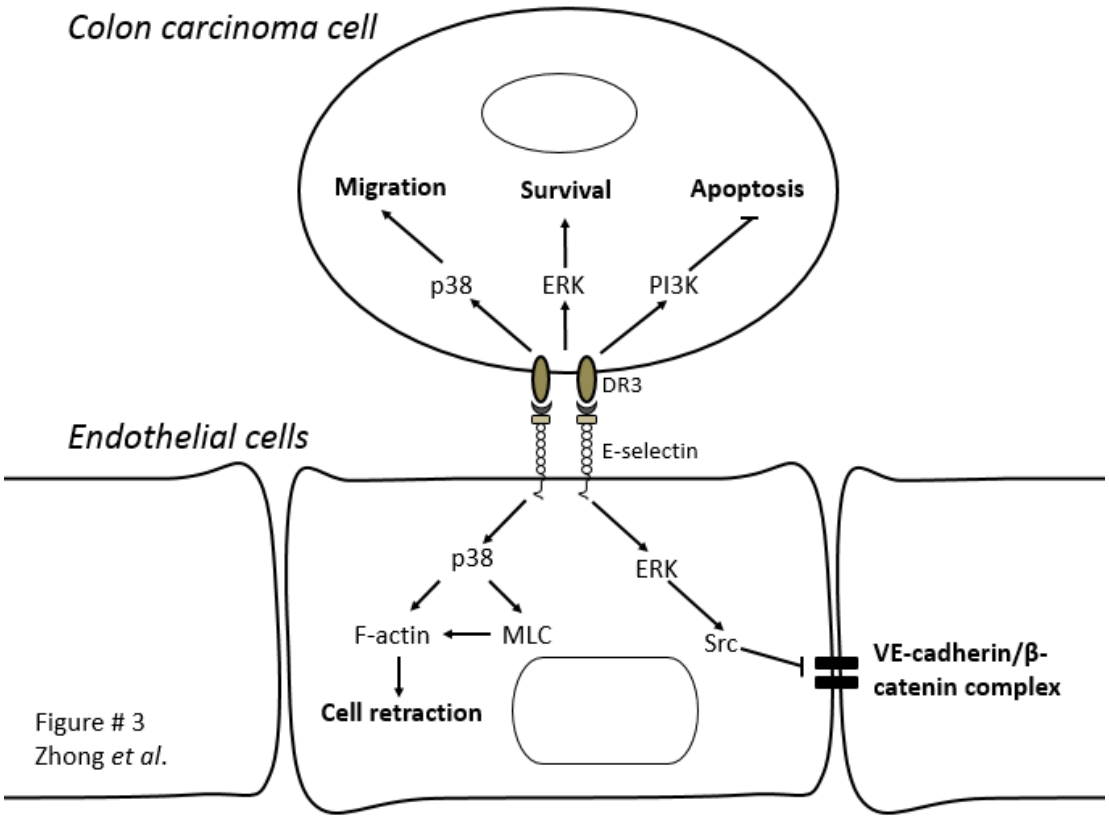


Figure 1-8. E-Selectin-Mediated Bidirectional Signaling in colon carcinoma cells and endothelial cells*. The adhesion of colon carcinoma cells to endothelial cells involves the binding of E-selectin on endothelial cells to death receptor-3 (DR3) on colon carcinoma cells. This interaction induces activation of PI3K, p38 and ERK MAP kinases in cancer cells, which increase their motile and survival potentials. Reciprocally, the interaction triggers the activation of p38 and ERK MAP kinases in endothelial cells, which results in myosin-light chain (MLC)-mediated cell retraction, and dissociation of the VE-cadherin-β-catenin complex, and thereby destruction of adherent junctions leading to increased endothelial permeability and extravasation of cancer cells.*

1.1.6.1.2 Clinical Relevance of E-selectin in Cancer

The finding that cancer cells are recruited by E-selectin-expressing endothelial cells is of significant clinical importance and opens several therapeutic avenues.

Targeting E-selectin and its ligands

Various strategies targeting E-selectin and its ligands are promising to suppress E-selectin-mediated cancer cell adhesion. For example, antibodies against E-selectin can impair lung metastasis of colon carcinoma in mice. E-selectin-targeting aptamer (ESTA) is able to reduce metastasis of breast cancer in mice. ESTA is safe as an antagonist as it can be applied at high doses without causing overt side effects. It is of particular interest for the prevention of metastasis of ER(-)/CD44(+) breast cancers. Similarly, SDA, a DNA aptamer antagonizing E- and P-selectins also exhibits anti-adhesive effect for colorectal cancer and leukemia *in vitro*. In mice, encouraging results have been obtained with colon cancer metastasis by using cimetidine to inhibit E-selectin expression. In clinical trials, cimetidine treatment dramatically improves the 10-year cumulative survival of colorectal cancer patients. Moreover, atrial natriuretic peptide (ANP) is capable of reducing E-selectin expression and preventing recurrence in patients with non-small cell lung cancer. E-selectin is also a target for inhibition of angiogenesis. Knocking down vascular E-selectin in mice inhibited the recruitment of endothelial progenitor cells to the tumor, thus reduced angiogenesis and tumor growth in human melanoma xenograft murine model (Läubli and Borsig, 2010). Another approach for reducing E-selectin-mediated adhesion is to target ligands of E-selectin. Antibodies against sialyl Lewis-a/x determinants inhibited the formation of metastasis by human pancreatic and gastric cancers in nude mice. The antisense-cDNA for fucosyltransferase genes (FUT III/VI), enzymes producing sialyl Lewis saccharides, suppressed metastatic colonization by colon cancer cells in mice. Along the same lines, celecoxib, an inhibitor of cyclooxygenase-2, impaired the expression of sialyl Lewis-a on colon cancer cells and reduced metastasis. Still in mice, re-introduction of the glycosyltransferase

B4GALNT2, which synthesizes “normal” saccharides instead of sialyl Lewis-a/x, prevented dissemination of gastric carcinoma (Bird et al., 2006; Khatib et al., 2005).

Soluble E-selectin as a diagnostic marker

A soluble form of E-selectin (sE-selectin) is generated by enzymatic cleavage or when activated endothelial cells shed their damaged parts. The concentration of sE-selectin is directly correlated with its cell surface expression. sE-selectin limits E-selectin-mediated rolling by competing for binding sites on the leukocyte, thus downregulates the inflammatory response. sE-selectin can be used as a marker of activation of endothelium and is therefore useful for diagnosis of acute inflammation and metastasis. Specifically, for breast cancer patients, high sE-selectin level is associated with liver metastasis. In patients with non-small cell lung cancer, high sE-selectin level correlates with poor prognosis when cancer cells express sialyl Lewis-a/x. Increased sE-selectin also characterizes patients with chronic lymphatic leukemia. For patients suffering from oral cavity cancer, higher level of E-selectin correlates with higher risk of cancer transformation and relapse. Hence, the determination of blood sE-selectin on tumor biopsies is of prognostic value (Gout et al., 2008).

E-selectin-mediated capture

Highly metastatic circulating cancer cells express mucins with increased sialyl Lewis-a/x (such as MUC1), and these mucins consistently expose their core epitope. Nanostructured surface coated with E-selectin offers a method to selectively capture viable cancer cells from blood samples. This E-selectin-mediated capture allows fast analysis and elimination of circulating cancer cells. For instance, microtube surface with E-selectin-functionalized liposomal

doxorubicin specifically captured breast adenocarcinoma MCF7 cells from the perfusion, and induced significant cancer cell death.

E-selectin as a receptor for targeted delivery

E-selectin can serve as a receptor for the delivery of anti-inflammatory drugs, anti-cancer drugs, and imaging markers in endothelial cells. For this purpose, antibodies against E-selectin, or artificial ligands of E-selectin are conjugated to the surface of polymeric particles. These immunoparticles are used to encapsulate the agent, so they can selectively bind to E-selectin-expressing endothelial cells and get internalized, together with the agent inside them. This technique allows the specific delivery of drugs to the pro-inflammatory microenvironment harboring tumor cells. Naturally, immunoparticles targeting E-selectin can also directly compete with cancer cells to bind to E-selectin. Based on these principles, intravenous injections of two E-selectin-targeting drug-carrying immunoparticles: P-(Esbp)-DOX and P-(Esbp)-KLAK, inhibited primary tumor growth and metastasis of lung carcinoma in mice. Moreover, the "drug free" immunoparticle P-(Esbp) also exhibited anti-metastatic effects by competing with circulating lung carcinoma cells. By targeting E-selectin, we can also carry out targeted gene therapy, if viral vectors are encapsulated. ESTA-conjugated multistage vector (ESTA-MSV) can carry therapeutic anti-STAT3 siRNA to bone marrow vascular endothelium of mice, and infect breast cancer cells there. *In vitro*, anti-E-selectin lipoplexes can deliver anti-VE-cadherin siRNAs to inflamed primary vascular endothelial cells originating from different vascular beds, which are generally difficult to transfect (Jubeli et al., 2012).

Overall, E-selectin-mediated endothelial adhesion plays a key role in metastasis, which opens new avenues for therapeutic interventions aiming at inhibiting the fatal complication of cancer.

1.1.4.2 P-selectin and cancer metastasis

P-selectin as E-selectin, belongs to a family of calcium-dependent, type I transmembrane, carbohydrate-binding glycoproteins (Feng et al., 2017). In resting endothelial cells, P-selectin is localized in Weibel–Palade bodies and is only translocated to cell surface following pro-inflammatory stimulation. P-selectin can then bind to its ligand glycoprotein ligand-1 (PSGL-1) on the surface of leukocytes and circulating cancer cells. Both E-selectin and P-selectin recruit circulating cancer cells to endothelium to initiate their rolling process. However, given that the majority of cancer cells of epithelial origin lacks PSGL-1, P-selectin is less required for cancer cell adhesion and less studied in the metastatic context compared to E-selectin (Kannagi et al., 2004). Increased expression of P-selectin has been reported in invasive breast cancer (Fox et al., 1995; Gorelik et al., 2001) and gastric cancer (Mayer et al., 1998). In addition, its expression on intra-tumoral vessels is correlated with poor survival in melanoma patients (Schadendorf et al., 1995), and with leukocyte infiltration in gastric cancer (Mayer et al., 1998). In P-selectin deficient mice, colon cancer growth and lung metastasis were significantly decreased (Kim et al., 1998). Along these lines, blocking endothelial P-selectin binding to heparin decreased lung metastasis *in vivo* (Ludwig et al., 2006).

However, decreased endothelial P-selectin is associated to the progression and metastasis of melanoma and colon cancer (Nooijen et al., 1998; Peeters et al., 2005). These observations can be explained by decreased infiltrating leukocytes. In other words, down-regulation of endothelial P-selectin allows tumors to evade inflammatory system (Peeters et al., 2005). Since tumor malignancy is positively correlated with the level of soluble P-selectin (sP-selectin) (bladder cancer (Coskun et al., 2006), breast cancer (Blann et al., 2001), haematological cancer (Blann et al., 2001), lung cancer (Roselli et al., 2002), colorectal cancer (Ferroni et al., 2004) and

lymphoma (Syrigos et al., 2004), the reduction of endothelial P-selectin may be caused by increased shedding of P-selectin into circulation.

In vitro, PSGL-1 negative breast cancer cells can still adhere to and roll on P-selectin-coated surface, and further analysis identified CD24 as a new ligand for P-selectin (Aigner et al., 1998). CD24 not only mediates P-selectin-dependent rolling *in vivo* (Friederichs et al., 2000), but also regulate the adhesion of colon cancer cells to platelets in vitro (McCarty et al., 2000), supporting its involvement in at least two aspects of cancer metastasis (Baumann et al., 2005).

1.1.4.3 ICAM-1/VCAM-1 and cancer metastasis

Selectins mediates the rolling of cancer cells adhering to them, while intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) modulate the firm adhesion of circulating cancer cells to endothelium. ICAM-1 and VCAM-1 are both glycoproteins belonging to the immunoglobulin superfamily that binds to integrin ligands on adhering cells. Like E-selectin, the roles of these CAMs in cancer metastasis have been scrutinized. Endothelial ICAM-1 is higher in cancer patients than in healthy subjects (Araki et al., 2001; Banks et al., 1993; Benekli et al., 1998; Sun et al., 1999; Taguchi et al., 1997). ICAM-1 and VCAM-1 are associated with metastasis, given that both CAMs are higher in breast cancer tissues and serum than in benign tumors (Regidor et al., 1998), and that ICAM-1 is increased in secondary tumors in nude mice (Sun et al., 1999).

As for soluble ICAM-1 (sICAM-1), it is higher in cancer patients and is negatively correlated with renal cell carcinoma patient survival. The latter correlation has not been observed for soluble VCAM-1 (sVCAM-1) or sE-selectin in patients with renal cell carcinoma. Nonetheless,

sVCAM-1 and sE-selectin are higher in patients suffering from lymph node metastasis of breast cancer (Byrne et al., 2000).

The adhesion of highly metastatic cancer cell lines depends on both E-selectin and VCAM-1 on endothelial cells (Moss et al., 2000). *In vitro,* ICAM-1 directly mediates the adhesion of small cell lung cancer cells to endothelial cells (Finzel et al., 2004). ICAM-1 also controls the endothelial adhesion and the TEM of circulating melanoma cells in an indirect manner through polymorphonuclear cells (PMNs), which bind to CAMs on endothelial cells and melanoma cells with integrins (Slattery and Dong, 2003).

ICAM-1 and VCAM-1 contribute to the molecular basis of the "seed and soil" hypothesis, a theory to explain the organ selectivity of cancer metastasis developed over 100 years ago. Liver is the preferred metastatic site for colon cancers. Gangopadhyay et al. have shown that colon cancer cells release carcinoembryonic antigen (CEA) to activate the production of cytokines including IL-1β, TNFα and IL-6 from Kupffer cells, which subsequently increase ICAM-1 on sinusoidal endothelial cells. Colon cancer cells are retained in liver sinusoid in an ICAM-1-dependent manner (Gangopadhyay et al., 1998; Auguste et al., 2007). On the other hand, organ-specific increase of VCAM-1 (in lung, brain, heart and liver) is correlated with organ-specific metastasis of melanoma cells (Auguste et al., 2007; Harris et al., 2008).

1.2 microRNA

Among the regulators of gene expression, the evolutionarily conserved small non-coding RNA molecules called microRNAs (miRNAs) have recently emerged as key mediators of the process. To generate their functional single-stranded ~22 nucleotides long form, they are firstly

transcribed as long primary miRNAs (pri-miRNAs) by RNA polymerase II. Pri-miRNAs are then processed by Drosha-DGCR8 complex in the nucleus to produce precursor miRNAs (pre-miRNAs), which are exported to the cytoplasm by exportin-5 (XPO5) to be cleaved by Dicer, producing miRNAs that are loaded into miRNA-induced silencing complex (miRISC). Through base pairing with the 3' untranslated region (3' UTR) of mRNA, miRNA guides the miRISC to its target, thereby repressing translation with or without causing mRNA degradation (*Figure 1-9*) (Huntzinger and Izaurralde, 2011).

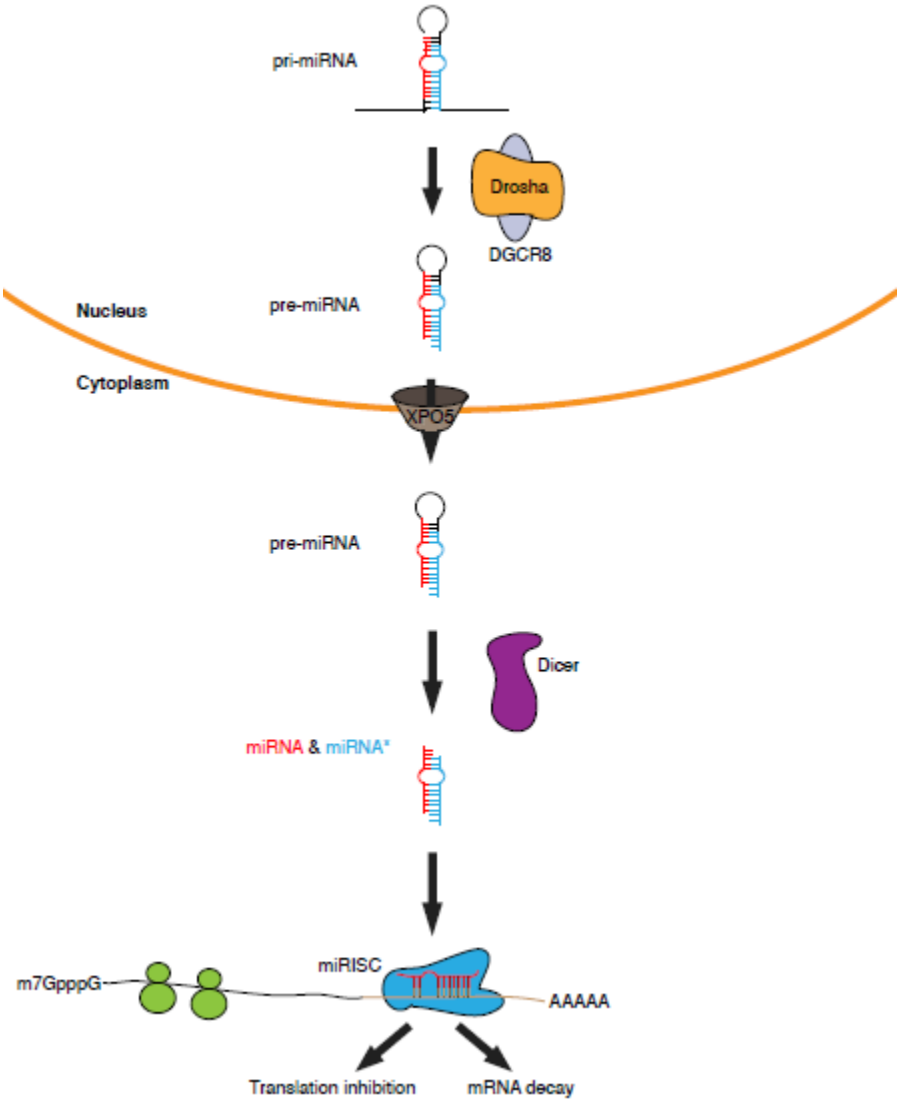


Figure 1-9. Biogenesis of microRNAs. *miRNAs are firstly transcribed as pri-miRNAs, which are processed by Drosha-DGCR8 complex to produce pre-miRNAs. Pre-miRNAs are exported to the cytoplasm by XPO5 to be cleaved by Dicer, producing miRNAs that are loaded into miRISC. Adapted from (Zhong et al., 2017: see Section 1.2.3)*

1.2.1 Regulation of miRNA biogenesis

The biogenesis of miRNAs is under tight control at multiple levels, including transcription, Drosha processing (microprocessing), Dicer processing, Argonaute (Ago) loading and miRNA turnover, as well as RNA modifications comprising editing, methylation and 3' end uridylation/adenylation (tailing). Non-canonical pathways for miRNA biogenesis, especially those that are independent of processing steps, are also emerging (reviewed in Ha and Kim, 2014).

1.2.1.1 Regulation of pri-miRNA transcription

miRNAs are transcribed from various genomic contexts. In humans, most miRNAs are encoded by introns of non-coding or coding transcripts, but some miRNAs are encoded by exonic regions. Several miRNA loci often locate close to each other, and are co-transcribed, constituting a miRNA cluster (Lee et al., 2002). Some miRNA genes reside in the introns of protein-coding genes and thus share the promoter of the host genes. However, most miRNA promoters have not been mapped yet. In this case, they can be inferred from chromatin immunoprecipitation followed by sequencing data (ChIP–seq) (Ozsolak et al., 2008).

Since RNA Pol II is responsible for transcribing miRNAs, RNA Pol II−associated transcription factors and epigenetic regulators control the process (Cai et al., 2004). Transcription factors p53, MYC and myoblast determination protein 1 (MYOD1) transactivate the miR−34, miR−17 and miR−1 clusters, while MYC and ZEB1/2 transcriptionally suppresses the miR−15a and miR−200 clusters, respectively (Kim et al., 2009). On the other hand, epigenetic control such as DNA methylation and histone modification also contribute to miRNA gene regulation. For such

instance, enhancer of zeste homolog 2 (EZH2) suppresses miR-31 expression by catalyzing histone H3 methylation on lysine 27 (Zhang et al., 2014b); whilst DNA methylation down-regulates miR-29b, a tumor suppressor miRNA to promote the progression of gastric cancer (Li et al., 2017a). Super-enhancers (SEs) are a new class of regulatory regions that consist of multiple enhancer-like elements occupied by high densities of transcription factors and mediator complexes and bearing active chromatin marks such as H3K27Ac (histone H3 lysine 27 acetylation), and they control cell identity and disease genes (Whyte et al., 2013). SEs link the transcription of multiple pri-miRNAs to their subsequent microprocessing by recruiting microprocessor. SEs together with H3K4me3 (histone H3 lysine 4 trimethylation) domains control the tissue-specific miRNA expression and is vital for cell identity (Duan et al., 2016; Suzuki et al., 2017).

1.2.1.2 Regulation of microprocessing

Following transcription, the pri-miRNA undergoes microprocessing by Drosha-DGCR8 complex in the nucleus (Figure 1-8). The RNase III Drosha crops the over 1 kb pri-miRNA into small a hairpin-shaped, ~65 nucleotide-long pre-miRNA. This cleavage defines the terminus and specificity of miRNA (Lee et al., 2003), and is mediated by multiple factors.

Some sequence motifs of pri-miRNA are involved in microprocessing, often through recruiting specific RNA binding proteins (RBPs) (Auyeung et al., 2013). The UG motif and the CNNC motif in the basal region, and the UGUG motif in the terminal loop of pri-miRNAs is commonly found in human miRNAs. Among them, the CNNC motif has been found to recruit the splicing factor SRp20, and DEAD-box RNA helicase p72 (DDX17), to increase the microprocessing (Mori et al., 2014). Similar additional auxiliary factors may contribute to microprocessing. G-

Quadruplexes (G4) are extremely stable DNA or RNA secondary structures formed by sequences rich in guanine. Between 9% and 50% of all pri-miRNAs contain at least one putative G4. Rouleau et al. reported that G4 locating near the Drosha cleavage site in three distinct pri-miRNAs (pri-miR-200c, pri-miR-451a, and pri-miR-497) influences their microprocessing either positively or negatively. G4-mediated microprocessing might have an important role to play in miRNA homeostasis (Rouleau et al., 2017). Alterations in RNA sequence and/or structure affect the microprocessing. Single nucleotide polymorphisms (SNPs) such as the C to T SNP in the first C of the CNNC motif in pri-miR-15a~16-1 reduces its microprocessing (Auyeung et al., 2013). RNA editing (the conversion of adenosine to inosine) also influences microprocessing. A certain adenosine deaminase (ADAR) edits the stem region of pri-miR-142, making it a poor substrate for Drosha (Yang et al., 2006) (Figure 1-10). The terminal loop of pri-miRNA is enriched with cis-elements that is subject to binding of regulatory protein factors including heterogeneous nuclear ribonucleoprotein A1 (HNRNPA1) and KH-type splicing regulatory protein (KSRP), which bind to the terminal loop of pri-mir-18a and pri-let-7, respectively, to facilitate microprocessing (Guil and Cáceres, 2007; Michlewski et al., 2008; Trabucchi et al., 2009). LIN28A and LIN28B bind to the same region of pri-let-7 to suppress microprocessing (Loughlin et al., 2011; Nam et al., 2011; Viswanathan et al., 2008). RBP heterodimer NONO-PSF binds a large number of pri-miRNAs to globally enhance microprocessing. NONO and PSF are brought together by and scaffolded around the long noncoding RNA (lncRNA) NEAT1, which orchestrates the efficient processing of potentially all miRNAs (Jiang et al., 2017).

Multiple mechanisms exist to control the level, activity and specificity of microprocessor. Notably DGCR8 stabilizes Drosha through protein–protein interactions (Han et al., 2009; Yeom et al., 2006). On the other hand, Drosha destabilizes DGCR8 mRNA by cleaving it at a hairpin in

the second exon (Chong et al., 2010; Han et al., 2009; Kadener et al., 2009). This cross-regulatory loop enables the homeostatic maintenance of microprocessor activity. The stability (Herbert et al., 2013; Tang et al., 2013), localization and activity of Drosha are tightly mediated by post-translational modifications. Glycogen synthase kinase 3β (GSK3β) phosphorylates Drosha to maintain its nuclear localization (Tang et al., 2010, 2011); while the p38 MAP kinase-mediated phosphorylation has the opposite effects (Yang et al., 2015). ERK MAP kinase phosphorylates and stabilizes DGCR8 (Herbert et al., 2013). The relationship between miRNA biogenesis and MAP kinases (p38, ERK and JNK) will be further discussed below. The acetylation of Drosha inhibits its degradation (Tang et al., 2013), and the deacetylation of DGCR8 by histone deacetylase 1 (HDAC1) increases its affinity to pri-miRNAs (Wada et al., 2012). SUMOylation at K707 of DGCR8 prevents its degradation via the ubiquitin proteasome pathway and influences its affinity with pri-miRNAs. This SUMOylation of DGCR8 is mediated by SUMO (small ubiquitin-like modifier) and can be promoted by its ERK-activated phosphorylation (Zhu et al., 2015), and a protein factor called p14ARF (Zhu et al., 2016). The previously mentioned KSRP is a component of microprocessor complex. It is SUMOylated by SUMO1 at K87. This modification inhibits KSRP interaction with the pri-miRNA/Drosha-DGCR8 complex, possibly by exporting KSRP into cytoplasm, to hinder microprocessing. miRNAs harboring short G-rich stretches in their terminal loops (TL-G), such as the let-7 family, are the most affected miRNAs by KSRP SUMOylation (Yuan et al., 2017). Drosha can be targeted by the final product of miRNA biogenesis in a feedback loop. For such instance, in lung cancer tissues, up-regulated miR-128-3p mediates the depletion of Drosha and promotes lung cancer cell migration, and might contribute to general miRNAs down-regulation in malignant

transformation. miR-128-3p also has the potential to target Dicer, though the functional impact of this aspect is not clear yet (Frixa et al., 2017).

Several Drosha/DGCR8 cofactors contribute to additional layers of regulation of microprocessing. Receptor-activated SMAD proteins (R-SMADs) SMAD1-3 and SMAD5, and p53 promote microprocessing through their interaction with p68 (Davis et al., 2008, 2010; Suzuki et al., 2009). TAR DNA-binding protein 43 (TDP43) interacts directly and stabilizes Drosha (Di Carlo et al., 2013; Kawahara and Mieda-Sato, 2012). Heme binding to DGCR8 is necessary for the activation of the latter and the precise recognition of pri-miRNA by specifically binding to the terminal loop near the 3' single-stranded segment. In this manner Heme enables the proper positioning of Drosha and DGCR8 on pri-miRNAs and is thus critical for the efficiency and fidelity of microprocessing (Partin et al., 2017). The Lupus Autoantigen (La) is an RNA-binding protein that stabilizes RNA polymerase III (Pol III) transcripts and supports RNA folding. La associates with DGCR8 in an RNA-dependent manner to promote microprocessing. La could be an important microprocessor component that regulates formation of the DGCR8-Drosha complex (Zheng et al., 2017). La also gates the loading of small RNAs into RISCs (see below). Mammalian apurinic/apyrimidinic endonuclease 1 (APE1) associates with the microprocessor during genotoxic stress and promotes the processing and stability of pri-miR-221/222, which targets the tumor suppressor PTEN and controls oncogenesis (Antoniali et al., 2017).

Pri-miRNA has been reported to be targeted by miRNA in the nucleus. For such example, nuclear miR-122 suppresses miR-21 maturation via binding to a 19-nt UG-containing recognition element in the basal region of pri-miR-21 and preventing the Drosha-DGCR8

microprocessor's conversion of pri-miR-21 into pre-miR-21. By doing so nuclear miR-122 interferes with the miR-21-targeted programmed cell death 4 (PDCD4) axis and promotes hepatocellular carcinoma cell apoptosis (Wang et al., 2017).

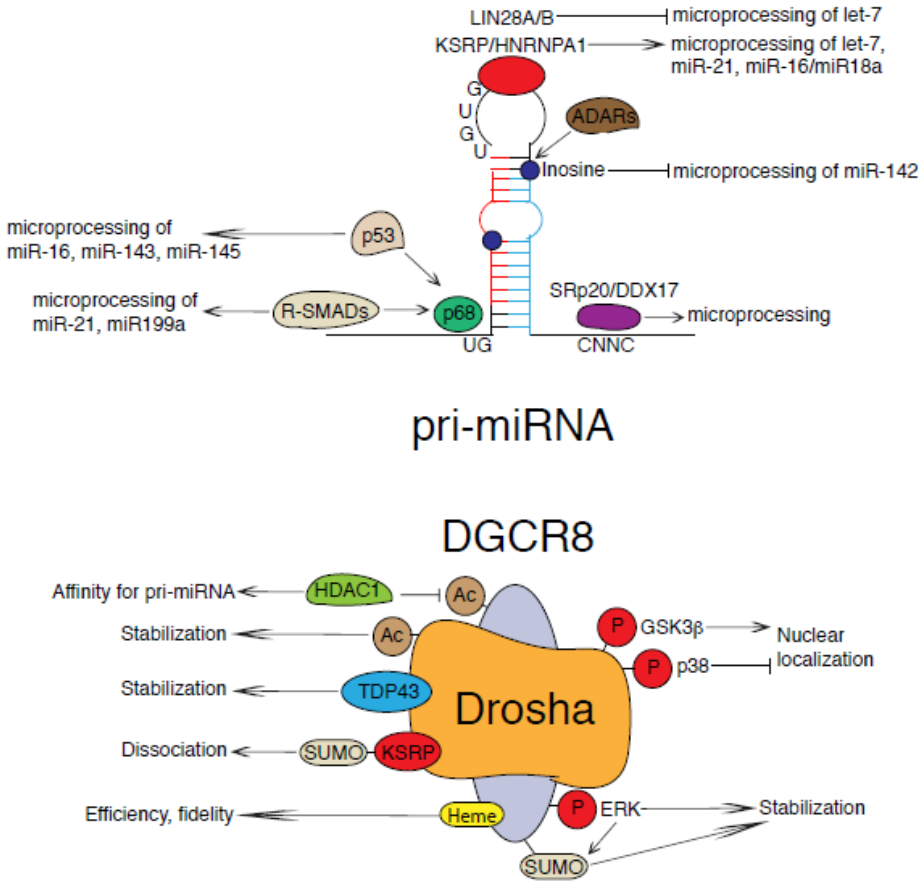


Figure 1-10. Regulation of microprocessing. Motifs in pri-miRNAs mediate microprocessing. *The UG, CNNC and UGUG motifs are commonly found in pri-miRNAs. CNNC motif recruits SRp20 and DDX17 to increase microprocessing. ADARs make pri-miR-142 a poor substrate for Drosha. The terminal loop of pri-miRNA binds HNRNPA1 and KSRP to enhance microprocessing. LIN28A/B bind to the same region of pri-let-7 to suppress microprocessing. Microprocessor is modulated by multiple mechanisms. GSK3β phosphorylates Drosha to maintain its nuclear localization, while the p38 phosphorylates Drosha to export it into cytoplasm. ERK phosphorylates and stabilizes DGCR8. The acetylation of Drosha inhibits its degradation, and the deacetylation of DGCR8 by HDAC1 increases its affinity to pri-miRNAs. SUMOylation of DGCR8 prevents its degradation and influences its affinity with pri-miRNAs.*

This SUMOylation can be promoted by its ERK-activated phosphorylation. SUMOylation of KSRP inhibits its interaction with microprocessor. Microprocessor cofactors mediate microprocessing. R-SMADs promote microprocessing through their interaction with p68. TDP43 interacts and stabilizes Drosha. Heme binds and activates to DGCR8.

1.2.1.3 Regulation of export to cytoplasm

Pre-miRNA from microprocessing is exported into the cytoplasm by XPO5-Ran•GTP (exportin 5-GTP-binding nuclear protein RAN•GTP) complex (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003).

The pro-survival PI3K-AKT pathway plays vital roles in XPO5 function. During cell cycle entry, XPO5 is post-transcriptionally induced in a PI3K-dependent manner (Iwasaki et al., 2013). Upon DNA damage, ATM-AKT axis phosphorylates the nucleopore protein NUP153, leading to its increased interaction with XPO5 and pre-miRNA export (Wan et al., 2013) (*Figure 1-11*). During melanoma development, XPO5 is induced through MEK-ERK signaling pathway and is responsible for the global up-regulation of miRNAs in melanoma cells, and increased XPO5 supports the malignant behavior of the cancer cells (Ott et al., 2016). The C-terminal-truncated XPO5 loses its function and might be the explanation for globally reduced miRNA in some tumors (Melo et al., 2010).

Exportin 1 (XPO1) has recently been reported to export a group of miRNAs during cellular quiescence in primary human fibroblasts. Since XPO5 is reduced during quiescence, the biogenesis of these quiescence-induced miRNAs is independent of XPO5 and depends instead on XPO1. Moreover, these pri-miRNAs are modified with a 2,2,7-trimethylguanosine (TMG)-cap by trimethylguanosine synthase 1 (TGS1), to be able to bind XPO1. At least one of these pri-miRNAs, pri-miR-34a is bound by an uncharacterized short form of cytoplasmic Drosha

(sDrosha), implying the existence of an alternative microprocessing pathway (Martinez et al., 2017).

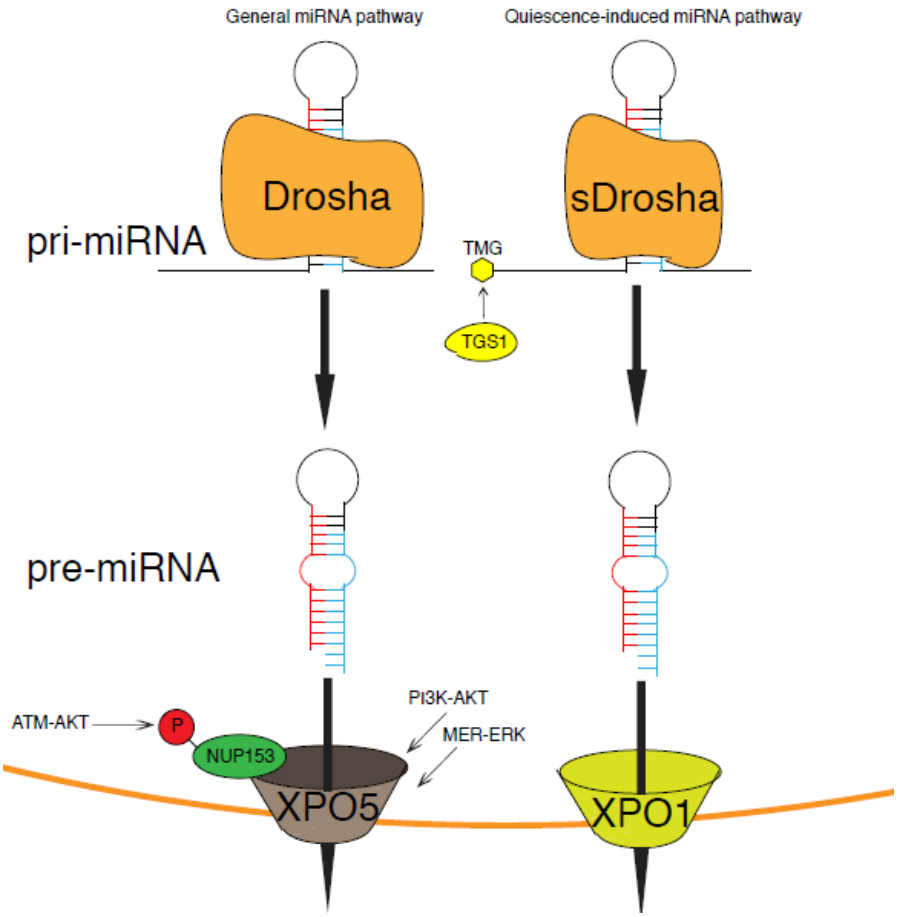


Figure 1-11. Regulation of export to cytoplasm. *MEK-ERK induces* ***XPO5****. PI3K post-transcriptionally induces XPO5 expression. Upon DNA damage, ATM-AKT axis phosphorylates NUP153, leading to its increased interaction with XPO5 and pre-miRNA export.* ***XPO1*** *exports quiescence-induced miRNAs. These pri-miRNAs are modified with a TMG-cap by TGS1, and processed by sDrosha.*

1.2.1.4 Regulation of processing by Dicer

In cytoplasm, the RNase III Dicer cleaves pre-miRNA near the terminal loop to liberate a small RNA duplex (Bernstein et al., 2001; Grishok et al., 2001).

RNA sequence and/or structure affect Dicer processing and the stability of its products. The untemplated nucleotidyl addition to the 3′ end of RNA (tailing) occurs both on pre-miRNA and mature miRNA (Ameres and Zamore, 2013). Uridylation of pre-let-7 is the most extensively studied tailing of pre-miRNAs. Let-7 family members are post-transcriptionally suppressed in embryonic stages (Pasquinelli et al., 2000; Suh et al., 2004). In addition to binding to the terminal loop of pri-let-7 and pre-let-7 and interfere with their processings (Loughlin et al., 2011; Nam et al., 2011; Viswanathan et al., 2008), LIN28 proteins recruit terminal uridylyl transferases (TUTases) TUT4 and TUT7 to oligouridylate of pre-let-7 (Hagan et al., 2009; Heo et al., 2008, 2009). This oligo-U tail blocks Dicer processing and facilitates miRNA decay by DIS3L2 (Chang et al., 2013; Heo et al., 2008; Ustianenko et al., 2013). In the absence of LIN28, TUT7, TUT4 and TUT2 induce monouridylation of pre-let-7 and increase Dicer processing (Heo et al., 2012). In this sense, LIN28 is the molecular switch converting TUT4 and TUT7 from biogenesis factors into negative regulators of let-7. KSRP interacts with the terminal loop of a group of pre-miRNAs and enhances their Dicer processing (Trabucchi et al., 2009). Apart from RNA tailing, ADAR1 edits pri-miR-151, and the edited pre-mir-151 cannot be efficiently processed by Dicer (Kawahara et al., 2007). The methylation of pre-miRNAs also interferes with Dicer processing. RNA methyltransferase BCDIN3D O-methylates the 5′ monophosphate of pre-miR-145 and pre-miR-23b, making them less accessible to Dicer (Park et al., 2011; Xhemalce et al., 2012).

The abundance of some pre-miRNAs is regulated at the stability level. Pre-miR-146a and -135b are cleaved at the terminal loop by the endoribonuclease MCP-induced protein 1 (MCPIP1) (Suzuki et al., 2011). Upon endoplasmic reticulum stress, the Ser/Thr protein kinase/endoribonuclease IRE1α cleaves four pre-miRNAs that target the pro-apoptotic caspase 2 (Upton et al., 2012) (*Figure 1-12*).

Multiple mechanisms exist to control the level, activity and specificity of Dicer. The transcription of Dicer is repressed by β-catenin in ovarian cancer cells, as well as the production of general miRNA population, including miR-29, an important oncogene. In this sense, β-catenin reduces Dicer to promote ovarian cancer epithelial-to-mesenchymal transition (EMT) and metastasis (To et al., 2017). Tumor suppressor glutaminase 2 (GLS2) interacts with and stabilizes Dicer, to facilitate miR-34a maturation and repress the expression of Snail. GLS2 thus inhibits malignancies of human hepatocellular carcinoma (HCC) by repressing the EMT via the Dicer-miR-34a-Snail axis (Kuo et al., 2016). Hypoxia-inducible factor-1α (HIF-1α) interacts with Dicer in multiple types of cancer cell lines and different human tumors. This interaction reduces Dicer by facilitating its ubiquitination by the E3 ligase Parkin, thereby enhancing autophagy-mediated degradation of Dicer, which further suppresses the maturation of tumor suppressors miRNAs including let-7 and miR-200b. Expression of HIF-1α might be the explanation for frequently down-regulated Dicer in human cancers and metastases (Lai et al., 2017). Dicer can be targeted by the final product of miRNA biogenesis in a feedback loop. For example, miR-122 promotes the metastasis of clear-cell renal cell carcinoma by targeting Dicer (Fan et al., 2018); human Dicer1 mRNA is a direct target of let-7, and these negative feedback loops contribute to Dicer homeostasis (Forman et al., 2008; Tokumaru et al., 2008).

The 5′-3′ exonucleases XRN2 binds pre-miR-10a in a Dicer-independent manner to accelerate miR-10a maturation, which promotes EMT and metastasis of lung cancer (Zhang et al., 2017). XRN2 plays a major role in miRNA turnover (see below, section 1.2.1.6).

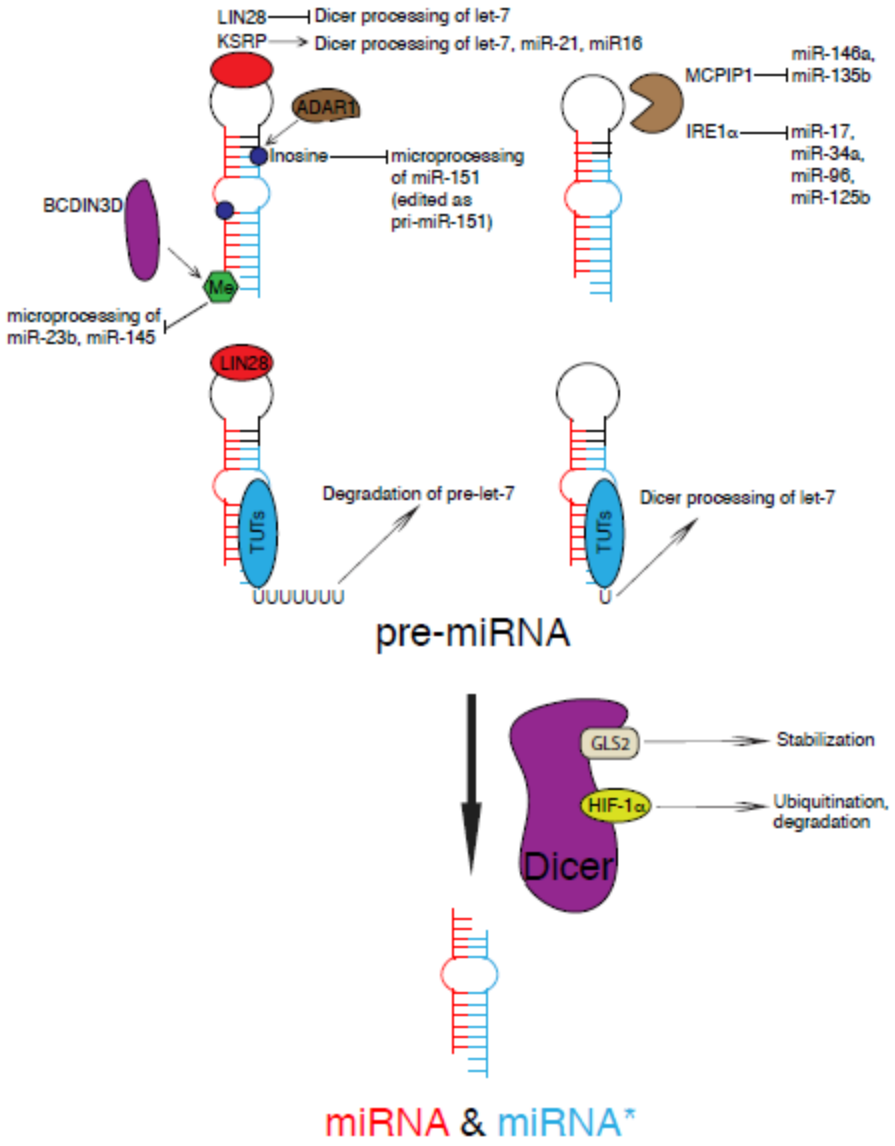


Figure 1-12. Regulation of processing by Dicer. RNA sequence and/or structure affect Dicer processing. *LIN28 binds to the terminal loop of pre-let-7 and interferes with its processings. LIN28 also recruits TUT4 and TUT7 to oligouridylate of pre-let-7, blocking its processing and facilitates its decay. Without LIN28, TUTs induce monouridylation of pre-let-7 and increase its processing. KSRP interacts with the terminal loop of a group of pre-miRNAs and enhances their processing. ADAR1 edits pri-miR-151, and the edited pre-mir-151 cannot be efficiently processed. BCDIN3D O-methylates the 5′ monophosphate of pre-miR-145 and pre-miR-23b, making them less accessible to Dicer. Dicer itself is regulated by several factors. GLS2 interacts with and stabilizes Dicer. HIF-1α interacts with and de-stabilizes Dicer.*

1.2.1.5 Regulation of Argonaute protein functions

The small RNA duplex generated by Dicer cleavage is subsequently loaded in an Argonaute protein to form an RNA-induced silencing complex (RISC) (Hammond et al., 2001; Mourelatos et al., 2002; Tabara et al., 1999). Argonaute proteins are under a large number of modulations, most of which have not yet been reported to affect miRNA levels but rather miRISC functions. However, since miRNA loading and release is closely linked to miRISC functions, some prominent examples of the regulation of Argonaute protein functions are discussed below.

As the key component of RISC, Argonaute proteins can be modulated by numerous modifications. Prolyl 4-hydroxylation of human Ago2 by type I collagen prolyl 4-hydroxylase (4PH) increases the stability of Ago2 and localization within processing bodies (P-bodies) (Qi et al., 2008; Wu et al., 2011a). Poly ADP-ribosylation of Ago2 takes place upon stress or viral infection, which inhibits the targeting ability of RISC (Leung et al., 2011; Seo et al., 2013). Argonaute proteins are also subject to degradation by the ubiquitin–proteasome pathway. In mouse embryonic stem cells, LIN41 acts as an E3 ubiquitin ligase for Ago2 (Rybak et al., 2009). Phosphorylation is the most studied type of post-translational modifications of Ago2. MAP kinase-activated protein kinase 2 (MAPKAPK2) (Zeng et al., 2008) and Akt3 (Horman et al., 2013) phosphorylate Ago2 at Ser387, resulting in its localization to P-bodies and translational repression, respectively. The importance of this phosphorylation was further validated by Bridge et al., who showed that Akt3 induces this phosphorylation to initiate LIMD1 binding, a scaffold protein recruiting TNRC6A (trinucleotide repeat-containing gene 6A) and downstream effectors for translational repression/mRNA destabilization. This Serine is conserved through Ago1-4, and Ago3 has an additional glutamic acid residue (E390) on the same interaction interface, which

allows Ago3 to bind to the LIM domain containing proteins irrespective of Akt signaling (Bridge et al., 2017). Under hypoxia, epidermal growth factor receptor (EGFR) phosphorylates Ago2 at Tyr393, resulting in the dissociation of Ago2 from Dicer and the reduction of processing of some pre-miRNAs (Shen et al., 2013) (*Figure 1-13*). The phosphorylation at Tyr529 on Ago2 was proposed to reduce small RNA binding and target repression, though the responsible kinase remains to be discovered (Mazumder et al., 2013; Rüdel et al., 2011). The C-terminal S824-S834 of Argonaute proteins is hyper-phosphorylated at five Serine residues in human and *C.elegans*. This hyper-phosphorylation does not affect miRNA binding, localization, or cleavage activity of Ago2, but rather mRNA binding, and it plays a role at late stages of gene silencing since the cluster remains unphosphorylated on Ago2 mutants that cannot bind miRNAs or mRNAs (Quévillon Huberdeau et al., 2017). S824-S834 hyper-phosphorylation is mediated by CSNK1A1, followed by rapid dephosphorylation by the ANKRD52-PPP6C phosphatase complex. Interestingly, although the hyper-phosphorylation inhibits target mRNA binding, inactivation of this phosphorylation cycle globally impairs miRNA-mediated silencing. This is because non-phosphorylatable Ago2 is overall occupied by an expanded target repertoire, potently reducing the active pool of Ago2 on a per-target basis. In this sense, S824-S834 hyperphosphorylation cycle stimulated by target engagement maintains the global efficiency of miRNA-mediated silencing (Golden et al., 2017). MAP kinases also phosphorylate Ago2 at different sites (see Section 1.2.2).

Some protein co-factors contribute to the fine-tuning of Argonaute protein functions. The nucleoporin Nup358 binds to Argonaute through its SUMO-interacting motifs (SIMs) and promotes the association of target mRNA with miRISC. Nup358 localizes to the nuclear pore complex and to the cytoplasmic annulate lamellae (AL), and these structures dynamically

associate with two mRNP granules: processing bodies (P bodies) and stress granules (SGs), suggesting that the coupling of miRISC with the target mRNA could occur at nuclear envelope, AL, or specialized domains within the endoplasmic reticulum. In addition, Argonaute proteins interact with SIMs derived from many other SUMO-binding proteins, suggesting their implication in miRISC function (Sahoo et al., 2017). The phosphatase PIR-1 was first reported to be responsible for the accumulation of a distinct class of siRNAs synthesized by an RNA-dependent RNA polymerase (RdRP) in *C. elegans*. The mammalian PIR-1 homolog, dual specificity phosphatase 11 (DUSP11) is an RNA triphosphatase. DUSP11 converts the 5' triphosphate of miRNA precursors to a 5' monophosphate, promoting the loading of derivative 5p miRNAs into Argonaute proteins via a Dicer-coupled 5' monophosphate-dependent strand selection mechanism. This activity of DUSP11 promotes Argonaute association with noncanonical viral miRNAs (Burke et al., 2016). The autoantigen La ensures correct tRNA maturation and gates the loading into RISCs by preventing the mischanneling of tRNA fragments onto Argonaute proteins (Hasler et al., 2016).

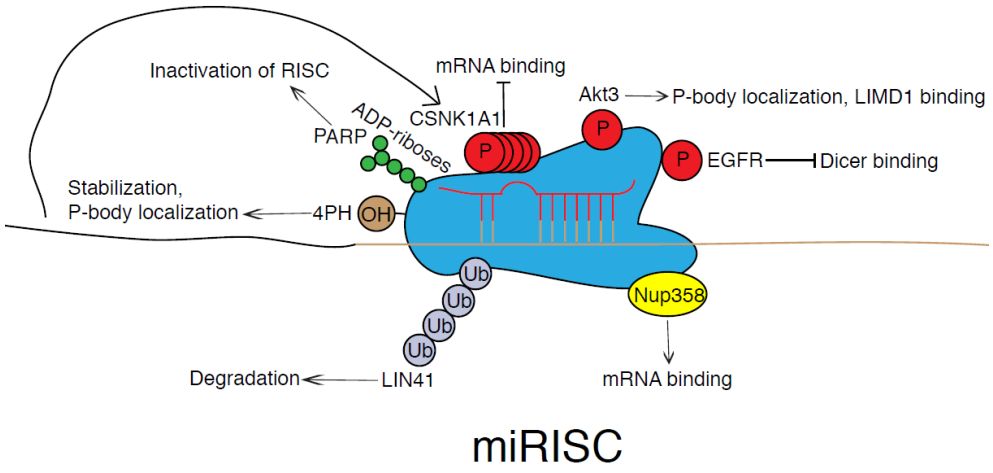


Figure 1-13. Regulation of Argonaute protein functions. Ago2 is modulated post-translationally. *Prolyl 4-OH of Ago2 by 4PH increases its stability and localization within P-*

bodies. Poly ADP-ribosylation of Ago2 inhibits the targeting ability of RISC. LIN41 acts as an E3 ubiquitin ligase for Ago2. Akt3 phosphorylates Ago2, resulting in its P-body localization, translational repression, and LIMD1 binding. EGFR phosphorylates Ago2, resulting in the dissociation of Ago2 from Dicer. mRNA engagement leads to the CSNK1A1-mediated C-terminal hyper-phosphorylation of Ago2, which in turn inhibits mRNA binding. Protein co-factors contribute to the fine-tuning of Argonaute functions. Nup358 binds to Argonaute promotes the association of target mRNA.

1.2.1.6 Regulation of miRNA stability

Mature miRNAs are highly stable once they are loaded into RISC, because both ends are protected by Argonaute proteins (Elkayam et al., 2012; Schirle and MacRae, 2012). For exonuclease-mediated decay to occur, miRNAs may need to be unloaded first.

The turnover of mature miRNA is carried out by several exonucleases. Human 3′–5′ exonuclease PNPT1 cleaves miR-221, miR-222 and miR-106 in melanoma cells (Das et al., 2010) (*Figure 1-14*). However, the 5′–3′ exonucleases XRN-1 and XRN-2 are the more studied enzymes that degrade miRNAs (Chatterjee and Grosshans, 2009; Chatterjee et al., 2011). XRN1 mediates the rapid decay of engulfed extracellular miRNA (ex-miRNA) in cancer cells, which are secreted by polymorphonuclear leukocytes. At least one of the ex-miRNAs, ex-miR-223-3p is functional in cancer cells and mediates EMT reprogramming. Due to XRN-1, engulfed ex-miRNAs are less stable than endogenous miRNA (Zangari et al., 2017). XRN2 shows some miRNA specificity in *C. elegans* (Miki et al., 2014). In *C. elegans,* the decapping scavenger protein DCS-1 interacts with XRN-1 to promote microRNA degradation in an independent manner from its decapping scavenger activity (Bossé et al., 2013). This activity of DCS-1 is conserved on its human ortholog DcpS, which acts as a nucleocytoplasmic shuttling protein.

Unlike in *C. elegans*, DcpS cooperates with XRN2 in human to degrade miRNAs (Meziane et al., 2015). Another decapping protein, HPat also associates with miRISC, suggesting a possible involvement in miRNA turnover (Barišić-Jäger et al., 2013)

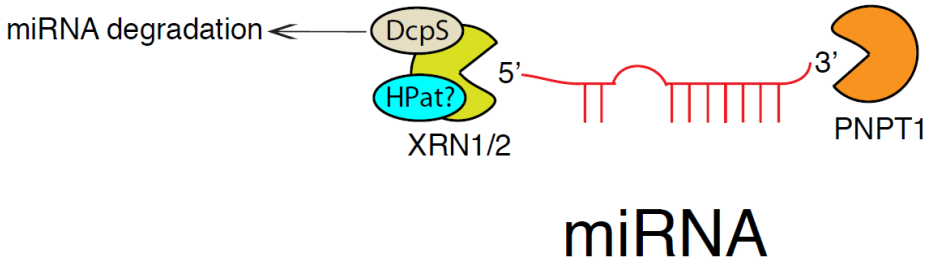


Figure 1-14. Regulation of miRNA stability. *The 3′–5′ exonuclease PNPT1 cleaves miR-221, miR-222 and miR-106 in melanoma cells. The 5′–3′ exonucleases XRN-1 and XRN-2 are the major enzymes degrading miRNAs. DcpS cooperates with XRN2, while its C.elegans homolog DCS-1 cooperates with XRN1, to degrade miRNAs. HPat also associates with miRISC, suggesting a possible involvement in miRNA turnover.*

3′ non-templated nucleotide addition (tailing), mainly uridylation and adenylation, also occurs on miRNAs. Profiling of small RNAs during early development in zebra fish revealed widespread miRNA tailing, which undergoes developmental stage-specific regulation (Wei et al., 2012). 3′ adenylation is enriched in cells and 3′ uridylation is enriched in exosomes secreted by them, so tailing might be linked to exosomal sorting of miRNAs (Koppers-Lalic et al., 2014).

In recent years, an increasing number of TUTases that uridylate miRNAs have been identified. TUT4 uridylates the cytokine-targeting miR-26b and the uridylation appears to derepress cytokine in human epithelial cell lines (Jones et al., 2009) (*Figure 1-15*). TUT7 and TUT4 mono-uridylate a group of miRNAs containing a common sequence motif. Interestingly, depleting these TUTases leads to reduced 3′ mono-uridylation and increased 3′ mono-adenylation of these miRNAs, without affecting their abundance (Rissland et al., 2007; Thornton et al., 2014). In

mammal cells, during the regulation of a target, the miRNA itself may be subject to regulation by its target, which enhances the uridylation and 3′ trimming of the miRNA (Ameres et al., 2010; Baccarini et al., 2011).

Unlike uridylation, adenylation occurs mainly after Dicer processing, and the consequences of miRNA adenylation depend on the cellular context. miR-122 is monoadenylated by the non-canonical poly(A) RNA polymerase GLD-2 and stabilized (Katoh et al., 2009), and GLD-2 is responsible for the 3′ adenylation of another group of miRNAs, though instead of stabilizing these miRNAs this adenylation reduces miRNA activity (Burroughs et al., 2010). On the other hand, adenylation of miR-21 by PAPD5 leads to its 3′–5′ digestion by poly(A)-specific ribonuclease (PARN) (Boele et al., 2014) (*Figure 1-15*).

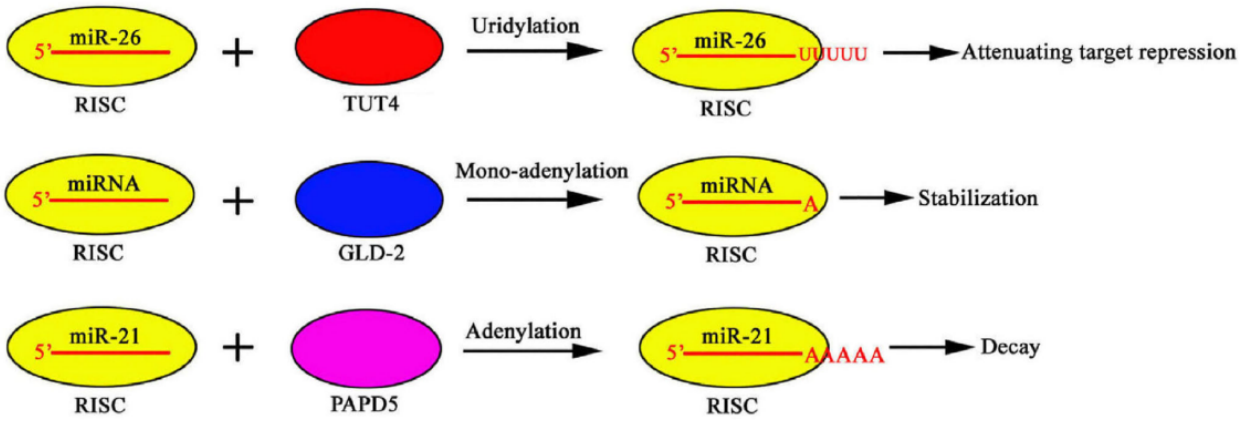


Figure 1-15. Uridylation and adenylation of miRNAs. *TUT4 uridylates miR-26 to attenuate target repression. Several miRNAs are monoadenylated by GLD-2 and stabilized. Adenylation of miR-21 by PAPD5 leads to its 3′–5′ digestion by PARN* (Song et al., 2015).

1.2.2 MAP kinase pathways and microRNA pathway

Mitogen-activated protein kinases (MAP kinases) are a group of highly conserved serine/threonine-specific protein kinases. MAP kinase pathways are involved in directing cellular responses to physiological and stress stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines, thus regulating proliferation, gene expression, differentiation, mitosis, cell survival and apoptosis (Dhillon et al., 2007; Kim and Choi, 2010; Murphy and Blenis, 2006; Sun et al., 2015). In mammals MAP kinases include p38 MAP kinase (p38), c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (Corre et al., 2017; Hommes et al., 2003; Pearson et al., 2011).

JNK, p38 and ERK5 are often considered as stress-activated protein kinases, or SAPKs. Nevertheless, they are also activated by growth factors (Drew et al., 2012; Hayashi and Lee, 2004; Rose et al., 2010). The induction of the SAPK (JNK and p38) pathways involves the activation in cascade of small GTPases, upstream of MAPKKK and MAKK (Cuenda and Rousseau, 2007) (*Figure 1-16*). In the case of ERK5, it has also been shown that the pathway is activated by Src kinase in response to oxidative stress (Abe et al., 1997). While there is a large degree of specificity within the different MAP kinase pathways, significant overlaps exist between them, allowing cross-talk and feedback signaling events between MAP kinase pathways (Rose et al., 2010). For instance, both ERK and p38 pathways are activated by VEGF (Vascular Endothelial Growth Factor) downstream of VEGFR2 (Lamalice et al., 2004; Rousseau et al., 2000; Takahashi et al., 2001). As for shared targets, both p38 and JNK share the downstream transcription factor ATF2 (Breitwieser et al., 2007).

The ERK cascade is the best known of MAP kinase pathways. It is mostly considered as a mitogenic and survival pathway following its activation by growth factors, though it is also

induced by stress including reactive oxygen species (ROS) or ROS-generating agents (Daou and Srivastava, 2004; Sun et al., 2015). The ERK pathway is activated through the binding of agonists to tyrosine kinase receptors, which results in auto-phosphorylation of tyrosyl residues on the receptors, hence creating docking sites for adapter proteins and enzymes. Then, follows the activation in cascade of the GTPase Ras, the MAP kinase kinase kinase (MAPKKK) Raf, the MAP kinase kinases (MAPKK) MEK-1/2 and finally the MAP kinases (MAPK) ERK1/2. ERK in its turn phosphorylates a number of cytoplasmic and nuclear proteins including transcription factors (Dhillon et al., 2007) (*Figure 1-16*).

MAP kinases mediate the microRNA pathway via either phosphorylating miRNA pathway factors (Drosha, DGCR8, Dicer and Ago2) (Table 1-1), or modulating the transcription of miRNAs (*Figure 1-16*). An ever-increasing large number of miRNAs are being reported to be regulated by MAP kinases at the transcriptional level, but only such examples in endothelial cells will be discussed in the following paragraphs (Table 1-2).

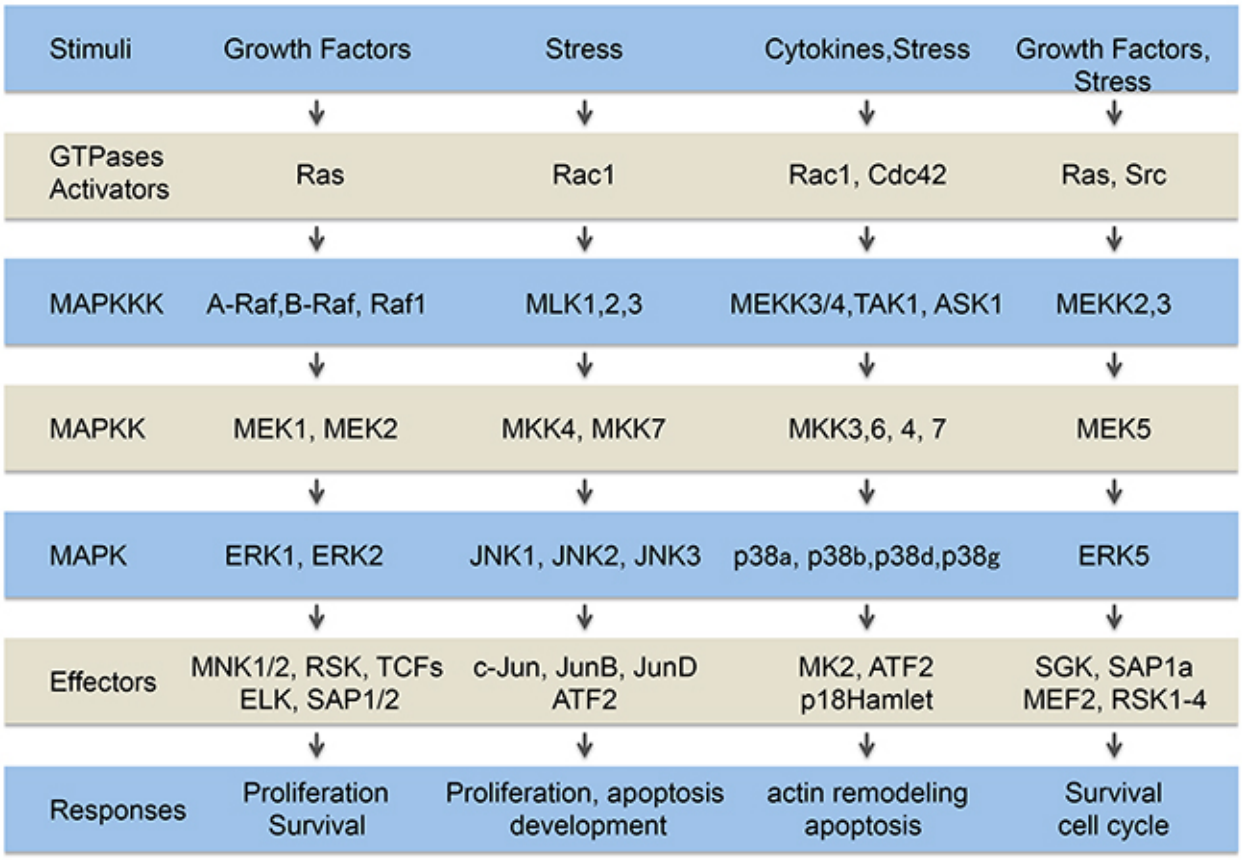


Figure 1-16. The MAP kinase pathways. *The MAP kinase module locates downstream of activators (usually GTPases) connected to tyrosine kinase receptors, cytokines receptors and stress sensors. The signals eventually converge on different cytoplasmic and nuclear effectors (transcription factors). MAPK: mitogen-activated protein kinase; MAPKK/MKK: mitogen-activated protein kinase kinase; MAPKKK: mitogen-activated protein kinase kinase kinase. Adapted from (Corre et al., 2017).*

1.2.2.1 p38 and microRNA pathway

p38 is one of the two major MAP kinases phosphorylating miRNA pathway factors. Stressful conditions such as heat and oxidative stress (H_2O_2) induce the p38-mediated S355 phosphorylation in the N-terminus of Drosha, which reduces its interaction with DGCR8, and promotes its nuclear export and subsequent degradation by calpain (Yang et al., 2015). p38 in

this sense mediates stress-induced inhibition of Drosha function, and the reduction of Drosha sensitizes cells to stress and death. In contrast, increasing the level of Drosha using vectors attenuated stress-induced death. Taken together, in response to stress, p38 phosphorylates and destabilizes Drosha to inhibit Drosha-mediated cellular survival (Yang et al., 2015). The S387 phosphorylation of the RISC component Argonaute 2 (Ago2) is one of the most investigated post-translational modifications of miRNA pathway factors. According to Zeng et al., Ago2-S387 is the major Ago2 phosphorylation site *in vivo*, which can be induced by sodium arsenite and anisomycin, two p38 activators. Along these lines p38 inhibitor SB203580 greatly diminished Ago2 phosphorylation. The direct substrate of p38, MAPKAPK2 (MAPK-activated protein kinase-2) phosphorylated Ago2 at S387 in an *in vitro* test. This phosphorylation is indispensable for the localization of Ago2 to processing bodies and corroborates a potential regulatory mechanism for RNA silencing (Zeng et al., 2008).

In endothelial cells, miRNAs have been proposed as novel regulators of vascular inflammation and dysfunction. p38 regulates the transcription of a large number of such miRNAs to fine-tune endothelial activation. TNFα down-regulates miR-149 in Eahy926 endothelial cells and human umbilical vein endothelial cells (HUVECs) through p38 MAP kinase. miR-149 mimic transfection counteracts the TNFα-induced expression of MMP-9, iNOS and IL-6. p38 in this sense, represses miR-149 to de-repress molecules associated with TNFα-induced endothelial dysfunction (Palmieri et al., 2014). MAP kinase-miRNA axis also controls the mechanical stimulation of endothelial cell functions in that inhibiting p38, JNK or ERK attenuates shear stress-induced miR-23b and -27b. Inhibiting these two miRNAs using antagomirs reduces the shear stress-induced activation of p38, JNK or ERK. Furthermore, overexpressing miR-23b and -27b overrides the suppressive effects of ERK inhibitors on shear stress-induced ERK activation,

suggesting a feed-forward loop whereby MAP kinases mediate the shear regulation of miRNA expression, which in turn enhances the shear stress-induced MAP kinase signaling events in endothelial cells. The authors proposed that this feed-forward loop regulates endothelial cell proliferation (He et al., 2012).

1.2.2.2 JNK and microRNA pathway

Unlike p38 and ERK, JNK has not been reported to phosphorylate miRNA pathway factors. However, in endothelial cells JNK has been reported to mediate the expression of miRNA via its downstream transcription factors. Tumor necrosis factor superfamily 15 (TNFSF15), a vascular endothelial cell anti-angiogenic cytokine, inhibits vascular endothelial cell growth factor (VEGF) production in a mouse endothelial cell line. Mechanistically TNFSF15 activates JNK-GATA3 pathway-mediated expression of miR-29b, which targets VEGF, and thus plays a pivotal role in inhibiting neovascularization. This mechanism might be the explanation for up-regulated VEGF in diseases including cancers (Zhang et al., 2016a).

1.2.2.3 ERK and microRNA pathway

ERK is the most extensively studied MAP kinase for phosphorylating miRNA pathway protein factors. Herbert et al. identified 23 phosphorylation sites on DGCR8 and proposed ERK as the responsible kinase. DGCR8 phosphorylation thus might respond to and integrate extracellular cues. Functionally, DGCR8 phosphorylation stabilizes DGCR8 at the protein level and increases Drosha, since stabilizing the latter is the canonical function of DGCR8. HeLa cells expressing phospho-mimetic DGCR8 exhibited increased proliferation accompanied by a pro-growth

miRNA expression profile (Herbert et al., 2013). XPO5 is not exempted for MAP kinase-mediated phosphorylation. ERK suppresses pre-miRNA export from the nucleus through phosphorylating XPO5 at T345/S416/S497. This phosphorylation initiates the prolyl isomerase Pin1-mediated conformational alteration of XPO5, which reduces pre-miRNA loading. The authors proposed this mechanism as the possible explanation for down-regulated miRNAs in cancers, and showed that at least in liver cancer cells, the ERK-mediated XPO5 inhibition reduces miR-122, increases microtubule dynamics, and boosts in tumor development and drug resistance. Their clinical specimens further showed that XPO5 phosphorylation is associated with poor prognosis for liver cancer patients (Sun et al., 2016). Drake et al. have shown that ERK phosphorylates *C. elegans* Dicer on evolutionarily conserved S1705 and S1833, within RNAse IIIb domain and dsRNA binding domain, respectively, and that phosphorylation of these residues is necessary and sufficient to trigger Dicer's nuclear translocation in worm, mouse, and human cells. In the female germ line of worm, the phosphorylation on either site inhibits Dicer function and dampens small RNA repertoire. ERK-mediating phosphorylation and inhibition of Dicer take place during meiosis I for proper oogenesis. This inhibition is relieved before fertilization for proper embryogenesis. In this manner ERK-Dicer nexus controls oocyte-to-embryo transition and couples extracellular stimuli to small RNA pathway (Drake et al., 2014). Secretion of miRNAs in extracellular vesicles is a recently discovered form of intercellular communication. In isogenic colon cancer cell lines, overactive mutant KRAS acts through the MAPKK I and II--ERK axis to phosphorylate Ago2 at S387, and this phosphorylation inhibits the association of Ago2 with multivesicular endosomes (MVEs) and its subsequent secretion in exosomes. This KRAS-MAPKK-ERK axis regulates the exosomal sorting of at least let-7a, miR-100, and miR-320a (McKenzie et al., 2016).

ERK also mediates the transcription of several miRNAs through its downstream transcription factors in endothelial cells, especially in response to VEGF. VEGF has long been reported to down-regulate tumor necrosis factor superfamily-15 (TNFSF15), a negative modulator of angiogenesis. Recent findings show that VEGF stimulates the production of miR-20a through PI3K and ERK pathways, and miR-31 through PI3K pathway. Since the two miRNAs directly target TNFSF15, the inactivation of either Erk or PI3K restores TNFSF15 expression (Deng et al., 2017b). Pin and colleagues revealed that the VEGF-induced p38 activation and cell migration are modulated by overexpression of Ago2, and that VEGF-induced miR-20a inhibits actin remodeling, cell migration and angiogenesis. They further showed that miR-20a targets MKK3 to repress VEGF-induced p38 activity in a feedback loop to negatively regulate angiogenesis (Pin et al., 2012). Moreover, in their recent publication Marquez and colleagues revealed repressed expression of miR-20a, and increased expression of its targets E2F1 and ARHGAP1 in endothelial cells during colorectal liver metastasis, and that targeting liver sinusoidal endothelial cells with miR-20a-loaded nanoparticles reduced murine colon cancer metastasis to the liver. In vitro analysis linked this function of miR-20a to its capacity to repress endothelial cell migration and sebsequent tumor infiltration (Marquez et al., 2018). Elevating miR-20a or miR-31 in HUVECs leads to enhancement of capillary-like tubule formation in *in vitro* angiogenesis assay, whereas reducing miR-20a and miR-31 results in its inhibition, corroborating that miR-20a and miR-31 mediate VEGF-induced down-regulation of TNFSF15 and angiogenesis (Deng et al., 2017b). VEGF also induces ERK-mediated transcription the miR-17-92 cluster (encoding miR-17, -18a, -19a/b, -20a, and miR-92a), which is necessary for endothelial cell proliferation and angiogenic sprouting *in vitro*. Postnatal inactivation of miR-17-92 cluster in mice endothelium (miR-17-92 iEC-KO mice) blunted physiological retinal angiogenesis during development and

diminished VEGF-induced ear angiogenesis and tumor angiogenesis. Further analysis identified phosphatase and tensin homolog (PTEN) as a target of the miR-17-92 cluster and a crucial mediator of miR-17-92-induced endothelial cell proliferation. VEGF-ERK-induced miR-17-92 cluster contributes to the angiogenic switch of endothelial cells and participates in the regulation of angiogenesis (Chamorro-Jorganes et al., 2016). Doebele and colleagues demonstrated that endothelial miR-17/20a inhibits endothelial sprouting *in vitro* and *In vivo* through targeting Janus kinase 1 (JAK1) and several other several proangiogenic genes. However, the inhibition of miR-17/20a did not affect tumor angiogenesis *in vivo* (Doebele et al., 2010).

Protein factor	MAP kinase	Amino acid(s)	Function(s)
Drosha	p38	S355	Reduce interaction with DGCR8; cytoplasmic localization and degradation
DGCR8	ERK	23 amino acids	Stabilize DGCR8 and Drosha
XPO5	ERK	T345, S416 and S497	Reduce pre-miRNA exportation
Dicer	ERK	S1705 and S1833	nuclear localization
Ago2	p38	S387	P-body localization
	ERK	S387	exosomal secretion

Table 1-1. miRNA pathway factors and MAP kinases.

MAP kinase	Stimulus	Impact	miRNA	Target(s)	Function(s) of the miRNA
p38	TNFα	Inhibit	miR-149	MMP-9, iNOS and IL-6	Repress TNFα-induced endothelial dysfunction
	Shear stress	Induce	miR-23b and -27b		Enhance the shear stress-induced MAP kinase signaling and endothelial cell proliferation
JNK	TNFSF15		miR-29b	VEGF	Inhibit neovascularization
	Shear stress		miR-23b and -27b		Enhance the shear stress-induced MAP kinase signaling and endothelial cell proliferation
ERK	VEGF		miR-20a	TNFSF15	Enhance angiogenesis
			miR-17-92 cluster	PTEN	Promote endothelial cell proliferation and angiogenesis
	Shear stress		miR-23b and -27b		Enhance the shear stress-induced MAP kinase signaling and endothelial cell proliferation

Table 1-2. MAP kinases mediate the transcription of miRNAs.

1.2.3 microRNAs mediating endothelial cell adhesion molecules

In response to pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β), endothelial cells express adhesion molecules such as E-selectin on their surface. The Sialyl-Lewis carbohydrate ligands found on leukocytes then loosely attach to E-selectin, which permits their rolling on the endothelium. The rolling leukocytes are then activated by chemokines locally released by macrophages and endothelial cells, to express integrins on their surface, which allow firmer adhesion to other endothelial cell adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), initiating their transendothelial migration into injured tissues (Walzog and Gaehtgens, 2000) (*Figure 1-15*). Hence, by mediating the extravasation

of leukocytes, E-selectin, ICAM-1 and VCAM-1 play critical roles in the development of inflammation-associated diseases such as atherosclerosis, sepsis and cancer.

The transcription of all these three cell adhesion molecules is driven predominantly by the pro-inflammatory transcription factor, nuclear factor of κ light polypeptide gene enhancer in B cells (NF-κB) (7). The major form of NF-κB is the p65/p50 heterodimer. Under basal conditions, it is sequestered in the cytoplasm by the Inhibitor of κB (IκB), until TAK1 phosphorylates and activates IκB kinase (IKK), which in turn phosphorylates IκB, causing the β-transducin repeat containing E3 ubiquitin protein ligase (βTRC) to mediate ubiquitination and proteasomal degradation of IκB. Freed NF-κB then enters the nucleus and binds to its target genes, including cell adhesion molecule genes, pro-inflammatory cytokine/chemokine genes, and pro-survival genes, to initiate their transcription(Cheng et al., 2014; Hayden and Ghosh, 2004) (*Figure 1-17*).

The microRNAs (miRNAs) are single-stranded non-coding RNAs of ~21 nucleotides long. They are firstly transcribed as primary miRNAs (pri-miRNAs) mainly by the RNA polymerase II. Pri-miRNAs are processed by Drosha-DGCR8 complex in the nucleus to generate precursor miRNAs (pre-miRNAs), which are exported to the cytoplasm to be cleaved by Dicer, producing duplexes containing the passenger strand and the guide strand miRNAs. The passenger strand is degraded, and the guide strand is loaded onto an Argonaute protein to form, with other factors, the miRNA-induced silencing complex known as miRISC (*Figure 1-17*). Through base pairing with the 3' untranslated region (3' UTR) of mRNAs, miRNAs guide miRISC to repress the translation of their target mRNAs, with or without causing mRNA decay. The base pairing between the nucleotides 2–7 of the

miRNA (seed region) and its target, is most of the time essential for altering protein production (Ha and Kim, 2014).

Given the importance of NF-κB in modulating inflammatory genes, a host of miRNAs converges to resolve this transcriptional pathway (*Figure 1-17*). In addition, the translation of E-selectin, ICAM-1 and VCAM-1 is under the direct repression of several miRNAs..

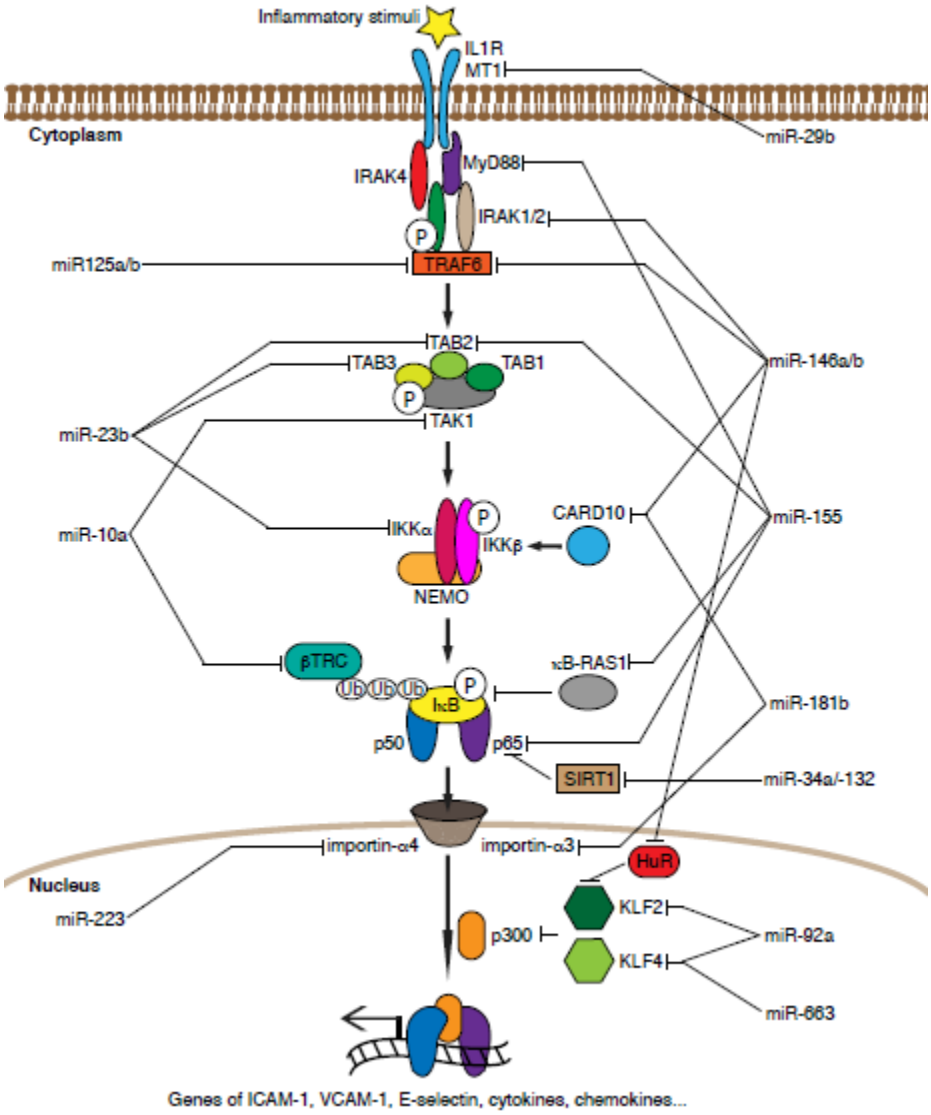


Figure 1-17. The miRNA network modulating NF-κB signaling. *In endothelial cells, the major form of NF-κB is the p65/p50 heterodimer, which is sequestered in the cytoplasm by IκB under*

basal conditions. In response to inflammatory signaling, the activated IKK complex phosphorylates IκB, causing the βTRC-mediated ubiquitination and proteasomal degradation of the latter. Freed NF-κB then enters the nucleus and bind to the genes of cell adhesion molecules, chemokines, and cytokines. KLF2 and KLF4 compete with NF-κB for access to the transcriptional co-activator, p300, and thus act as inhibitors of NF-κB. A host of miRNAs converges to modulate the NF-κB pathway and their targets are shown, as discussed in the text. Adapted from (Cheng et al., 2014).

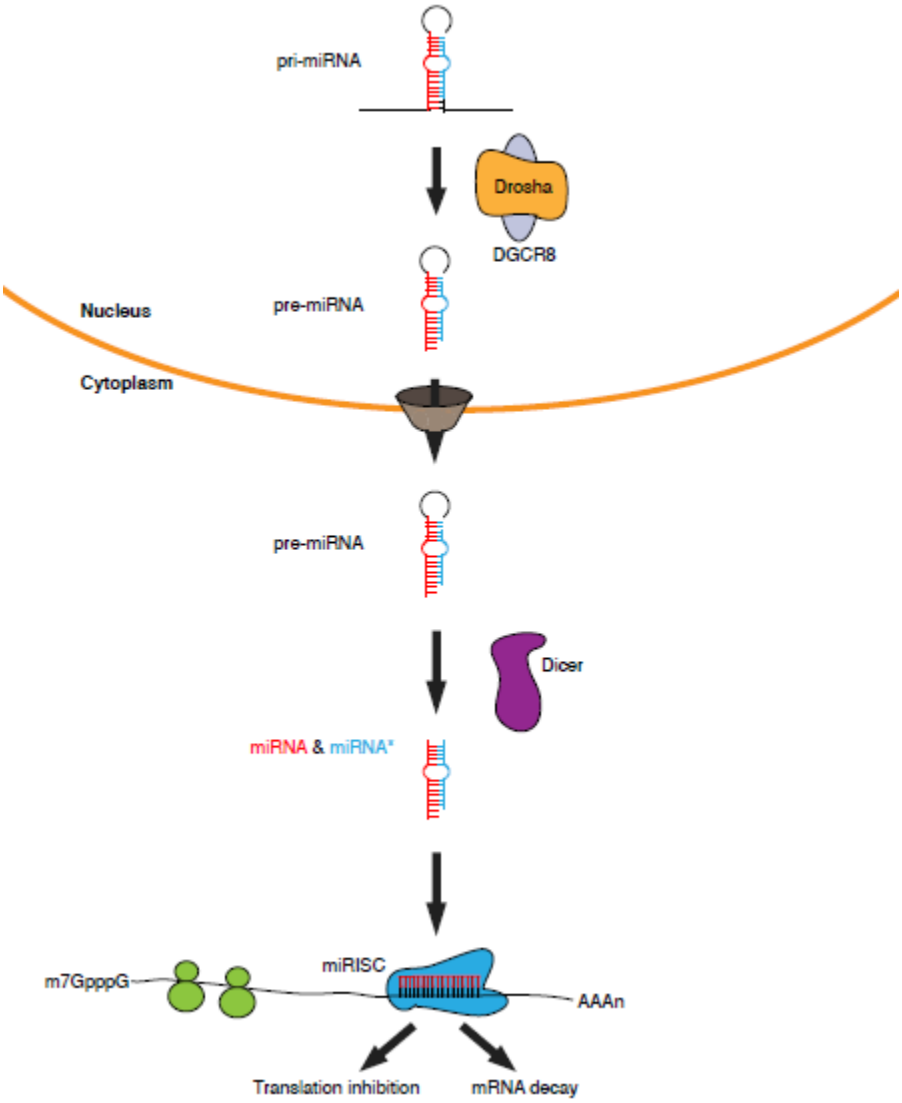


Figure 1-18. The biogenesis of miRNAs. *The miRNAs are firstly transcribed as pri-miRNAs. Pri-miRNAs are processed by Drosha-DGCR8 complex to generate pre-miRNAs, which are exported to the cytoplasm to be cleaved by Dicer, producing miRNAs. Through partially complementary base pairing with the 3' UTR of mRNAs (brown), miRNAs guide the miRISC to repress the translation of their target mRNAs, with or without causing mRNA decay.*

1.2.3.1 miRNAs modulating the expression and functions of cell adhesion molecules via targeting the NF-κB pathway

The NF-κB pathway consists of an activating receptor complex upstream of a cytoplasmic and nuclear effector machinery. Several miRNAs act at these three levels of the pathway to regulate inflammation by modulating the expression and functions of endothelial cell adhesion molecules including E-selectin, ICAM-1 and VCAM-1 (Fig 2). Certain miRNAs act specifically on one of the three components of the NF-κB pathway, whereas others, such as miR-146a have multiple targets along the NF-κB axis. These latter will be categorized according to their major targets in this review.

miRNAs targeting the receptor complex of NF-κB pathway

The NF-κB receptor complex consists of membrane receptors, notably interleukin-1 receptor (IL1R) and melatonin receptor-1 (MT1), and receptor adaptors including TRAF6, IRAKs and MyD88. These members of the NF-κB pathway are targeted by different sets of miRNAs.

At the receptor level, it has been shown that high fat diet, a stimulus associated with atherosclerosis, stimulates the expression of miR-29b in mice, which targets MT1 to modulates NF-κB activity and ICAM-1 expression. In turn, this mediates the high fat diet-induced endothelial apoptosis and permeability (Zhu et al., 2014).

On the other hand, the receptor adaptors TRAF6, IRAK1 and IRAK2 are targeted by miR-146a. Pro-inflammatory cytokines induce the transcription of miR-146a, and its accumulation coincides with the resolution of NF-κB pathway and thereby precludes the

expression of E-selectin, ICAM-1 and VCAM-1, as well as monocyte adhesion to endothelial cells in *vitro*. Ultimately, this results in impaired inflammation. Along these lines, miR-146a$^{-/-}$ mice have enhanced endothelial inflammatory responses(Cheng et al., 2013; Yin et al., 2016). Additionally, thrombin and lipopolysaccharide (LPS) are inducing the expression of miR-146a that targets caspase recruitment domain-containing protein 10 (Card10), an adaptor protein implicated in the activation of the IKK complex, to reduce inflammation(Cowan et al., 2014; Rau et al., 2014). Another mechanism that reduces LPS-induced inflammation is the Angiopoietin 1 (Ang-1)-miR-146b axis. Ang-1 activates the expression of the latter, which targets IRAK1 and TRAF6, to disrupt LPS-induced toll-like receptor 4 (TLR4) signaling, the downstream NF-κB activity, and the expression of E-selectin, ICAM-1, VCAM-1, TNFα, IL-1β, IL-6 and IL-8(Echavarria et al., 2015).

Oxidized low-density lipoprotein (ox-LDL) can de-repress NF-κB activity through reducing miR-125a. In human brain microvessel endothelial cells (HBMECs), ox-LDL reduces the expression of miR-125a, the function of which is to counteract the effects of ox-LDL on inflammation, namely on the expression of ICAM-1 and VCAM-1, and leukocyte adhesion. *In vitro* miR-125a promotes HBMEC proliferation, migration and tube formation(Pan et al., 2017). Though the direct targets of miR-125a in HBMECs require further studies, the target of miR-125b in mouse heart is TRAF6, an adaptor molecule in the NF-κB pathway. In mouse heart, transfection with lentivirus expressing miR-125b (LmiR-125b) significantly attenuates the expression of ICAM-1 and VCAM-1, the accumulation of immune cells, and cardiac dysfunction caused by sepsis. LmiR-125b also reduces serum TNFα and IL-1β in mice, which further weakens endothelial inflammation(Ma et al., 2016a).

miRNAs targeting the cytoplasmic axis of NF-κB pathway

In the cytoplasm, signal elicited by the NF-κB receptor complex is transduced through the TAK1-TAB, IKK and IκB axis to release NF-κB into nucleus. Multiple miRNAs target this cytoplasmic axis to modulate NF-κB activity.

Numerous miRNAs are down-regulated by inflammatory stimuli to de-repress NF-κB activity. The first discovery of the kind is miR-181b, which is rapidly reduced by TNFα and thrombin(Sun et al., 2012), and it shares the capacity of miR-146a to target and inhibit Card10 (Lin et al., 2016). Intriguingly, in response to thrombin, but not to TNFα, the reduced expression of Card10 by miR-181b is associated with a decreased activation of the NF-κB pathway in endothelial cells, which suggests a stimulus-specific regulation of NF-κB signaling in these cells by miR-181b. Along these lines, downregulation of Card 10 by thrombin results in reduced expression of NF-κB target genes including VCAM and E-selectin. In addition, miR-181b also targets importin-α3, an importer protein required for the nuclear translocation of NF-κB (Sun et al., 2012). The downstream expression of E-selectin, ICAM-1 and VCAM-1 is hence diminished by miR-181b. Accordingly, miR-181b exhibits anti-inflammatory effects in a mouse model of sepsis by decreasing leukocyte recruitment and damage to the lung, and prolonging the survival (Sun et al., 2012). Interestingly, in a clinical setting, miR-181b is down-regulated in the circulation during vascular inflammatory diseases such as coronary artery disease (CAD) and sepsis (Lin et al., 2016), indicative of a potential role in these pathologies. miR-23b targets TAK1 cofactors TAB2 and TAB3, and IKKα in lymphocytes(Hu and O'Connell, 2012). In endothelial cells,

LPS down-regulates miR-23b and allows the expression of NF-κB, E-selectin, ICAM-1, VCAM-1, TNFα and IL-6, suggesting that the miRNA-target relationship might be conserved in endothelial cells(Wu et al., 2015).

Some miRNAs inducible by pro-inflammatory cytokines and viral infection exert more pleiotropic functions as they both enhance and decrease NF-κB activity. miR-155 is one such example, which is highly expressed in human umbilical vein endothelial cells (HUVECs) and is inducible by pro-inflammatory cytokines. This miRNA targets pro-inflammatory molecules as diverse as p65, AT1R, Ets-1, MAP3K10 and endothelial NO synthase (eNOS) to repress the expression of ICAM-1, VCAM-1 and CCL2, and to impair the adhesion of T lymphocytes (Ambros, 2004; Cerutti et al., 2016; Wu et al., 2014; Zhu et al., 2011a). On the other hand, in mouse bone marrow stromal endothelial cells, Notch signaling reduces miR-155, which targets an NF-κB inhibitor, κB-Ras1, to enhance pro-inflammatory cytokine production and myelo-proliferation(Wang et al., 2014). Thus, depending on endothelial cell types, miR-155 has pleiotropic roles in controlling NF-κB activity. This pleiotropism is observed in immune cells as well, where miR-155 antagonizes the NF-κB signaling by targeting the receptor adaptor molecule MyD88 in macrophages, and the TAK1 cofactor TAB2 in dendritic cells(Ceppi et al., 2009; Huang et al., 2010), while it drives NF-κB-dependent pro-inflammatory responses in macrophages through an unknown mechanism (Donners et al., 2012; Nazari-Jahantigh et al., 2012). In endothelial cells, miR-155 and the previously mentioned miR-146a depend on the formation of super enhancers mediated by NF-κB and BET bromodomain protein for their transcription and potentially Drosha processing (Duan et al., 2016; Suzuki et al., 2017). Similar to miR-155, miR-132 is another pleiotropic miRNA that both counteracts and promotes endothelial inflammation. As an

inflammation promoter, miR-132 increases NF-κB signaling by targeting SIRT1, a p65 deacetylator and inhibitor, to promote lipid metabolism-dependent inflammatory processes including ICAM-1 expression(Zhang et al., 2014a). As an inflammation inhibitor, Kaposi's sarcoma-associated herpes virus (KSHV)-induced miR-132 targets the transcriptional co-activator of NF-κB: p300, which in turn modulates the transcription of miR-132. This feedback loop may contribute to the transient expression of miR-132(Lagos et al., 2010). Interestingly, miR-132 also targets the key component of miRISC, the Argonaute Ago2, in primary human dermal lymphatic endothelial cells. This miR-132-mediated Ago2 suppression impairs the levels of at least miR-146a and miR-221, which control respectively inflammatory and angiogenic responses of endothelial cells. In this sense, miRNA machinery autoregulates itself in endothelial cells through miR-132 (Leonov et al., 2015).

Endothelial cells are activated by various shear-stress-sensitive miRNAs that are induced by pulsatile blood flow (PS) and reduced by oscillatory (OS) blood flow. Upon the exposure to PS, Krüppel-like Factor 2 (KLF2)-mediated transcription of miR-30 is activated. This miRNA then targets Angiopoietin-2 (Ang-2), a pro-inflammatory autocrine regulator that activates the transcription of NF-κB(Fiedler et al., 2006), to inhibit the inflammatory activation of endothelial cells exposed to PS. By this means, miR-30 inhibits the expression of E-selectin, ICAM-1 and VCAM-1 *in vitro*(Demolli et al., 2015). Moreover, a profiling of miRNA levels in athero-protective regions in mouse reveals that PS in these regions increased miR-10a(Fang et al., 2010). Further *in vitro* investigations revealed that athero-susceptible OS inhibits while athero-protective PS increases miR-10a in endothelial cells, and targets the transcription factor Gata6 leading to a downregulation of adhesion

molecule VCAM-1(Hergenreider et al., 2012). As for the mechanism behind the expression of miR-10a, blood flows mediate its transcription through KLF2-retinoid acid receptor-α (RARα)-RA-responsive element (RARE) axis. Specifically, PS induces the expression, nuclear localization, and association of RARα with the binding enhancer retinoid X receptor-α (RXRα), to augment the RARα binding to RARE and hence miR-10a expression. In contrast, OS induces the association of RARα with histone deacetylases HDAC-3/5/7, which impairs the RARα-directed miR-10a expression(Lee et al., 2017). In cultured human aortic endothelial cells (HAECs), miR-10a impairs NF-κB-mediated expression of E-selectin, VCAM-1, MCP-1, IL-6 and IL-8, through targeting two key regulators of IκBα degradation: TAK1 and βTRC (Fang et al., 2010). In contrast to miR-30 and miR-10a, miR-34a is reduced by PS and increased by OS. Since miR-34a de-represses the NF-κB activity through its direct regulation of SIRT1, miR-34 is thus involved in the flow-dependent regulation of endothelial inflammation by affecting the NF-κB-mediated expression of ICAM-1 and VCAM-1(34).

miRNAs targeting the nuclear effectors of NF-κB pathway

Importins α4 and α5 are the nuclear transport receptors for p65 and STAT3, respectively, and both importins are targeted by miR-223. Mesangial cell-derived IL-6 decreases miR-223 to de-repress NF-κB and STAT3 pathways, as well as ICAM-1 expression and monocyte adhesion. These processes boost the activation of glomerular endothelial cells, which might lead to renal injuries and IgA nephropathy (IgAN). In patients suffering from IgAN, circulating endothelial cells isolated through a flow of anti-CD146, anti-CD45 and anti-CD133 magnetic beads have decreased miR-223, which may serve as an IgAN marker (Bao et al., 2014).

Like miR-34a, miR-92a is another miRNA reduced by PS and increased by OS(Wu et al., 2011b). Furthermore, miR-92a shares the function of miR-34a in boosting NF-κB activity. In human aortic endothelial cells, miR-92a targets KLF2 and KLF4, two endothelial transcription factors that inhibit NF-κB activity by competing for access to the transcriptional co-activator p300/CBP (SenBanerjee et al., 2004; Zhou et al., 2012). miR-92a thus enhances the expression of inflammatory markers including E-selectin, VCAM-1, CCL2 and eNOS, and contributes to leukocyte adhesion. *In vivo,* its expression correlates with athero-susceptibility in swine(Fang and Davies, 2012; Taganov et al., 2006). In a mouse model, miR-92a augmented during atherogenesis, and miR-92a inhibitor increased KLF2 and KLF4 while decreased phosphorylated p65, as well as atherosclerotic plaque formation(Loyer et al., 2014). These *in vivo* observations suggest that miR-92a enhances NF-κB activity at two levels: by targeting KLF2/4 and by promoting the phosphorylation of p65. Yet, the latter mechanism remains elusive.

A distinct set of miRNAs is up-regulated by OS in endothelial cells to sustain inflammation. miR-663 is induced by OS and is necessary for the efficient transcription of E-selectin and VCAM-1, and monocyte binding to endothelium *in vitro*. Although not a direct target of miR-663, the transcription of KLF4 is increased by miR-663 knockdown(Hamik et al., 2007; Ni et al., 2011). OS also induces the c-Jun/AP-1-mediated transcription of miR-21, which targets peroxisome proliferator-activated receptor-α (PPARα), an inhibitor of AP-1. This feed forward loop enables the sustained induction of miR-21, contributing to the pro-inflammatory responses of vascular endothelium, including the expression of VCAM-1 and CCL2, and the consequential adhesion of monocytes *in vitro*(Zhou et al., 2011). Mechanistically, miR-21 targets Grhl3 and leads to the de-phosphorylation of eNOS to enhance pro-inflammatory responses(Pourhoseini et al., 2015).

1.2.3.2 miRNAs targeting cell adhesion molecules

Recent studies indicate that endothelial cell adhesion molecules, notably E-selectin, ICAM-1 and VCAM, are direct targets of miRNAs in response to inflammatory stimuli.

The exclusive expression of E-selectin by endothelial cells enables the adhesion and rolling of leukocytes on the endothelium(Barthel et al., 2007). It has been reported that miR-31 directly targets E-selectin to hinder the endothelial adhesion and transendothelial migration of neutrophils *in vitro* (Suárez et al., 2010; Zhong et al., 2016). The transcription of miR-31 depends on IL-1β-induced p38/JNK-c-Jun/c-Fos/Gata2 pathways (Suárez et al., 2010; Zhong et al., 2016).

Endothelial cells also express ICAM-1, a glycoprotein belonging to the immunoglobulin superfamily, to firmly bind to β2 integrin on the surface of adhering cells (Hubbard and Rothlein, 2000). The first endothelial miRNA revealed to target ICAM-1 is the passenger strand of miR-17 (herein miR-17*). By this means miR-17* impedes the endothelial adhesion of neutrophils *in vitro*. miR-17* can be induced by TNFα, though a mechanism that still remains to be discovered (Suárez et al., 2010). TNFα also up-regulates miR-141, another miRNA targeting ICAM-1, which diminishes the endothelial adhesion of leukocytes *in vitro*. In a murine model, miR-141 reduced ICAM-1 in heart and alleviated myocardial ischemia-reperfusion (MI/R) injury and ischemic heart disease. These cardioprotective effects of miR-141 could be attributed to the decreased infiltration of macrophages into ischemic myocardium tissue (Liu et al., 2015).

miR-221 and miR-222 are two other miRNAs that target ICAM-1 in endothelial cells, where HIV transactivator of transcription (Tat) represses their expression through a NF-κB-dependent mechanism, leading to a decrease of ICAM-1 and thus, increase the endothelial adhesion of monocytes *in vitro*. On the other hand, Tat also activates ERK/MAPK-NF-κB-mediated transcription of ICAM-1. In this regard, Tat modulates ICAM-1 at both the transcriptional and the post-transcriptional levels. The importance of this Tat-mediated expression of ICAM-1 in HIV-associated cardiomyopathies has been validated in HIV transgenic rats (Duan et al., 2013). Intriguingly, miR-221 and miR-222 depletion in HUVECs affects the entire miRNA profile, possibly through altering the levels of the transcription factors Ets1/2 (Martinez and Walhout, 2009; Poliseno et al., 2006), ZEB2 (Chen et al., 2010) or STAT5a (Dentelli et al., 2010), or eNOS. miR-221 and miR-222 thus controls the entire miRNA profile through modulating the whole transcriptome (Suárez et al., 2007).

Comparable to those miRNAs, in human mammary epithelial cell-1 (HMEC-1), miR-22 has recently been reported to target ICAM-1. In this study, ATP and UTP increase the transcription of miR-22 by acting as extracellular signaling molecules and transduce signals through P2Y2-receptor. *In vitro*, ATP and UTP alleviate epithelial ICAM-1 expression and leukocyte adhesion through up-regulating miR-22(Gidlöf et al., 2015). Whether this mechanism is conserved in endothelial cells warrants further investigation.

Like ICAM-1, VCAM-1 is implicated in the firm adhesion to endothelial cells (Iademarco et al., 1993). The most scrutinized miRNA targeting VCAM-1 is the endothelial cell-specific miR-126, which represses leukocyte adhesion to endothelial cells and exhibits anti-inflammatory functions *in vitro* (Harris et al., 2008). In the kidney of mice, miR-126 contributes to the segmental, heterogenic expression of VCAM-1 by renal microvascular endothelial cells in response to systemic inflammatory stimuli (Asgeirsdóttir et al., 2012). The expression of miR-126 is regulated by factors as varied as estrogens, endotoxins, injuries, blood flow, and glucose metabolism. The estrogen 17β-estradiol (E2) up-regulates c-Src/Akt-mediated expression of the transcription factor Ets-1 to increase miR-126. By this means E2 decreases VCAM-1 and SPRED1, a protein inhibitor of mitogenic signaling and another target of miR-126, partially blocks monocyte adhesion, endothelial cell proliferation, migration and tube formation *in vitro*. Along these lines, miR-126 controls estrogen's anti-atherogenic effects and mitigates antherogenesis in $ApoE^{-/-}$ mice (Li et al., 2017c). Another factor that mediates miR-126 through modulating transcription factors is the endotoxin LPS, which down-regulates miR-126 at the transcriptional level via reducing its transcription factor KLF2. This down-regulation of miR-126 in endothelial cells results in de-repressed VCAM-1 and SPRED1, increased apoptosis, and decreased endothelial cell

proliferation and migration *in vitro*. *In vivo* over-expressing miR-126 with lentivirus attenuates LPS-induced vascular injuries and increases the survival rate of LPS-intoxicated mice (Chu et al., 2016). The down-regulation of miR-126 has also been observed following spinal cord injury (SCI) in mice. In these mice, mimic-miR-126 inhibits vascular inflammation and promotes angiogenesis by targeting VCAM-1, SPRED1 and PIK3R2 (negative regulator of PI3K/AKT pathway) in endothelial cells and thereby exerts a positive effect on the recovery from SCI (Hu et al., 2015). Blood flow also affects miR-126 level in that PS-responsive transcription factor KLF2 regulates several miRNAs including miR-126 in endothelial cells(Bartel, 2004; Nicoli et al., 2010; Wienholds et al., 2005). Further corroborating the PS-KLF2-miR-126 axis, a putative KLF2 binding site is located 1 kb upstream of the miR-126 transcriptional start site in human(Harris et al., 2010). In zebrafish, it has been demonstrated that the ortholog of KLF2, klf2a, regulates miR-126 to induce flow-stimulated angiogenesis(Nicoli et al., 2010). Considering that KLF2 also inhibits the transcription of VCAM-1 and E-selectin through inhibiting the NF-κB pathway(Lin et al., 2005; SenBanerjee et al., 2004), this transcription factor could be of great value to resolve inflammatory responses. Genetically speaking, miR-126 is expressed from intron 7 of epidermal growth factor-like domain 7 (EGFL7), an endothelial cell-secreted protein essential for vascular development. miR-126 is excised from EGFL7 pre-mRNA without affecting the splicing or the expression of its host gene since its knockdown did not alter EGFL7 at the mRNA or the protein level (Zhu et al., 2011b). Zhang et al. reported that the passenger strand of miR-126, miR-126* targets the chemokine CXCL12 to negatively affect VCAM-1 production(Zhang et al., 2013), a function shared by miR-126(Mondadori dos Santos et al., 2015). *In vitro*, both miR-126 and miR-126* are down-

regulated by TNFα and IFNγ in human brain endothelial cells, to allow an increase in E-selectin and VCAM-1 levels, and associated leukocyte adhesion (Cerutti et al., 2017).

1.2.3.3 miRNAs as paracrine regulators of inflammation

The presence of miRNAs in the whole blood can serve both as markers and modulators of inflammatory diseases. In a population study, positive correlations between circulating miR-1185 and the endothelial expression of E-selectin/VCAM-1 have been observed, and the expression of these cell adhesion molecules further correlates positively with arterial stiffness, a characteristic of atherosclerosis. In vitro assays confirmed the relationships between miR-1185 and E-selectin/VCAM-1, suggesting a miR-1185-E-selectin/VCAM-1-atherosclerosis axis, despite that the direct targets of miR-1185 in the scenario remain elusive(Deng et al., 2017a). Another study that measured the level of miR-122 in blood samples taken from patients and rats suffering from ischemic stroke, showed a correlation with the decrease of this miRNA with an increase in expression in the brain of direct target genes such as VCAM-1 and indirect target genes such as ICAM-1. Interestingly, elevating blood miR-122 level in rats reduced the expression of these genes and thus improved stroke outcomes(Liu et al., 2016).

miRNAs can be secreted from cells in various forms: associated with RNA-binding proteins such as Ago2 and nucleophosmin, within high/low-density lipoprotein (HDL/LDL) particles, and within microvesicles (MVs) including microparticles (MPs), exosomes, and apoptotic bodies (ABs)(Bayraktar et al., 2017). Circulating miRNAs can be used as biomarkers for inflammatory and cardiovascular diseases. Notably, circulating

inflammation-associated miR-126, miR-17, miR-92a, miR-155 and miR-181b are decreased in CAD patients (Fichtlscherer et al., 2010; Sun et al., 2014). In addition to their potential as diagnostic biomarkers, circulating miRNAs are capable of entering endothelial cells and regulate inflammatory responses. Along these lines, HDL has the capacity to transfer functional miRNAs. Purified HDL from plasma can suppress ICAM-1 expression by endothelial cells *in vitro* through transferring miR-223, which directly targets ICAM-1, into endothelial cells (Tabet et al., 2014).

Furthermore, the observation that some miRNAs can be selectively loaded or excluded from MVs(Li et al., 2013; Pigati et al., 2010), along with the report that blood MPs are increased during inflammatory pathologies (Bernal-Mizrachi et al., 2003; Bulut et al., 2009; Mallat et al., 2000; Nozaki et al., 2009), suggest that investigating MV miRNAs as paracrine regulators of vascular inflammation is particularly intriguing and attractive (**Fig. 4**). MVs are the major form of miRNA transfer in the vascular system. miR-126, which targets VCAM-1 and several other pro-inflammatory mediators(Harris et al., 2008), is the most studied miRNA as paracrine regulator in endothelial cells. It can be transferred between endothelial cells by endothelial MVs (EMVs). *In vitro*, the pro-resolution lipid mediator lipoxin (LX)A_4 bolsters both the production of miR-126 in donor endothelial cells and its EMV transfer to recipient endothelial cells, where EMV miR-126 abrogates the expression of VCAM-1 and SPRED1. EMV miR-126 thus inhibits vascular inflammation and promotes endothelial migration of recipient cells (Codagnone et al., 2017). Endothelial ABs (EABs) have also been reported to transfer miR-126 between neighboring endothelial cells. These miR-126 containing bodies restrain the production of CXCL12 by endothelial cells *in vitro*, and injection of the EABs alleviates atherosclerosis in mouse models (Zernecke et al., 2009).

Plasma miR-126 exhibits indirect anti-thrombotic properties and attenuates vascular inflammatory responses (expression of VCAM-1 and fibrinogen, leukocyte counts) by targeting tissue factor (TF) in monocytes(Witkowski et al., 2016). Endothelial MPs (EMPs) harboring miRNAs enable molecular cross-talk between endothelial cells. They are released by activated or apoptotic endothelial cells and can be taken up by adjacent endothelial cells. EMPs significantly reduce endothelial ICAM-1 and monocyte adhesion both *in vitro* and in mice by transporting miR-222 between endothelial cells to target ICAM-1(Jansen et al., 2015). EMPs derived from glucose-treated endothelial cells (simulating diabetic conditions) contain lower miR-222 and have reduced anti-inflammatory capacity. This *in vitro* observation is in line with the diminished circulating miR-222 in patients with CAD, supporting a role of EMP miR-222 in the pathology (Jansen et al., 2015). Platelets can release exosomal miRNAs when they are challenged by stimuli such as thrombin and myocardial infarction (MI). Thrombin-activated platelet exosomes contain elevated miR-223. Upon entering HUVECs, miR-223 inhibits NF-κB and MAPK pathways, and ICAM-1 expression, possibly through targeting importin α4/α5. The author proposed that miR-223 is the key factor behind the protective effects of platelet-derived exosomes on endothelial inflammation and atherosclerosis(Li et al., 2017b). On top of that, platelets activated by MI release miR-22 and miR-320b, of which miR-320b can be transferred to endothelial cells to target ICAM-1, thus playing a crucial role in the pathogenesis of MI(Gidlöf et al., 2013) (*Figure 1-19*).

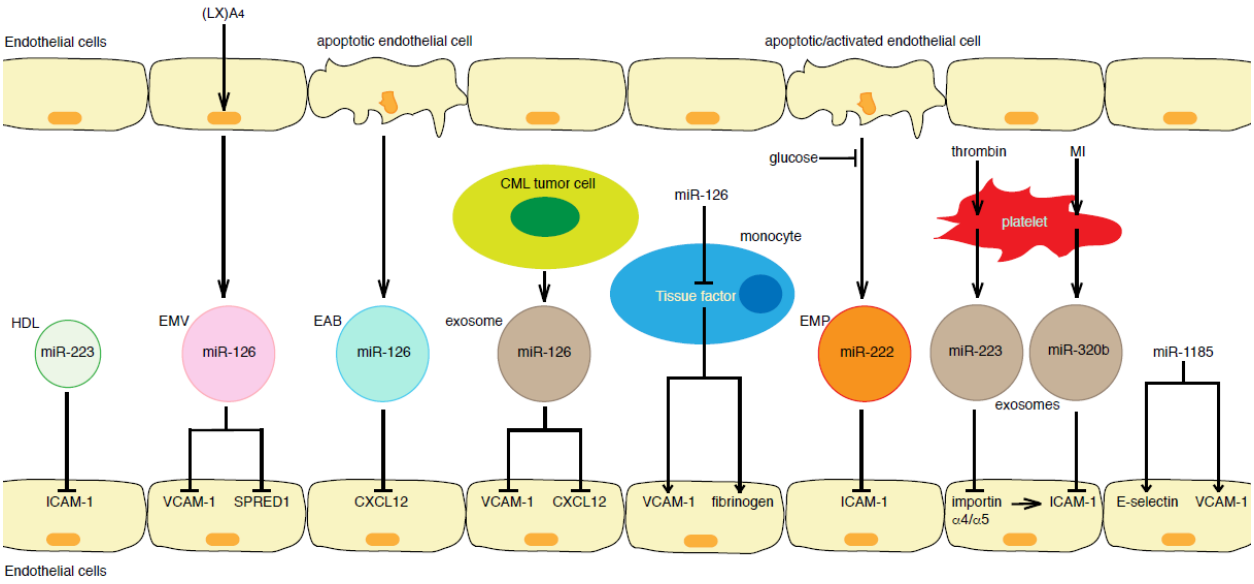


Figure 1-19. miRNAs as paracrine regulators of inflammation. *As described in the text, several miRNAs can be secreted from different types of cells. In most cases, endothelial cells are the recipients, unless mentioned otherwise. Plasma high-density lipoprotein (HDL) carries miR-223 to target ICAM-1. (LX)A_4 induces endothelial microvesicles (EMV)-mediated delivery of miR-126, which targets VCAM-1 and SPRED1. Endothelial apoptotic bodies (EABs) transfer miR-126 to target CXCL12. CML-tumor-secreted exosomal miR-126 targets VCAM-1 and CXCL12. Plasma miR-126 targets tissue factor in monocytes to indirectly reduce VCAM-1 and fibrinogen expressed by endothelial cells. Activated/apoptotic endothelial cells release endothelial microparticles (EMPs) containing miR-222 to target ICAM-1, in a process inhibited by glucose. Upon stimulation with thrombin, platelets derive exosomal miR-223, which targets importin α4/α5 and reduces ICAM-1. Platelets activated by myocardial infarction (MI) release miR-320b to target ICAM-1. Blood miR-1185 enhances the expression of E-selectin and VCAM-1 through an unknow mechanism (Zhong et al., 2018).*

1.2.3.4 miRNA-based therapeutics to treat inflammation-associated diseases

While miRNAs are emerging as therapeutic tools to treat vascular inflammation-associated diseases, numerous challenges remain due to their potential off-target effects. Therefore,

the development of carriers for their selective delivery to inflammatory endothelium is pressing. To deliver anti-inflammatory miRNAs miR-146a and miR-181b to inflamed endothelium, an E-selectin-targeting multistage vector (ESTA-MSV) has been developed. *In vitro*, miR-146a and miR-181b packaged in ESTA-MSV efficiently suppressed the expression of cell adhesion molecules and chemokines by inflamed endothelial cells. They also abolished monocyte adhesion to these endothelial cells. In mice, treatment with the vectors reduced vascular inflammation markers and atherosclerotic plaque size(Ma et al., 2016b). In parallel, coated cationic lipoparticles (CCLs) decorated with VHPK peptide targeting VCAM-1 have been engineered. These nanoparticles successfully delivered anti-miR-712 to inflamed mouse aortic endothelial cells *in vitro* and *in vivo*, where they rescued the expression of the targets of miR-712: TIMP3 and RECK, and prevented atheroma formation in a mouse model of atherosclerosis(Kheirolomoom et al., 2015). Another useful lipid molecule is the polyelectrolyte complex micelles, which cannot only encapsulate and protect nucleic acids, but also be modified to include targeting capabilities. It has been reported that VHPKQHR-containing micelles (targeting VCAM-1) and REKA-containing micelles (targeting fibrin) can deliver anti-miR-92a into endothelial cells in an attempt to treat atherosclerosis (Kuo et al., 2014). Although not for delivering miRNAs, a polymeric nanoparticle made of low-molecular-weight polyamines and lipids for carrying siRNAs to endothelial cells *in vivo* have been developed (112). These nanoparticles may be exploited for miRNA transfer. Instead of using exogenous carriers, siRNA-delivering exosomes have been derived from dendritic cells. The tissue-specific targeting is achieved by fusing the exosomal membrane protein Lamp2 of dendritic cells to the neuron-specific RVG peptide to

carry siRNAs to mouse brain (Alvarez-Erviti et al., 2011). We can expect this approach being adapted to deliver miRNAs to vascular endothelium.

In parallel, significant advances have been made to enhance the uptake and/or specificity of miRNAs to treat endothelial inflammation. Given the importance of miR-126 in protecting against inflammation and promoting angiogenesis, Rohde and colleagues developed a chimera for its delivery, by linking pre-miR-126 to an aptamer of the ubiquitously expressed transferrin receptor (transferrin receptor aptamer, TRA), which gave rise to efficient uptake and processing of pre-miR-126. *In vitro* TRA-miR-126 chimera repressed the endothelial expression of VCAM-1 and improved endothelial cell sprouting in a spheroid assay (Rohde et al., 2015). To improve the spatiotemporal accuracy and the specificity of E-selectin targeting, two artificial miRNAs, miR-E1 and miR-E2 complementary to E-selectin cDNA were cloned downstream of E-selectin promoter in plasmids, which suppressed E-selectin expression by HAECs and leukocyte adhesion, hence have the potential to prevent leukocyte infiltration in the brain(Yoshizaki et al., 2008). This target promoter-directed artificial miRNA expression system could be promising to specifically restrain inflammatory responses. Thus, the promising engineering progresses on delivery vectors and miRNAs *per se* substantiate the use of anti-inflammatory miRNAs as stable, specific and efficient therapeutic tools in a near future.

1.2.3.5 Conclusion and perspectives concerning the role of miRNAs in inflammation

There is now ample evidence that miRNAs modulate the vascular expression of endothelial cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 to mediate the extravasation of leukocytes during inflammation (*Table 1-3*). Numerous miRNAs regulate a network of genes in the NF-κB pathway, the major pathway driving inflammation (*Figure 1-17*) (Csiszar et al., 2008). Besides, all those three cell adhesion molecules can be directly targeted by miRNAs and this could limit the development of resistance to treatments. Since most of these miRNAs are modulated by inflammatory stimuli and blood flow, further research will likely reveal additional miRNAs providing extra layers of fine-tuning of vascular inflammation and inflammatory diseases such as atherosclerosis and sepsis, in response to these cues. *In vitro* experiments clearly indicate the pivotal roles played by endothelial miRNAs in inflammation through modulating cell adhesion molecules. However, most of the *in vivo* work linking miRNAs to inflammation to date has been conducted with miRNA mimics, miRNA inhibitors or genetic manipulation in mouse models. Cell adhesion molecule-targeting vectors exhibit high efficiency in delivering anti-inflammatory miRNAs to inflammatory sites, provide a more specific alternative for future *in vivo* studies, and hold great promises in treating inflammatory diseases. The discovery that miRNAs can be released from diverse types of cells to be carried by vectors especially microvesicles to enter endothelial cells added to the complexity of miRNA-mediated regulation of inflammation and underlines their potentials as paracrine regulators and biomarkers for pathologies such as atherosclerosis and diabetes (*Table 1-4*). Moreover, given that cancer cells can hijack the inflammatory process to extravasate and form metastases(Gout et al., 2008; St Hill, 2011; Stroka and Konstantopoulos, 2014), studies of miRNAs that regulate

the inflammatory process may lead as well to important new findings in the context of metastatic dissemination.

miRNA	Expression stimulus	Known regulator	Target	Secondary target	Function
miR-10a	PS and OS (I)	KLF2-RARα– or retinoid X receptor-α–RARE axis, HDAC-3/5/7 (I)	TAK1, β-TRC, GATA6, HOXA1	MCP-1, IL-6, IL-8, VCAM-1, E-selectin	Counteract the inflammatory response in atherosusceptible regions
miR-17*	TNF-α		ICAM-1		Counteract the inflammatory response
miR-21	Leptin		Grhl3	P-NOS3, ICAM-1 (E), VEGFR-2 (E), E-selectin (E)	Promote the inflammatory response, SED and NASH
	OS	c-Jun (AP-1)	PPAR-α, PPAR-γ	VCAM-1, CCL2	Sustain the inflammatory response under OS
miR-22	Extracellular ATP/UTP		ICAM-1		Counteract the inflammatory response
miR-23b	LPS (I), IL-17 (I)		TAB2, TAB3, IKK-α	NF-κB, TNF-α, IL-6, ICAM-1, VCAM-1, E-selectin	Counteract the inflammatory response; prevent autoimmune diseases including sepsis
miR-29b	High-fat diet		MT1	ICAM-1	Promote high-fat diet–stimulated endothelial permeability and apoptosis
miR-30	PS	KLF2	Ang-2	E-selectin, ICAM-1, VCAM-1	Counteract the inflammatory response under PS
miR-31	TNF-α, IL-1β	p38/JNK/c-Fos/Gata2 axis	E-selectin		Counteract the inflammatory response; inhibit the transendothelial migration of metastatic colon cancer cells
miR-34a	OS, PS (I)		SIRT1	VCAM-1, ICAM-1	Counteract the inflammatory response
miR-92a	OS, PS (I), low shear stress + ox-LDL		KLF2, KLF4	CCL2, VCAM-1, E-selectin, NOS3, P-p65	Promote the inflammatory response and atherosusceptibility
miR-125a	Ox-LDL (I)			ICAM-1, VCAM-1, ROS, NO (E), cleaved caspase-3	Counteract the effect of ox-LDL on apoptosis, senescence and inflammation; promote angiogenesis
miR-125b	Ox-LDL, PDGF		TRAF6, p53, Bax, Bak1, PODXL	ICAM-1, VCAM-1, transgelin, LOX-1, VE-cadherin, CCL2, IL-6	Counteract the inflammatory response; suppress proliferation and migration; modulate arteriosclerosis obliterans
miR-126	TNF-α (I), IFN-γ (I), LPS (I), PS, E_2, UnAG	c-Src/Akt, Ets1/2, KLF2	VCAM-1, SPRED1, PIK3R2, PAK1, CXCL12, VEGFA	RGS16 (E), CXCR4 (E), SDC-4 (E), SIRT1 (E), SOD-2 (E)	Counteract the inflammatory response; attenuate atherosclerosis
miR-126*	TNF-α (I) and IFN-γ (I)		CXCL12	E-selectin, VCAM-1	Counteract the inflammatory response

(Continued)

miRNA	Expression stimulus	Known regulator	Target	Secondary target	Function
miR-132	Kaposi's sarcoma-associated herpes virus	EP300	SIRT1, EP300, Ago2	SREBP-1c, FASN, HMGCR	Promote lipid metabolism–dependent inflammatory response and apoptosis; inhibit proliferation, viability and migration; counteract viral inflammation
miR-141	TNF-α		ICAM-1		Counteract the inflammatory response; attenuate myocardial ischemia-reperfusion injury
miR-146a	TNF-α, IL-1β, LPS	EGR-3, NF-κB	HuR, TRAF6, IRAK1, IRAK2, Card10	eNOS (E), IL-17, ICAM-1, VCAM-1, E-selectin	Counteract the inflammatory response
miR-146b	Ang-1		TRAF6, IRAK1	TNF-α, IL-1β, IL-6, IL-8, ICAM-1, VCAM-1, E-selectin	Counteract the inflammatory response
miR-155	Inflammatory cytokines	NF-κB, Notch signaling (I)	p65, AT1R, Ets-1, MAP3K10, eNOS	VCAM-1, ICAM-1, ROS, NO, caspase-3	Counteract/promote the inflammatory response
miR-181b	TNF-α (I)		Card10, importin-α3	TF, ICAM-1, VCAM-1, E-selectin	Counteract the inflammatory response
miR-221	IFN-γ (I), Tat (I)		ICAM-1	Ets1/2, ZEB2, STAT5a, eNOS	Counteract the inflammatory response; inhibit cholangiocyte interaction with effector lymphocyte
miR-222	Tat (I)	NF-κB	ICAM-1	Ets1/2, ZEB2, STAT5a, eNOS	Counteract the inflammatory response in HIV-associated cardiomyopathy; inhibit susceptibility of tumor cells to CTL-mediated lysis
miR-223	IL-6 (I)	NF-κB	Importin-α4 and -α5	NF-κB/STAT3 signaling, ICAM-1	Inhibit GEnC activation (proliferation and monocyte adhesion) in IgAN
miR-663	OS			KLF4, VCAM-1 (E), E-selectin (E)	Promote OS-induced inflammatory response and atherosclerosis

Table 1-3. Endogenous endothelial miRNAs mediating inflammation. *(I) indicates that the expression stimulus or the known regulator acts as inhibitor of the expression of the miRNA; (E) indicates that the miRNA acts as enhancer of its target or secondary target.*

miRNA	Source	Transfer stimulus	Target	Secondary target	Function
miR-122	Blood	Ischemic stroke (I)	VCAM-1, Nos2, Pla2g2a	ICAM-1, Alox5, Itga2b, Timp3, IL-1β, IL-2, Mmp8	Inhibit brain inflammation; improve stroke outcomes
miR-126	EMVs	LXA_4	VCAM-1, SPRED1		Counteract the inflammatory response; enhance endotheli migration
	Plasma	Antidiabetic treatment	TF	VCAM-1, fibrinogen	Counteract the inflammatory response and thrombosis indirectly
	EABs		CXCL12		Counteract the inflammatory response; prevent atherosclerosis
miR-222	EMPs	Glucose (I)	ICAM-1		Counteract the inflammatory response; mediate diabetic conditions and CAD
miR-223	Activated platelets exosomes	Thrombin		NF-κB/MAPK signaling, ICAM-1	Counteract the inflammatory response; prevent atherosclerosis
	HDL		ICAM-1		Counteract the inflammatory response
miR-320b	Platelets	MI	ICAM-1		Regulate pathogenesis of MI
miR-1185	Blood		VCAM-1 (E), E-selectin (E)		Mediate arterial stiffness and atherosclerosis
miR-E_1/-E_2	Artificial miRNA–expressing vectors		E-selectin		Counteract the inflammatory response

Table 1-4. Exogenous miRNAs mediating inflammation. *(I) Indicates that the expression stimulus or the known regulator acts as inhibitor of the expression of the miRNA; (E) indicates that the miRNA acts as enhancer of its target or secondary target.*

1.2.4 miRNAs as the cause and consequence of cancer-related epigenetic alteration

1.2.4.1 As cause of cancer-related epigenetic alteration

Growing evidence shows the substantial role of miRNAs in the control of several canonical epigenetic mechanisms. Specifically, miRNAs regulate at the post-transcriptional level many epigenetic factors, including but not limited to: DNMTs, PRC1 and PRC2, heterochromatin protein 1 (HP1), and HDACs. Deregulation of these proteins caused by aberrant expression of miRNAs could lead to the epigenetic silencing of tumor suppressor genes (Kazanets et al., 2016).

Deregulation of DNMTs in cancer is sometimes attributable to miRNAs. miR-29, downregulated in lung cancer, targets DNMT3A-B. Exogenous expression of miR-29 results in a decrease of global DNA methylation and in the re-expression of tumor suppressor genes in lung cancer and in acute myeloid leukemia (Garzon et al., 2009). miR-29a was found to modulate DNMT1 and DNMT3B in hepatocellular carcinoma (Braconi et al., 2011). Similarly, in cholangiocarcinoma, miR-148a and miR-152 target DNMT1 to affect the transcription of tumor suppressor genes Ras association domain family member 1 (*RASSF1A*) and cyclin-dependent kinase inhibitor 2A (*p16INK4a*) (Braconi et al., 2010). miR-148 was also reported to target the coding region of DNMT3B (Duursma et al., 2008). A viral miRNA, miR-K12-4-5p, encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) also regulate DNMT3A-B through targeting RBL2, which is a repressor of the former (Benetti et al., 2008).

HDACs are up-regulated in various types of cancer (Witt et al., 2009). In prostate cancer miR-449a is down-regulated and is responsible for the direct de-repression of HDAC1. The exogenous expression of miR-449a in prostate cancer cells affects cell growth and viability, in

part by targeting HDAC1 (Noonan et al., 2009). In different cancer cell lines, HDAC1 was demonstrated to act as a repressor of miR-449, suggesting a feedback loop (Buurman et al., 2012). In hepatocellular carcinoma, miR-145 negatively regulates HDAC2 expression and is down-regulated. Re-introduction of miR-145 reduces the tumorigenic potential of hepatocellular carcinoma cells *in vitro* and *in vivo*, recapitulating the effects of HDAC2 inhibition (Noh et al., 2013).

1.2.4.2 As consequence of cancer-related epigenetic alteration

In 2007, Weber et al. found that 47% of investigated human miRNA (47%) were associated with CpG islands (CGIs), suggesting that miRNAs were subject to transcriptional regulation by DNA methylation (Weber et al., 2007). The first evidence of regulation of miRNAs by DNA methylation came from a profiling of miRNA expression of the T24 bladder cancer cell line after treatment with the DNA de-methylating agent 5-Aza-2′-deoxycytidine (5-AZA), in combination with an HDAC inhibitor (4-phenylbutyric acid; 4-PBA). Where miR-127 was found to be up-regulated, with consequent down-regulation of its target, the proto-oncogene B-cell lymphoma 6 (BCL6) (Saito et al., 2006). Later, in the HCT116 colorectal cancer cell line, miR-124a, miR-373, *and* miR-517c were demonstrated to be transcriptionally inactivated by CGI promoter methylation following depletion of *DNMT1* and *DNMT3B* (Lujambio et al., 2007).

Several tumor-specific studies showed aberrant methylation of miRNA genes. Aberrant methylation of miRNA genes is a recurrent theme in cancer, which underlines their biological importance in general tumorigenic processes. Hyper-methylation-associated silencing of miR-9, miR-34b/c, and miR-148a is prevalent in metastatic cell lines of colon, melanoma, head and neck,

breast, lung carcinomas (Lujambio et al., 2008). While *miR-9*, *miR-34b/c*, *miR-124a*, and *miR-148a* hyper-methylation was confirmed in breast cancer cells, together with *let-7a*, *miR-10b*, *miR-125b*, *miR126*, *miR-152*, *miR-195/497* and *miR-200* family. Moreover, down-regulation by methylation of *miR-149* was reported in clinical cases of chemoresistant breast cancer (Ramassone et al., 2018). In gastric cancer (GC) cell lines *miR-34b/c* and *miR-181c* were found to be epigenetically silenced by CGI hyper-methylation. This was postulated to contribute to the activation of NOTCH4 and KRAS proto-oncogenes, targets of these miRNAs (Suzuki et al., 2010). *miR*-9 has been reported aberrantly methylated in ovarian, renal, liver, lung, colorectal cancer, and multiple myeloma. Its silencing allows up-regulation of important oncogenic products, such as cyclin G1 (CCNG1) and epidermal growth factor (EGF) (Selcuklu et al., 2012). *miR-34*s are similarly methylated in several type of cancers, and their silencing affects cellular stemness by targeting CD44 and NOTCH1 (Liu et al., 2011).

The first evidence of deregulation of miRNA due to histone modification in cancer cells was reported by Scott et al. in 2006. These authors treated SKBr3 breast cancer cells with an HDACs inhibitor and observed aberrant expression of 27 miRNAs (Scott et al., 2006). In chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), HADCs are overexpressed, leading to epigenetically silenced miR-15a and miR-16 (Zhang et al., 2012). The oncogenic miR-155 has been found to be epigenetically repressed in breast cancer by BRCA1, which recruits HDAC2 to the miR-155 promoter (Chang et al., 2011). Wang et al. demonstrated that in HCC HDAC1 and HDAC3 act as negative regulators of miR-224 expression, whereas the histone acetyl-transferase EP300 is a positive regulator. In normal cells, the *miR-224* locus is transcriptionally quiescent by HDAC1 and HDAC3, while during cellular transformation, it is activated by the overexpression of EP300, which could represent a potential drug target to

reverse miR-224 overexpression in HCC (Wang et al., 2012). Histone methylation also plays a significant role in miRNA regulation in cancer. In an osteosarcoma cell line, miR-449a/b is epigenetically repressed through the tri-methylation of the lysine 27 on the histone H3 (H3K27me3), which is reversible by epigenetic drug treatment (Yang et al., 2009). In addition, EZH2 downregulates multiple miRNAs through mediating H3K27me3 in cancer. Such miRNAs include miR-139-5p, miR-125b, miR-101, let-7c, and miR-200b in HCC, miR-181a, miR-181b, miR-200b, miR-200c, and miR-203 in prostate cancer, and miR-31 in prostate cancer (Au et al., 2012; Cao et al., 2011; Zhang et al., 2014).

1.3 Hypothesis and objectives

Extravasation of circulating cancer cells is a key event of metastatic dissemination that is initiated by the adhesion of cancer cells to vascular endothelial cells. It requires interactions between adhesion receptors present on endothelial cells and their ligands on cancer cells. Notably, E-selectin is an endothelial cell adhesion receptor induced by inflammation that binds to Death Receptor-3 and other receptors expressed by colon cancer cells to enable their extravasation. This research project intends to uncover the mechanisms that regulate E-selectin expression and functions. The central hypothesis is that miRNAs by targeting E-selectin expression directly or indirectly can modulate the metastatic abilities; i.e. the adhesion to endothelial cell monolayer and the transendothelial migration.

Three objectives were pursued during my doctoral studies:

1. To identify miRNAs that modulate the expression of E-selectin, and to characterize the mechanism of the regulation. The miRNAs can either target E-selectin directly at the post-transcriptional level, indirectly at the transcriptional level by targeting certain transcription factors, or even at the turnover level by affecting its internalization and degradation.
2. Since miRNAs that mediate the expression of E-selectin might be induced by the same pro-inflammatory stimulus that activates E-selectin expression, the mechanism of the pro-inflammatory cytokine-mediated expression of miRNAs is investigated. The signaling pathways, especially the role of MAP kinase pathways are revealed using their respective small molecule inhibitors; the involvement of transcription factors is examined with shRNA-mediated gene silencing.

3. Most importantly, to scrutiny the miRNA-mediated regulation of E-selectin-dependent metastatic abilities of colon cancer cells. Colon cancer cell-endothelial cell adhesion assay is carried out, followed by transendothelial migration assay in Boyden chambers.

Following the present introductory chapters, the readers will find the development of these three goals in the main Chapters 2 and 3 of the thesis, focusing on two sets of miRNAs modulating E-selectin at the transcriptional and the post-transcriptional levels respectively, followed by a general discussion in Chapter 4, and conclusion and perspectives in Chapter 5.

Chapter 2. p38 and JNK pathways control E-selectin-dependent extravasation of colon cancer cells by modulating miR-31 transcription

Foreword

The present chapter is constituted by an article published in the journal 'Oncotarget' (Zhong et al., 2016). I performed most of the experiments presented in the manuscript. Bryan Simoneau carried out the adhesion assay and the trans-endothelial migration assay of colon cancer cells. Drs. Simard and Huot supervised the work and the writing.

p38 and JNK pathways control E-selectin-dependent extravasation of colon cancer cells by modulating miR-31 transcription

Liang Zhong, Bryan Simoneau, Jacques Huot* and Martin J. Simard*

Laval University Cancer Research Center, Hôtel-Dieu de Québec (CHUQ), Quebec City, QC G1R 2J6, Canada

Corresponding author:

Jacques Huot, Martin J. Simard

Email : Jacques.Huot@fmed.ulaval.ca, Martin.Simard@crchudequebec.ulaval.ca

Key words: c-Fos, c-Jun, GATA2, IL-1β, metastasis, endothelial cells

2.1 Résumé

L'extravasation des cellules cancéreuses circulantes est la clef de la dissémination métastatique qui est initiée par l'adhérence de ces cellules aux cellules endothéliales vasculaires. Elle nécessite l'interaction entre les récepteurs d'adhérence comme E-sélectine sur les cellules endothéliales et leurs ligands sur les cellules cancéreuse. Notamment, E-sélectine influence le potentiel métastatique des cancers du sein, de la vessie, de l'estomac, du pancréas et du côlon, des leucémies et lymphomes. Ici, on montre qu'un microARN, miR-31, cible directement le mRNA de E-sélectine. La transcription de miR-31 est activée par IL-1β et cette activation dépend de p38 et JNK MAP kinases et les facteurs de transcription en aval, GATA2, c-Fos et c-Jun. L'inhibition de E-sélectine médiée par miR-31 diminue le potentiel métastatique de cellules de cancer du côlon en réduisant leur adhérence à l'endothélium, et leur migration transendothéliale. Ces résultats soulignent pour la première fois qu'un microARN médie l'extravasation des cellules du cancer du côlon dépendante de l'E-sélectine.

2.2 Abstract

Extravasation of circulating cancer cells is a key event of metastatic dissemination that is initiated by the adhesion of cancer cells to vascular endothelial cells. It requires the interaction between adhesion receptors such as E-selectin present on endothelial cells and their ligands on cancer cells. Notably, E-selectin influences the metastatic potential of breast, bladder, gastric, pancreatic, and colorectal carcinoma as well as of leukemia and lymphoma. Here, we show that E-selectin expression induced by the pro-inflammatory cytokine IL-1β is directly and negatively regulated by miR-31. The transcription of miR-31 is activated by IL-1β. This activation depends on p38 and JNK MAP kinases, and their downstream transcription factors GATA2, c-Fos and c-Jun. The miR-31-mediated repression of E-selectin impairs the metastatic potential of colon cancer cells by decreasing their adhesion to, and migration through, the endothelium. These results highlight for the first time that microRNA mediates E-selectin-dependent extravasation of colon cancer cells.

2.3 Introduction

The metastatic process consists of sequential interrelated steps, all of which must be completed successfully to cause a secondary tumor (Gout and Huot, 2008). In particular, the adhesion of cancer cells to endothelium of blood vessels is a prerequisite for extravasation of circulating cancer cells and their metastatic dissemination. This adhesive event requires specific interactions between adhesion receptors present on vascular endothelial cells and their ligands on cancer cells. E-selectin is an inducible member of the selectin adhesion receptor family that is expressed exclusively by endothelial cells stimulated by pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) (Gout et al., 2008). The physiological role of E-selectin is to mediate the adhesion and subsequent rolling of leukocytes on the endothelium. Thereafter, the rolling leukocytes bind more firmly to other cell adhesion molecules (ICAM and VCAM), which lead to their extravasation into inflamed tissues (Vestweber and Blanks, 1999). Cancer cells can hijack the inflammatory process and use E-selectin to extravasate and form metastases (Laferrière et al., 2004). Several studies suggest that E-selectin is a key determinant of metastasis. The binding efficiency of colon cancer cell lines to E-selectin is proportional to their respective metastatic potential (Sawada et al., 1994) and hence, anti-E-selectin antibodies are capable of reducing experimental liver metastasis (Brodt et al., 1997). Other cancer types showing a strong E-selectin-dependent metastatic pattern include breast, bladder, gastric, pancreatic, and colorectal carcinoma as well as some leukemia and lymphoma (Gout et al., 2008; Reymond et al., 2013). The expression of E-selectin relies on the activation of JNK, p38 and PI3K pathways. However, apart from its degradation taking place in lysosomes (Subramaniam et al., 1993), little is known about mechanisms that downregulate E-selectin and inhibit E-selectin-mediated extravasation.

MicroRNAs (miRNAs) are single-stranded, evolutionarily conserved, small (~21 nucleotides long) non-coding RNAs. At first, they are transcribed as primary miRNAs (pri-miRNAs), mainly from intergenic or intronic regions by RNA polymerase II. Pri-miRNAs are processed by Drosha-DGCR8 complex in the nucleus to produce precursor miRNAs (pre-miRNAs), which are exported to the cytoplasm to be cleaved by Dicer, producing mature miRNAs that are loaded into the miRNA-induced silencing complex (miRISC). Through base pairing with the 3' untranslated region (3' UTR) of mRNA, miRNA guides the miRISC to its target, thereby repressing translation with or without causing its degradation. Interestingly, a recent finding suggested that, the expression of E-selectin is regulated by miR-31 during inflammation (Suárez et al., 2010). It is therefore possible that miR-31 contributes to the regulation of E-selectin-dependent extravasation of cancer cells as well.

In this study, we show that IL-1β induces the expression of miR-31 in endothelial cells, which directly targets the 3'UTR of E-selectin mRNA and inhibits its expression. We also report that IL-1β-induced an increase of miR-31 expression that results from activation of both p38 and JNK MAP kinases, and their downstream transcription factors GATA2, c-Fos, and c-Jun. Specific inhibition of miR-31 is associated with increased E-selectin-dependent adhesion and transendothelial migration of colon cancer cells.

2.4 Results

IL-1β induces the expression of miR-31, which affects E-selectin abundance

E-selectin is an adhesion receptor expressed exclusively by endothelial cells upon stimulation by inflammatory cytokines, such as TNFα and IL-1β (Laferriere et al., 2001). Following exposure of human umbilical vein endothelial cells (HUVECs) to IL-1β, the expression of E-selectin is greatly induced at 4h and decreased to near null level at 24h (*Figure 2-1a*). On the other hand, the expression of miR-31 was significantly increased following exposure to IL-1β, especially at 24h, which is inversely correlated with the observed decrease of E-selectin (*Figure 2-1b*). To further confirm this inverse correlation, endothelial cells were transfected with the inhibitor of miR-31 (henceforth anti-miR-31), which clearly increased the level of E-selectin (*Figure 2-1c*). This finding was further confirmed in another type of endothelial cells (human liver sinusoidal microvascular endothelial cells or HLSMECs, *Supplemental Figure 2-1a*) even if the endogenous level of miR-31 was significantly higher in those cells (Supplemental *Figure 2-1b*). Taken together, these findings indicate that miR-31 is an important regulator of E-selectin expression in endothelial cells.

To investigate whether miR-31 directly targets E-selectin by binding to the 3'UTR of its mRNA, we carried out a dual-luciferase reporter assay. The 3'UTR of E-selectin mRNA was cloned downstream of the *Renilla* luciferase reporter gene in an expressing vector that constitutively expresses firefly luciferase. The plasmid was transfected into HEK293T cells, a cell line constitutively expressing miR-31 (Lin et al., 2013; Meziane et al., 2015). After 48 hours, we measured the *Renilla* luciferase activity in transfected cells and observed that the *Renilla* luciferase expression under the control of the wt 3'UTR of E-selectin mRNA, was significantly

lower than the cells transfected with *Renilla* luciferase reporter construct with mutations in the miR-31 binding site within E-selectin 3'UTR (*Figure 2-1d*). To further confirm the miR-31-dependent regulation of E-Selectin 3'UTR, we co-transfected these cells with either control or inhibitor of miR-31 (anti-miR-31). When assessing the *Renilla* luciferase activity, we observed that only the reporter carrying the wt E-selectin 3'UTR was significantly relieved of the repression by miR-31 (*Figure 2-1d*).

Taken together, these findings suggest that miR-31 represses the expression of E-selectin through binding to the 3'UTR of its mRNA.

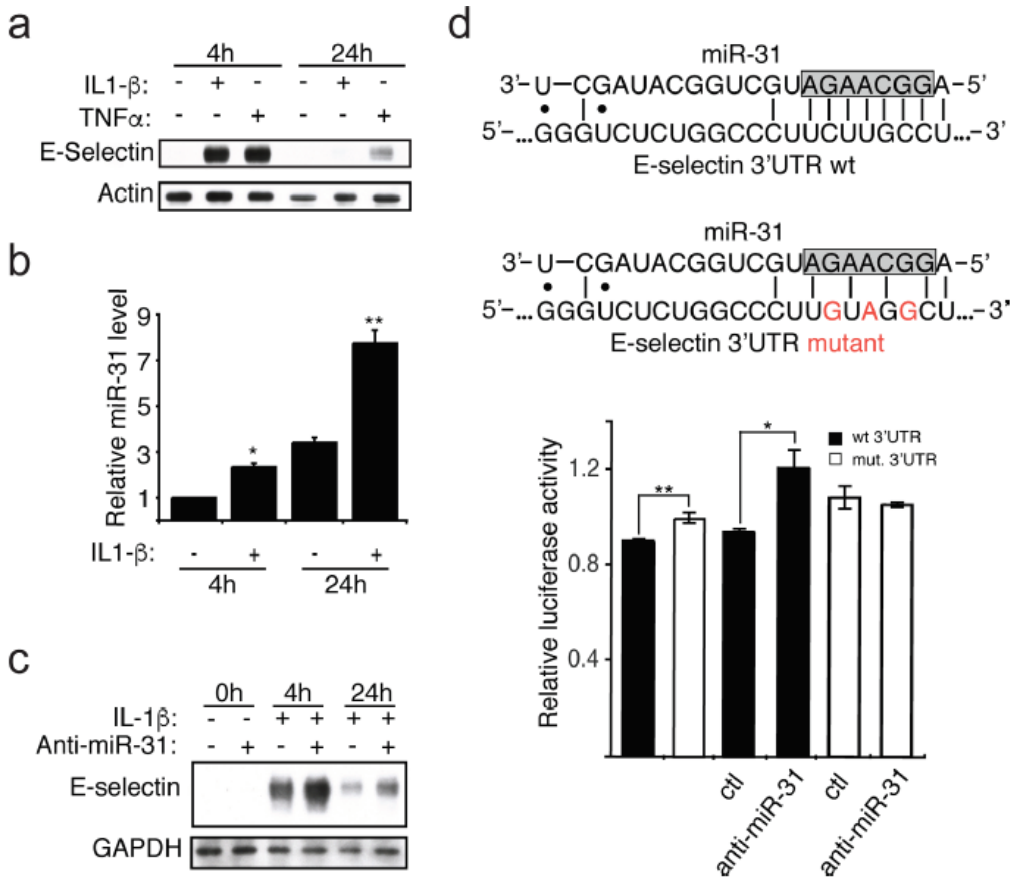
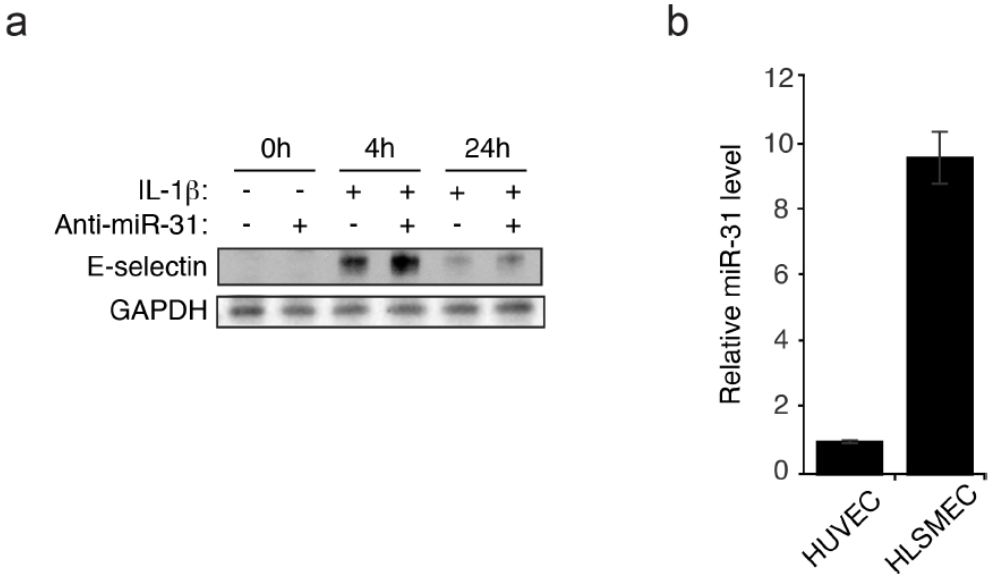


Figure 2-1. IL-1β induces the expression of miR-31, which affects E-selectin abundance. *(a) The monolayer of HUVEC endothelial cells was treated with IL-1β (20ng/ml) or TNFα (10ng/ml) for indicated hours. Western blotting monitored the expression of E-selectin and Actin used as*

*loading control. (**b**) The miR-31 level was measured by quantitative reverse transcription-PCR and the snRNA U6 was used as the normalization control. (**c**) Endothelial cells were transfected with 30nM of either anti-miR-31 (+) or corresponding inhibitor negative control (-) before the addition of IL-1β. The Western blots shown are representative of three independent experiments. The endogenous GAPDH was used as loading control. (**d**) Top: The representation of miR-31 base-pairing with either E-selectin wild-type (wt) or mutated (mut) 3' UTR sequence. The nucleotides in the gray box represent the seed region of miR-31, region important for target interaction. Bottom: E-selectin 3'UTR mediated reporter assays. Vector expressing luciferase reporter under the regulation of either wild-type (black bars) or mutated (white bars) E-selectin 3'UTR were transfected into HEK293T cells. 48 hours after transfection, relative luciferase activities were measured. Another set of reporters transfected cells were also transfected with 30nM of either anti-miR-31 or corresponding inhibitor negative control (ctl) to further test the contribution of miR-31 in E-selectin regulation. The error bars shown in panel b and d represent standard errors of three and six independent experiments, respectively. The significance was analyzed using a Student's t-test (*p <0.05; **p<0.01).*



Supplementary Figure S2-1: miR-31 affects E-selectin level in human liver sinusoidal microvascular endothelial cells. *a. HLSMEC endothelial cells were transfected with 75nM of either anti-miR-31 (+) or corresponding inhibitor negative control (-) before the addition (+) of IL-1β (20ng/ml). Western blotting monitored the expression of E-selectin and endogenous*

GAPDH was used as loading control. The Western blot shown a representative of five independent experiments. b. Comparative analysis of miR-31 miRNA levels in HUVEC and HLSMEC endothelial cells. The miR-31 level was measured by quantitative reverse transcription-PCR and the snRNA U6was used as the normalization control. The error bars represent standard errors of three independent experiments.

IL-1β induces the transcription of miR-31 via p38/JNK pathways

Since the IL-1β treatment of endothelial cells lead to an increase of the expression of miR-31, this raised the question whether the transcription of miR-31 is affected. When evaluating the level of the transcript of miR-31 gene locus called primary miR-31 or pri-miR-31, we observed an increase in its level when cells were treated with IL-1β, indicating that exposure to this cytokine induces the transcription of miR-31 (*Figure 2-2a*).

The expression of E-selectin is regulated by several signaling pathways that are activated by IL-1β, including ERK, JNK, PI3K and p38 pathways (Laferriere et al., 2002; Prasad et al., 2005; Tanigawa et al., 2013). In order to investigate whether the activation of these pathways in response to IL-1β also regulates the transcription of miR-31, endothelial cells were treated with IL-1β in the presence or the absence of inhibitors of MEK1/2 (PD098059), JNK (SP600125), PI3K (LY294002) or p38 (SB203580). Thereafter, the activation of ERK was determined by measuring its phosphorylation whereas activities of the other three kinases were assayed by evaluating the phosphorylation of their downstream targets: c-Jun (JNK), Akt (PI3K) and HSP27 (p38). The levels of pri-miR-31 and of mature miR-31 were determined concomitantly. We observed that IL-1β treatment increased the activation of each pathway: p38 and JNK (*Figure 2-2b and c*) as well as ERK and PI3K (*Supplemental Figure 2-3a and b*). The levels of pri-miR-31 and miR-31 were independent of ERK and PI3K given that they were not affected by PD098059

and LY294002 treatment, respectively (*Supplemental Figure 2-3*). In contrast, inhibiting JNK1 with SP600125, or p38 with SB203580 were associated with decreased transcription and expression of miR-31 (*Figure 2-2b* and *c*, *Supplemental Figure 2-2*). These results strongly suggest that IL-1β acts through JNK and p38 to induce the transcription of miR-31.

We next investigated which transcription factors downstream of p38 and JNK were involved in regulating the transcription of miR-31. Based on the publicly available Chromatin ImmunoPrecipitation Sequencing (ChIP-seq) data from UCSC Genome Browser, we selected three transcription factors that are associated with the regulatory region of miR-31 gene that could be involved in transcribing miR-31, namely c-Jun, c-Fos and GATA2. C-Jun can be activated by JNK (Minden et al., 1994) while c-Fos and GATA2 can be activated by p38 (Baumgartner and Baccarini, 2014; Chen et al., 2006; Tanos et al., 2005; Zarubin and Han, 2005). To determine whether these transcription factors are involved in the production of miR-31, lentiviral vectors expressing shRNAs were employed to silence these transcription factors in endothelial cells individually, and knockdown efficacy was assessed by Western blotting (*Figure 2-3*). We observed that the knockdown of all three transcription factors significantly decreased the level of pri-miR-31 and thus, that of miR-31 (*Figure 2-3* and *Supplemental Figure 2-4*). These data suggest that upon IL-1β treatment of endothelial cells, the activation of c-Jun mediated by JNK as well as the activation c-Fos and GATA2 by p38 lead to an increase of miR-31 expression.

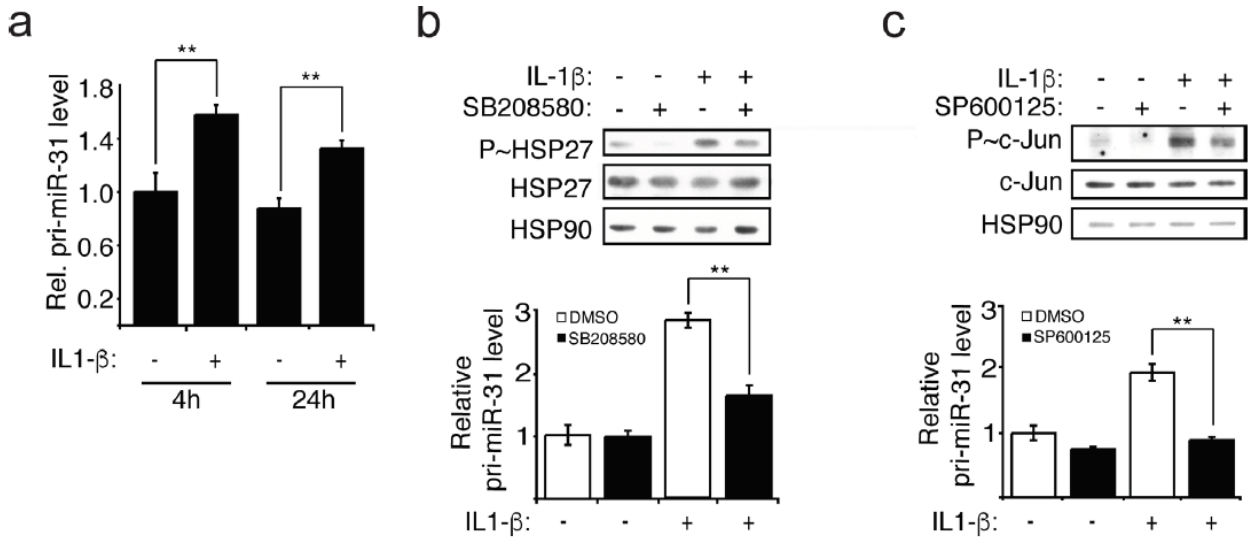


Figure 2-2. IL-1β induces the transcription of miR-31 via p38 and JNK. *(**a**) The pri-miR-31 levels were measured relative to GAPDH mRNA using quantitative reverse transcription-PCR (qRT-PCR). (**b-c**) HUVEC endothelial cells were pre-treated with 5μM of p38 inhibitor SB203580 or 10μM of JNK inhibitor SP600125 for 1 hour before the addition of IL-1β (20ng/ml). The inhibitions were confirmed by Western blotting showing decreased of phospho-HSP27 (P~HSP27) and phospho-c-Jun (P~c-Jun), downstream of p38 and JNK, respectively. The endogenous HSP90 was used as loading control. Pri-miR-31 levels relative to GAPDH mRNA were determined by qRT-PCR. The Western blots are representative of four independent experiments. The error bars represent standard errors of four independent experiments and significance was analyzed using a Student's t-test (*p <0.05; **p<0.01).*

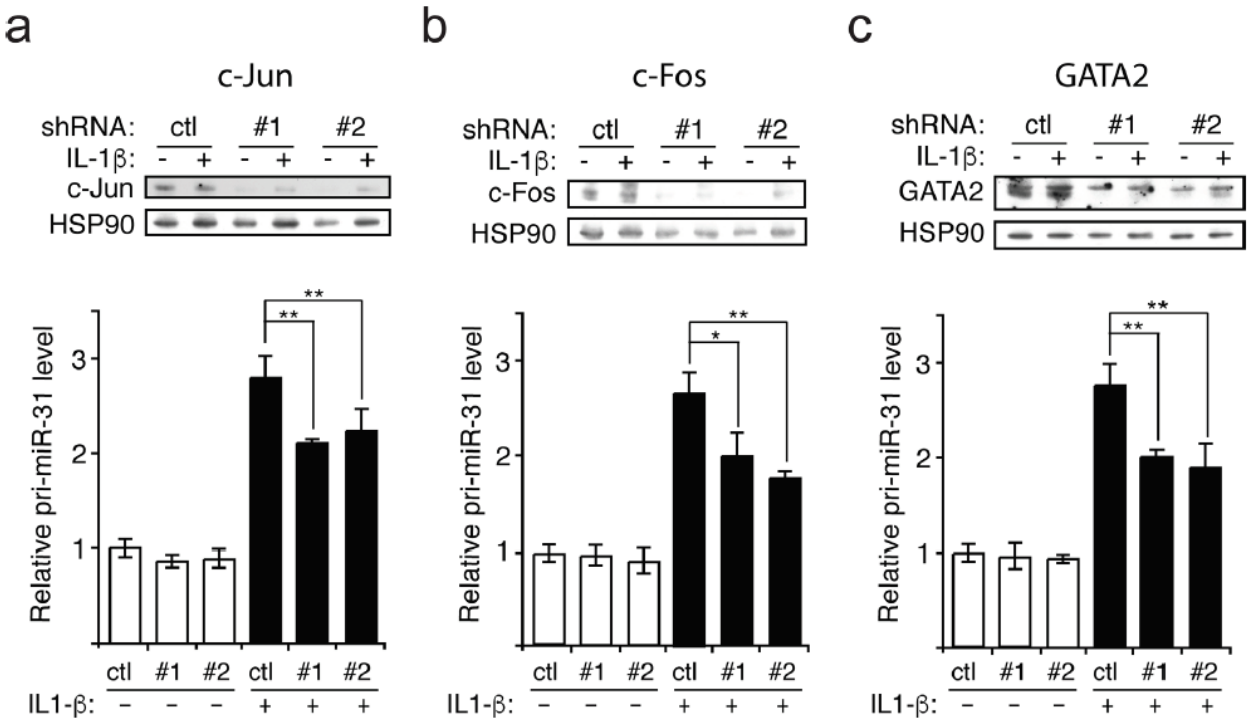
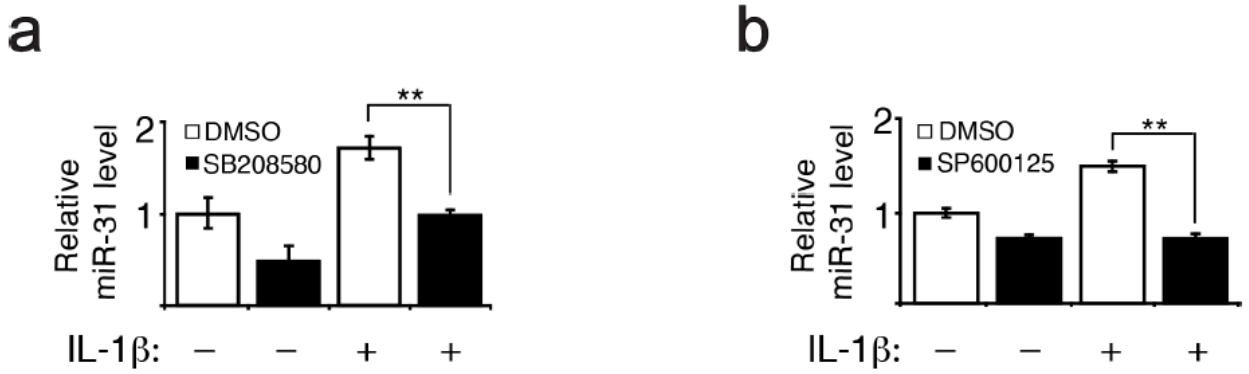
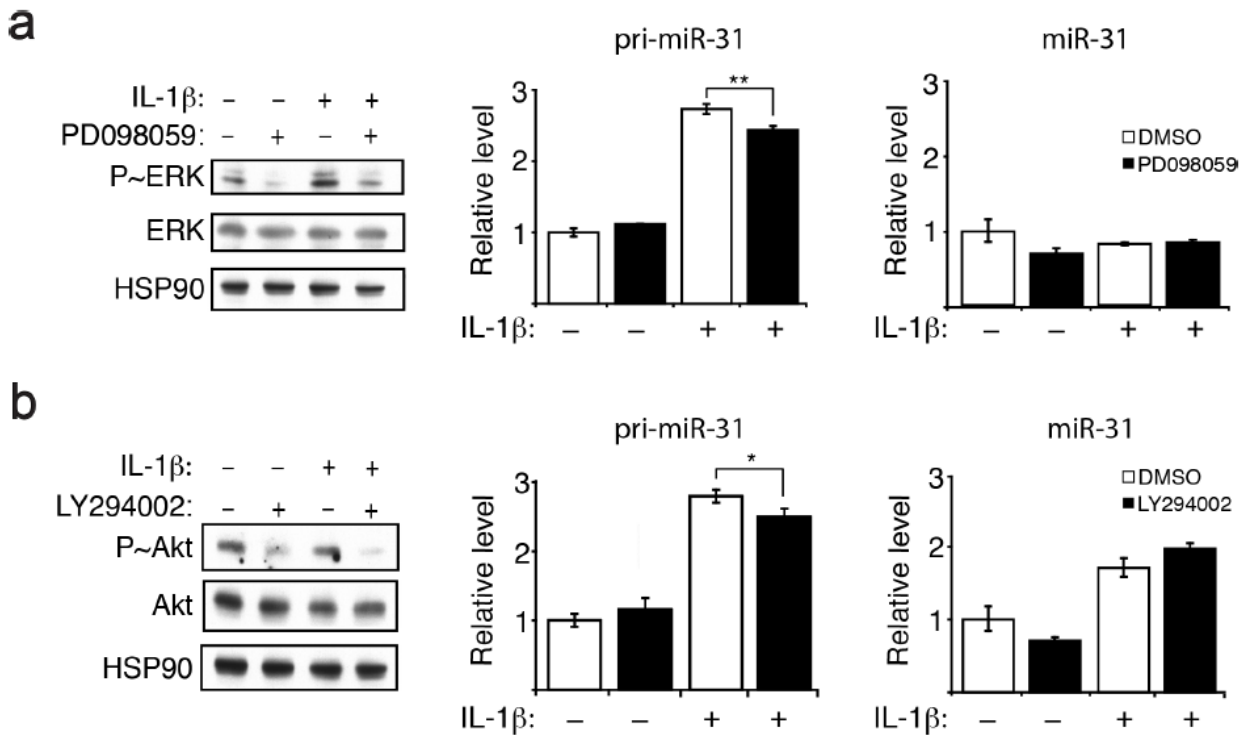


Figure 2-3. IL-1β induces the transcription of miR-31 via c-Jun, c-Fos and GATA2. *(a-c) Upon knockdown with lentivirus expressing either control (scramble shRNA) or two different shRNAs (#1 and #2) targeting each transcription factor (c-Jun, c-Fos and GATA2), the level of primary miR-31 (pri-miR-31) transcript relative to GAPDH mRNA (control) was determined by qRT-PCR. The knockdowns for each transcription factor were validated by Western blotting and HSP90 was probed and used as loading control. The Western blots are representative of three independent experiments. The error bars represent standard errors of three independent experiments and significance was analyzed using a Student's t-test (*p <0.05; **p<0.01).*

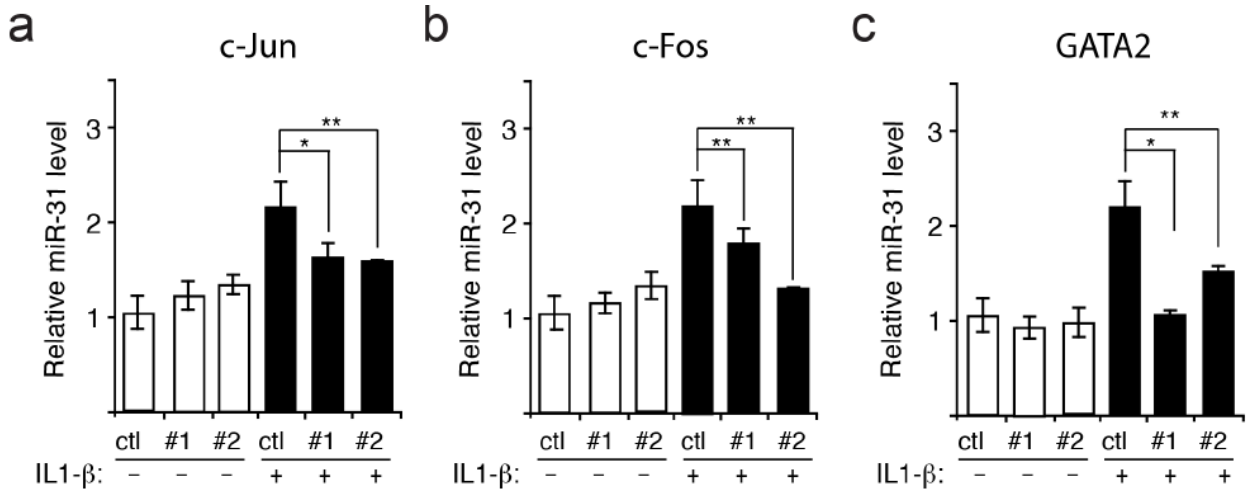


Supplementary Figure S2-2: IL-1β induces the production of miR-31 via p38 and JNK. *The miR-31 level from samples treatedwith either p38 inhibitor a. or JNK inbitor b. presented in Figure 2B-C was measured by quantitative reverse transcription-PCR and thesnRNA U6 was used as the normalization control. The error bars represent standard errors of three independent experiments and p valueswere obtained using Student's t-test (**p<0.01).*



Supplementary Figure S2-3: The activation of the ERK and Akt signaling pathways by IL-1β do not affect miR-31 expression. *Endothelial cells were pre-treated with either control DMSO*

*or 50μM of ERK inhibitor PD098059 a. or 5μM of Akt inhibitorLY294002 b. for 1 hour before the addition of IL-1β (20ng/ml). The inhibition of those signaling pathways was confirmed by Western blotting monitoring phospho-ERK (P~ERK) and phospho-Akt (P~Akt), as indicated. The levels of primary miR-31 (pri-miR-31) andmiR-31 relative to GAPDH mRNA and snRNA U6 respectively, were determined by quantitative reverse transcription-PCR. The error bars represent standard errors of four independent experiments and p values were obtained using a Student's t-test (*p <0.05; **p<0.01).*



Supplementary Figure S2-4: IL-1β induces the transcription of miR-31 via c-Jun, c-Fos and GATA2. *The miR-31 level from samples upon shRNA knockdown targeting c-Jun a., c-Fos b. and GATA2 c. presented in Figure 3 was measured by quantitative real-time PCR and the snRNA U6 was used as the normalization control. The error bars represent standard errors of three independent experimentsand p values were obtained using a Student's t-test (*p <0.05; **p<0.01).*

Mir-31 modulates E-selectin-mediated adhesion of colon cancer cells to endothelial cells

Adhesion of colon cancer cells to endothelial cells expressing E-selectin is a prerequisite to their transendothelial migration (TEM) during metastatic dissemination (Faryammanesh et al., 2014;

Gout and Huot, 2008). In order to examine whether miR-31 could affect the adhesion of colon cancer cells to endothelial cells, we performed adhesion assays of HT29 and LoVo metastatic colon cancer cells on endothelial cells transfected with anti-miR-31 or its control. The inhibition of miR-31 was associated with an over two-fold increase in the adhesion of both HT29 and LoVo cells compared to the control inhibitor (*Figure 2-4a* and *b* respectively, left panels). The treatment with anti-miR-31 could no longer increase the adhesion of colon cancer cells when endothelial cells were not stimulated with IL-1β and thereby did not express E-selectin (*Figures 2-4a* and *2-1a*). Moreover, treating endothelial cells with E-selectin neutralizing antibody significantly reduced the effect of anti-miR-31 (*Figure 2-4a* and *b*, right panels), indicating that the increase in the adhesion of cancer cells mediated by inhibiting miR-31 was E-selectin-dependent. Overall, these results indicate that by regulating the expression of E-selectin, miR-31 is an important modulator of E-selectin-dependent adhesion of colon cancer cells to endothelial cells.

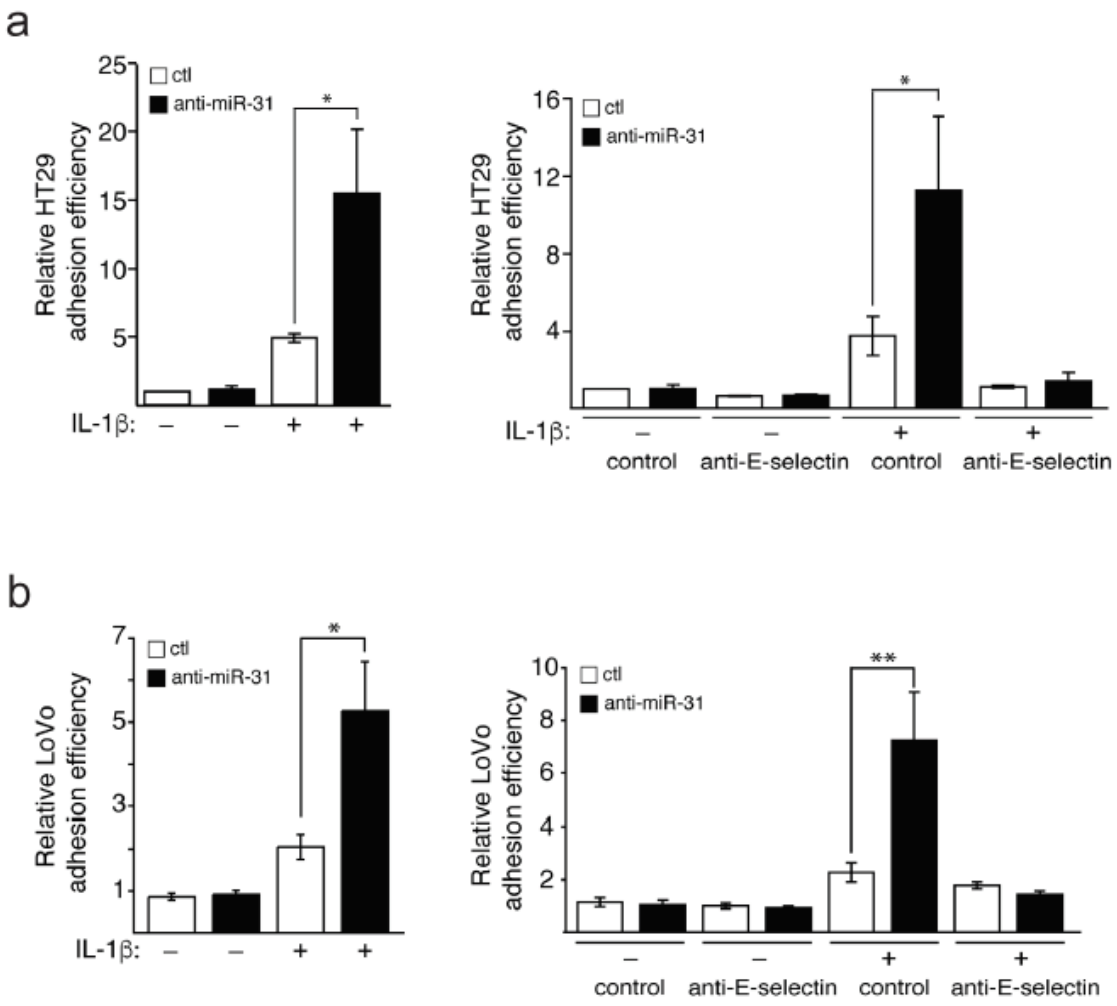


Figure 2-4. Mir-31 inhibits E-selectin-dependent adhesion of cancer cels to endothelial cells. *HUVEC endothelial cells were transfected and stimulated as described in Figure 1. Calcein AM-stained HT29 (**a**) or LoVo (**b**) metastatic cancer cells were added on a tight layer of endothelial cells and incubated for 30 minutes. Non-adhering cells were washed and those cells that adhere to endothelial cells were determined. To test the E-selectin dependence of the effect of miR-31 on cancer cells adhesion, anti-E-selectin antibody or MOPC21 antibody (used as control) was added to endothelial cells one hour before the addition of HT29 and LoVo cells (**a** and **b** respectively, right panels). The error bars represent standard errors of three independent experiments and p values were obtained using a Student's t-test (*p <0.05, **p <0.001).*

MiR-31 modulates E-selectin-mediated transendothelial migration of colon cancer cells

The TEM of cancer cells is associated with their motile and survival potentials (Laferriere et al., 2001) , which are enhanced by their binding to E-selectin (Gout et al., 2006). Since miR-31 is involved in modulating the expression of E-selectin and the adhesion of HT29 cells to endothelial cells, we next studied whether miR-31 modulates TEM of HT29 and LoVo cells by determining the capacity of these cells to cross a tight layer of endothelial cells transfected with anti-miR-31 or its control, in a Boyden chamber. We observed that the TEM of both HT29 and LoVo cells was increased by about two-fold upon inhibition of miR-31 (*Figure 2-5a* and *b* respectively, left panels). Pre-treating E-selectin expressing endothelial cells with anti-E-selectin antibody almost completely abolished the increase in TEM supporting the essential role played by E-selectin in the process (*Figure 2-5a* and *b*, right panels).

Taken together, these data are consistent with the fact that miR-31 is an important modulator of the metastatic process by targeting E-selectin.

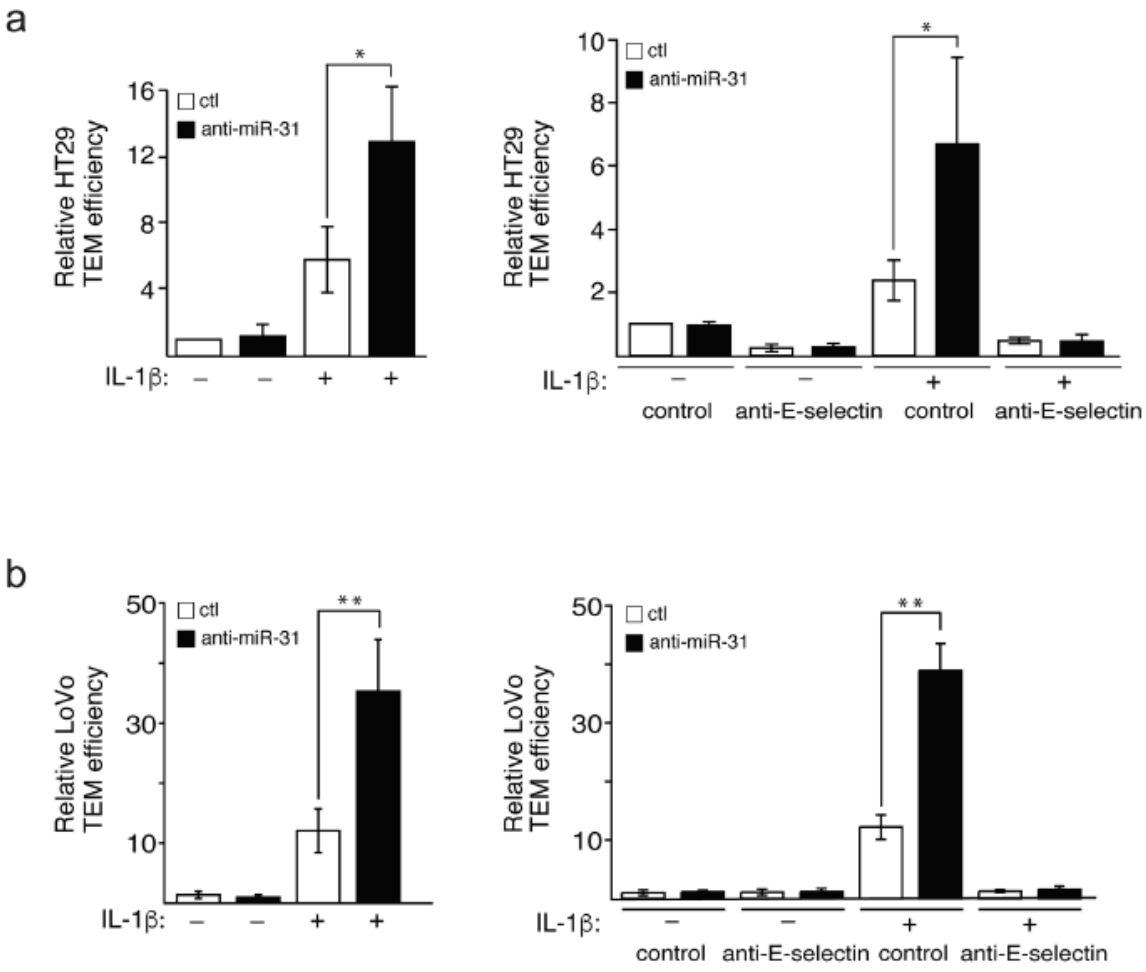


Figure 2-5. Mir-31 inhibits E-selectin-dependent transendothelial migration of cancer cells. *Fluorescent HT29 (**a**) or LoVo (**b**) cells that penetrated a tight layer of endothelial cells were counted. To test the E-selectin dependence of the effect of miR-31 on cancer cells adhesion, anti-E-selectin antibody or MOPC21 antibody (used as control) was added to endothelial cells one hour before the addition of HT29 and LoVo cells (**a** and **b** respectively, right panels). The error bars represent standard errors of three independent experiments and p values were obtained using a Student's t-test (*p <0.05, **p <0.001).*

2.5 Discussion

Several lines of evidence indicate that cancer cells hijack the inflammatory system and interact with E-selectin to extravasate and form metastases. Nevertheless, despite the importance of E-selectin in metastatic progression, little is known about the mechanisms that downregulate its expression and stop the E-selectin-mediated adhesion process. Here, we show that miR-31 is induced by IL-1β and that its expression is inversely correlated with that of E-selectin and E-selectin-mediated metastatic potential of colon cancer cells. We further report that the expression of miR-31 in response to IL-1β is stimulated at the transcriptional level in a process involving JNK and p38 MAPK pathways and their downstream transcription factors c-Jun, c-Fos and GATA 2.

The conclusion that miR-31 regulates the expression of E-selectin is supported by four independent but complementary observations. First, IL-1β-induced expression of miR-31 is inversely correlated in HUVECs with that of E-selectin. Second, inhibiting miR-31 is associated with an increase of E-selectin level both in HUVECS and HLSMECs. Lastly, altering the predicted binding site of miR-31 in the 3'UTR of E-selectin mRNA hinders its repression, supporting a direct role of miR-31 in E-selectin regulation.

An important novelty of our study is the identification of p38 and JNK as major pathways regulating the expression of miR-31. This is supported by the observation that both pathways are induced by IL-1β and that their inhibition impairs the IL-1β-induced increase in miR-31. The regulation occurs at the transcriptional level given that: (1) IL-1β increased the level of pri-miR-31 and; (2) a decreased level of miR-31 in the presence of inhibitors is accompanied by decreased pri-miR-31 (down to basal level without IL-1β). Chromatin ImmunoPrecipitations followed by sequencing (ChIP-seq) data from UCSC Genome Browser show that c-Jun, c-Fos

and GATA2 are transcription factors bound to regulatory sites of the genome of miR-31. They can act as transcription factors regulating miR-31 following IL-1β stimulation. Along these lines, the knockdown of c-Fos, c-Jun and GATA2 in endothelial cells reduced the IL-1β-induced transcription and production of miR-31. Intriguingly, these transcription factors are all known targets of JNK and p38, of which c-Jun can be activated by JNK MAPK, while c-Fos and GATA2 can be activated by p38 MAPK (Baumgartner and Baccarini, 2014; Chen et al., 2006; Minden et al., 1994; Tanos et al., 2005; Zarubin and Han, 2005). This is consistent with our finding that inhibiting p38 and JNK impairs the transcription of miR-31 in response to IL-1β. It has been reported that AP1 (heterodimer of c-Jun and c-Fos) and GATA2 may act cooperatively in regulating gene transcription, which is in line with our data (Kawana et al., 1995). It is worth mentioning that both p38 and JNK have also been shown to modulate the transcription of E-selectin (Laferriere et al., 2002), a target of miR-31. Recent mathematical modeling and single-cell reporter assays show that the key function of miRNAs is to regulate the "noise", or variability, of protein expression, in order to confer precision and stability to protein expression (Schmiedel et al., 2015). In this regard, in physiological conditions, JNK and p38 may be of pivotal importance in the expression and the fine-tuning of E-selectin, by mediating its transcription and controlling its translation through miR-31 regulation.

In accordance with our finding that miR-31 regulates the expression of E-selectin, inhibiting miR-31 is associated with increased adhesion and transendothelial migration of colon cancer cells to and through endothelial layer, both of which are abolished by E-selectin neutralizing antibody. These findings support our argument that miR-31 has anti-metastatic properties against colon cancers. MiR-31 has already been shown to have anti-metastatic properties for breast cancers via targeting GNA13 and WAVE3 (Rasheed et al., 2015; Sossey-Alaoui et al., 2011). It

has also been reported to inhibit gastric carcinogenesis and progression by targeting Smad4 and SGPP2 (Ruoming et al., 2015). Alternatively, miR-31 can also reduce gastric tumor cell invasion and metastasis by targeting integrin α5 to indirectly affect the PI3K/AKT pathway (Zhang et al., 2016b). In brief, miR-31 has previously been shown to reduce the metastatic potential of cancer cells by targeting signaling and cytoskeletal remodeling proteins in cancer cells. Our findings now show for the first time that miR-31 can also exert its anti-metastatic ability by targeting E-selectin, an adhesion molecule in endothelial cells. Hence, miR-31 may be a key player in the metastatic process of not only colorectal carcinoma, but also breast, bladder, gastric, and pancreatic carcinoma, leukemia and lymphoma, which all depend on E-selectin for their extravasation (Gout et al., 2008; Reymond et al., 2013) .

In conclusion, our study highlights miR-31 as a key player regulating E-selectin-dependent colon cancer metastasis. Our findings further raise the possibility that miR-31 may be maintained at a low level in endothelial cells constantly expressing E-selectin, thus promoting TEM of cancer cells during metastasis. In this context, increasing the expression of miR-31 in endothelial cells may be envisioned as an approach to reduce metastases of cancer cells which extravasate in an E-selectin-dependent manner into organs as various as liver, bone marrow, skin, and lung (Gout et al., 2008; Reymond et al., 2013). In corollary, it might be expected that a low level of miR-31 in endothelial cells could serve as a biomarker of pro-metastatic states.

2.6 Materials and methods

Reagents and antibodies

PD098059, LY294002, SP600125 and SB203580 chemical inihbitors were obtained from Sigma (St Louis, MO). Calcein-AM was obtained from Invitrogen Molecular Probes (Burlington, ON). Dimethylsulfoxyde was purchased from Fisher (Montreal, QC). IL-1β was obtained from R&D Systems (Minneapolis, MN). Anti-phospho-ERK1/2 MAPK (T^{202}/Y^{204}) mouse antibody, anti-phospho-Akt (S^{473}) rabbit antibody, anti-Akt rabbit antibody, anti-phospho-c-Jun (S^{63}) rabbit antibody, anti-c-Jun rabbit antibody were obtained from Cell Signaling Technology (Beverly, MA). Anti-E-selectin mouse monoclonal antibody was obtained from R&D Systems (Minneapolis, MN). Anti-GAPDH mouse antibody was obtained from Novus Biologicals (Oakville, ON). Anti-actin rabbit antibody was obtained from Sigma (St Louis, MO). Anti-ERK1/2 rabbit antibody, anti-phospho-HSP27 rabbit antibody, anti-HSP27 rabbit antibody were kind gifts from Dr. Jacques Landry (CRCHU de Québec-Université Laval, QC). Anti-mouse/rabbit-IgG-horseradish-peroxidase (HRP) goat antibodies were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cells

Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion of umbilical veins from undamaged fresh cords, as described (Huot et al., 1997). The cords were obtained under protocols approved by the CRCHU de Québec-Université Laval Ethic Committee. HUVECs at passages ≤5 were grown to form monolayer in EGM2 endothelial cell growth medium (Lonza, Allendale, NJ) in gelatin-coated tissue culture flasks. Human liver sinusoidal

microvascular endothelial cells (HLSMECs, Cell Systems, WA) were grown to form monolayer in CSC complete medium (Cell Systems, WA) in gelatin-coated tissue culture flasks.

HT29 (ATCC) colorectal adenocarcinoma cells were cultivated in McCoy 5A medium (Sigma, St Louis, MO) supplemented with 10% foetal bovine serum (FBS). HEK293T human embryonic kidney cells (ATCC) were cultivated in DMEM (Lonza, Allendale, NJ) supplemented with 10% FBS. LoVo (ATCC) colorectal adenocarcinoma cells were cultivated in Ham's F12 nutrient mixture (Life Technologies, Carlsbad, CA) supplemented with 10% FBS.

All cells were cultivated at 37°C in 5% CO_2 humidified atmosphere.

Plasmids, shRNAs, miRNA mimics and inhibitors

MiRIDIAN microRNA inhibitors were obtained from Dharmacon (Lafayette, CO, USA). These antimiRs bind to microRNAs by complementary sequences and thus block their capacity to silence mRNA target without affecting the level of microRNAs (Hutvágner et al., 2004). PsiCHECK2 vectors containing wild type SELE 3'UTR was a kind gift from Dr. Yajaira Suarez (Yale School of Medicine, New Haven, CT). This vector has a *Renilla* luciferase open reading frame upstream of E-selectin 3'UTR, and a constitutively expressed firefly luciferase open reading frame. All constructs were confirmed by sequencing. Lentiviral particles containing shRNAs against c-Jun (TRCN0000039589, TRCN0000039590), c-Fos (TRCN0000016004, TRCN000016007) and GATA2 (TRCN0000019264, TRCN0000019265) were obtained from Dr. Stéphane Gobeil (CRCHU de Québec-Université Laval, QC).

Transfection and infection

Endothelial cells were transfected using X-tremeGene HP Transfection Reagent following manufacturers' protocol (Roche Life Science, Laval, QC). HEK293T cells were transfected with calcium-phosphate-mediated transfection method with HEPES buffered saline of Sigma (St Louis, MO). Endothelial cells were infected by lentivirus in the presence of 8 μg/mL hexadimethrine bromide (Sigma, St Louis, MO).

RNA extraction and quantification

Cells were lysed in TRIzol (Invitrogen, Carlsbad, CA) to extract total RNA following manufacturer's protocol. The quality of RNA was assessed on agarose gel and spectrometry.

The reverse transcription was performed with TapMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using specific primers (Life Technologies, Carlsbad, CA) for microRNAs, or random primers for pri-microRNAs. The quantification was carried out with Universal PCR Master Mix (Life Technologies, Carlsbad, CA) and specific probes from the same company.

Western blotting

Cells were lysed using SDS-PAGE loading buffer without reducing agents. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Antibodies were applied

according to their manufacturers' protocols. Blots were developed with SuperSignal West Pico Substrate (Fisher, Montreal, QC).

Luciferase reporter assay

Previously described psiCHECK2 vectors were transfected into HEK293T cells with calcium-phosphate-mediated transfection method. After 48 hours, cells were lysed and the luciferase activity was evaluated using Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luciferase activities were measured by Luminoskan Ascent Microplate Luminometer (Thermo Scientific, Waltham, MA).

Adhesion assay

Endothelial cells were plated on gelatin-coated wells and left to grow to confluence. HT29 cells were labeled with calcein-AM for 30 min at 37 °C, then were added to IL-1β-activated endothelial cells for 30min. The endothelial layer was washed twice with PBS and the attached cells were quantified by measuring the fluorescence emission with Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific). To study the E-selectin-dependence of the adhesion, neutralizing anti-E-selectin antibodies were introduced one hour before adding HT29 or LoVo cells.

Transendothelial migration assay

Cell migration was investigated using a modified Boyden chamber assay. Endothelial cells were grown to confluence on a 5.0μm-pore-sized gelatinized polycarbonate membrane separating the two compartments of a 6.5mm migration chamber (Transwell, Costar, MA). After IL-1β-mediated activation of endothelial cells for 4 hours, calcein-AM stained HT29 cells suspended in migration buffer (medium199, 10mM HEPES pH7.4, 1.0mM $MgCl_2$, 0.5% BSA) were added to the monolayer of endothelial cells, previously washed with the same buffer. After five hours, cells on the upper face of the membrane were scraped with a cotton swab. The number of HT29 and LoVo cells that have migrated to the lower face of the filter was counted using an inverted fluorescence microscope.

Chapter 3: p38 activation induces production of miR-146a and miR-31 to repress E-selectin expression and inhibit transendothelial migration of colon cancer cells

Foreword

The present chapter is constituted by a manuscript published in *Scientific Reports*. I have done all the experiments and wrote the manuscript. Drs. Simard and Huot supervised the work and the writing.

p38 activation induces production of miR-146a and miR-31 to repress E-selectin expression and inhibit transendothelial migration of colon cancer cells

Liang Zhong, Jacques Huot* and Martin J. Simard*

Laval University Cancer Research Center, Hôtel-Dieu de Québec (CHUQ), Quebec City, QC G1R 2J6, Canada

Corresponding author:

Jacques Huot, Martin J. Simard

Email : Jacques.Huot@fmed.ulaval.ca, Martin.Simard@crchudequebec.ulaval.ca

Key words: microRNA, NF-κB, metastasis

3.1 Résumé

L'extravasation des cellules cancéreuses circulantes est un mécanisme cellulaire primordial à la dissémination métastatique qui est initiée par l'adhérence de ces cellules aux cellules endothéliales vasculaires. Elle nécessite l'interaction entre les récepteurs d'adhérence comme E-sélectine sur les cellules endothéliales et leurs ligands sur les cellules cancéreuse. Ici, on montre que l'expression de E-sélectine est ciblée par miR-146a et -181b, qui répriment son expression indirectement en ciblant la voie de NF-κB en amont. L'inhibition de E-sélectine médiée par miR-146a/-181b diminue le potentiel métastatique de cellules de cancer du côlon en réduisant leur adhérence à l'endothélium, et leur migration transendothéliale. La voie des MAP kinases joue un rôle pivot dans la transcription de miR-146a en réponse à l'IL-1β, étant donné que les MAP kinases p38, ERK et JNK médient sa transcription. Les facteurs de transcription en aval de ces MAP kinases, GATA2, c-Fos et c-Jun, modulent la transcription de miR-146a. L'inhibition de p38 augmente l'activité de NF-κB au moins partiellement par miR-146a. L'inhibition de p38 augmente aussi l'expression de E-sélectine au niveau post-transcriptionnel en diminuant miR-31. En réponse à l'IL-1β, p38 MAP kinase réprime donc l'expression de E-sélectine aux niveaux transcriptionnel et post-transcriptionnel, via miR-146a et miR-31, respectivement. Ces résultats révèlent un nouveau mécanisme par lequel p38 inhibe l'expression de E-sélectine par les microARN suivant une stimulation pro-inflammatoire.

3.2 Abstract

Extravasation of circulating cancer cells determines their metastatic potentials. This process is initiated by the adhesion of cancer cells to vascular endothelial cells through specific interactions between endothelial adhesion receptors such as E-selectin and their ligands on cancer cells. In the present study, we show that miR-146a and -181b impede the expression of E-selectin by repressing the activity of its transcription factor NF-κB, thereby impairing the metastatic potentials of colon cancer cells by decreasing their adhesion to, and migration through, the endothelium. Among the two microRNAs, only miR-146a is activated by IL-1β, through the activation of p38, ERK and JNK MAP kinases, as well as their downstream transcription factors GATA2, c-Fos and c-Jun. Inhibiting p38 MAP kinase increases NF-κB activity, at least partially via miR-146a. Inhibiting p38 also increases the expression of E-selectin at the post-transcriptional level via decreasing miR-31, which targets E-selectin mRNA and also depends on p38 for its expression. In response to IL-1β, p38 MAP kinase hence represses the expression of E-selectin at the transcriptional and the post-transcriptional levels, via miR-146a and miR-31, respectively. These results highlight novel mechanisms by which p38 downregulates the expression of E-selectin through different microRNAs following inflammatory stimuli associated to cancer progression.

3.3 Introduction

Metastasis depends on sequential interrelated steps (Gout and Huot, 2008). Notably, the adhesion of circulating cancer cells to the endothelium of blood vessels is a prerequisite for their extravasation. This adhesive event is initiated by specific interactions between endothelial adhesion receptors such as E-selectin, and their ligands on cancer cells. E-selectin is expressed exclusively by endothelial cells stimulated by pro-inflammatory cytokines including interleukin-1β (IL-1β) (Gout et al., 2008). In the inflammatory context, E-selectin triggers the adhesion and the subsequent rolling of leukocytes to and on the endothelium, thus initiating their extravasation into inflamed tissues (Vestweber and Blanks, 1999). Cancer cells including breast, bladder, gastric, pancreatic and colorectal carcinoma, as well as leukemia and lymphoma can hijack this inflammatory process to extravasate and form metastases (Gout et al., 2008; Reymond et al., 2013; Vestweber and Blanks, 1999). Accordingly, several lines of evidence suggest E-selectin as a key determinant of metastasis of colon cancer cells. In particular, the binding efficiency of colon cancer cells to E-selectin is proportional to their respective metastatic potential (Sawada et al., 1994) and an anti-E-selectin antibody is capable of reducing orthotopic liver metastasis of colon cancers (Brodt et al., 1997). The canonical model indicates that E-selectin relies on the activation of NF-κB, JNK and p38 pathways for its transcription (Prasad et al., 2005; Read et al., 1997; Subramaniam et al., 1993; Zhong et al., 2016). However, the precise regulation of its transcription and translation following inflammatory stimuli is still largely unknown. Notably, the role of microRNAs in the signaling network governing the expression of E-selectin is ill-defined.

Among the regulators of gene expression, the evolutionarily conserved small non-coding RNA molecules called microRNAs (miRNAs) have recently emerged as key mediators of the process. To generate their functional single-stranded ~21 nucleotides long form, they are firstly transcribed as long primary miRNAs (pri-miRNAs) by RNA polymerase II. Pri-miRNAs are then processed by Drosha-DGCR8 complex in the nucleus to produce precursor miRNAs (pre-miRNAs), which are exported to the cytoplasm to be cleaved by Dicer, producing miRNAs that are loaded into miRNA-induced silencing complex (miRISC). Through base pairing with the 3' untranslated region (3' UTR) of mRNA, miRNA guides the miRISC to its target, thereby repressing translation with or without causing mRNA degradation (Ha and Kim, 2014).

We previously reported that one of the miRNAs, miR-31, post-transcriptionally represses the expression of E-selectin by targeting its mRNA(Zhong et al., 2016). Moreover, recent reports revealed a number of miRNAs repressing the expression of E-selectin by hindering the inflammatory process. Among them, miR-146a has been shown to repress the pro-inflammatory NF-κB and JNK pathways by targeting the pro-inflammatory receptor adaptors as varied as Card10, TRAF6, IRAK1 and IRAK2, thereby deterring the expression of E-selectin (Cheng et al., 2013; Hou et al., 2009; Rau et al., 2014; Taganov et al., 2006). MiR-181b also impairs the activity of the NF-κB pathway and the expression of E-selectin by targeting Card10 (Lin et al., 2016), as well as importin-α3, an importer protein required for the nuclear translocation of NF-κB (Sun et al., 2012). MiR-10a is another miRNA impeding NF-κB-mediated E-selectin expression, through targeting two key regulators of IκBα degradation: MAP3K7 and βTRC (Fang et al., 2010). MiR-30a represses E-selectin expression by targeting Ang2, a protein enhancing the expression of multiple adhesion receptors (Demolli et al., 2015), and miR-92a

reduces E-selectin via targeting endothelial transcription factors KLF2 and KLF4 (Fang and Davies, 2012). However, none of these miRNAs that exhibit anti-inflammatory properties have been scrutinized in a metastatic context, to investigate their involvement in E-selectin-mediated extravasation of cancer cells.

In this study, we found that miR-146a and -181b inhibit NF-κB-mediated expression of E-selectin and act as potent repressors of E-selectin-dependent metastatic abilities of colon cancer cells. Among these two miRNAs, IL-1β induces only miR-146a at the transcriptional level, through p38, JNK and ERK MAP kinase pathways. Inhibiting p38 MAP kinase increases the activity of NF-κB at least partially by decreasing miR-146a. In addition, inhibiting p38 augments the expression of E-selectin at the post-transcriptional level through decreasing miR-31, a miRNA targeting E-selectin mRNA (Zhong et al., 2016).

3.4 Results

miR-146a and -181b repress the transcription of E-selectin

To find repressors of E-selectin-dependent metastatic potentials of colon cancer cells, we first evaluated the role of miRNAs known as modulators of the inflammatory responses, namely miR-10a, -30a, -92a, miR-146a and -181b, in the regulation of E-selectin expression in human umbilical vein endothelial cells (HUVECs) using their respective inhibitors (henceforth anti-miRs), together with anti-miR-31 (positive control). Although anti-miR-10a mildly increased E-selectin mRNA (*Figure 3-1b*), a corresponding increase was not observed for the protein (*Figure 3-1a*). On the contrary, anti-miR-146a and -181b significantly increased E-selectin expression to

levels comparable to that of anti-miR-31, the inhibitor of a miRNA repressing E-selectin expression by directly targeting its mRNA(Zhong et al., 2016) (*Figure 3-1a*). The effect of miR-146a on the regulation of E-selectin was further confirmed in human liver sinusoidal microvascular endothelial cells (HLSMECs; *Supplementary Figure S3-1a*), but the blockage of miR-181b did not affect E-selectin level in these cells that express very high level of this miRNA (~20 fold more miR-181b detected in HLSMECs compared to HUVECs; *Supplementary Figure S3-1b*). In HUVECs, anti-miR-146a and -181b also significantly increased E-selectin mRNA compared to the control and anti-miR-31 (*Figure 3-1b*), suggesting that in contrast to miR-31, miR-146a and -181b regulate E-selectin at the transcriptional level.

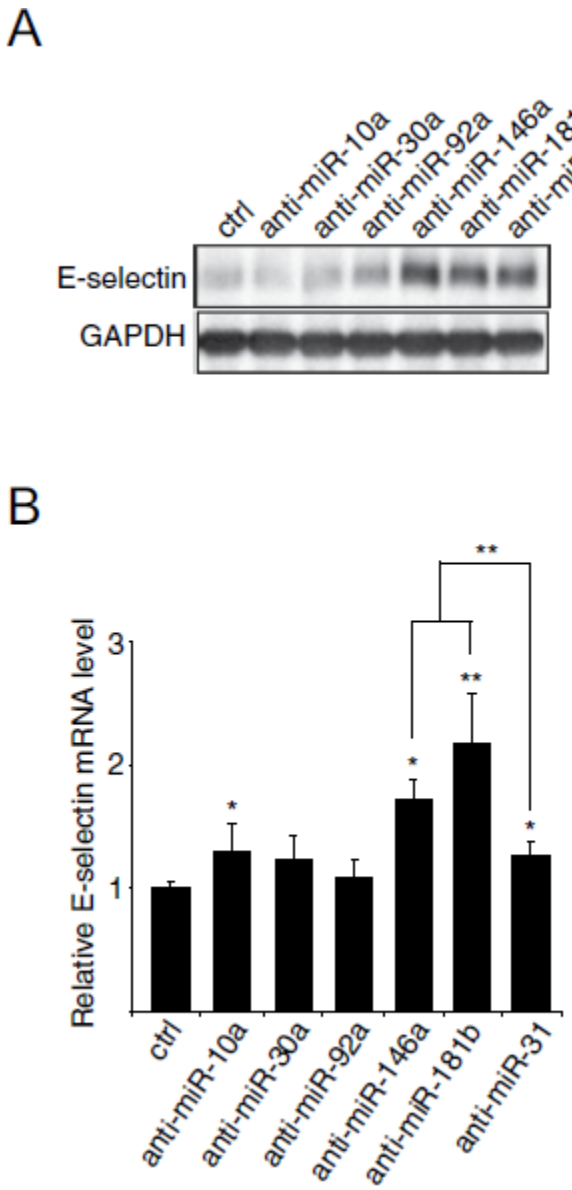


Figure 3-1. miR-146a and -181b repress the transcription of E-selectin. A. B. *Human Umbilical Vein Endothelial Cells (HUVECs) cultivated as monolayers were transfected with 50nM of miRNA inhibitors or controls, before being treated with IL-1β (20ng/ml) for four hours.* ***A.*** *The expression of E-selectin was monitored by Western blotting. GAPDH was used as loading control.* ***B.*** *RT-qPCR monitored the level of E-selectin mRNA relative to GAPDH mRNA. The Western blot is representative of four independent experiments. The quantification are the mean values of four independent experiments, the error bars represent standard errors of four independent experiments, and the significance was analyzed using a Student's t-test. The p-values are calculated comparing to the ctrl, unless indicated otherwise (*p <0.05; **p<0.01).*

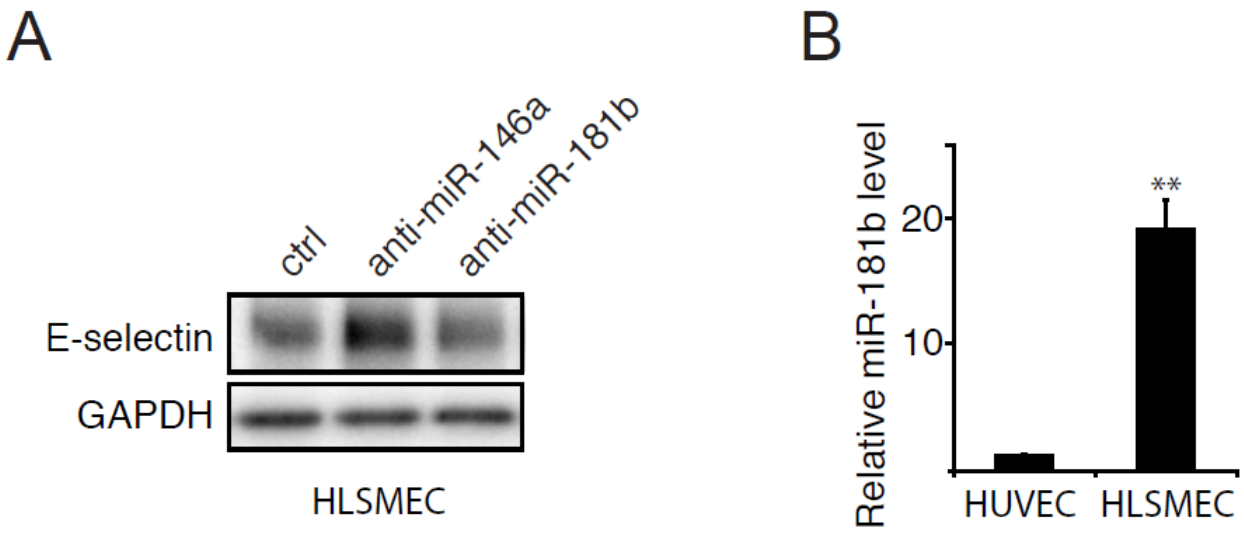


Figure S3-1. miR-146a represses the transcription of E-selectin in HLSMECs. A. *HLSMECs cultivated as monolayers were transfected with 50nM of miRNA inhibitors or controls, before being treated with IL-1β (20ng/ml) for four hours. The expression of E-selectin was monitored by Western blotting. GAPDH was used as loading control.* ***B.*** *HLSMECs and HUVECs were treated with IL-1β (20ng/ml) for four hours. The miR-146a level was measured by RT-qPCR and the snRNA U6 was used as the normalization control. The Western blot is representative of four independent experiments. The quantifications are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test. The p-values are calculated comparing to the ctrl, unless indicated otherwise (*p <0.05; **p<0.01).*

miR-146a and -181b repress the transcription of E-selectin by inhibiting NF-κB signaling

MiR-146a has been reported to hamper the activation of the NF-κB and JNK pathways, two major pathways controlling the transcription of E-selectin(Cheng et al., 2013), while miR-181b particularly subdues NF-κB pathway[13,14]. To study whether the two miRNAs act on NF-κB and/or JNK pathways to affect the transcription of E-selectin, the activities of both pathways in HUVECs were evaluated using phospho-specific antibodies: anti-phospho-NF-κB-p65 (S536)

and anti-phospho-c-Jun (S63)(Eritja et al., 2017; Morgan et al., 2008). Both miRNA inhibitors greatly increased phosphorylated NF-κB-p65 (P~p65), but neither increased phosphorylated c-Jun (P~c-Jun) (*Figure 3-2a*). We further investigated whether the effect of the miRNA inhibitors depends on NF-κB activity. To this end, we treated HUVECs with an inhibitor of its upstream activator IKK(Waelchli et al., 2006), and found that the inhibitor greatly reduced the transcription and expression of E-selectin as well as totally abolished the ability of both miRNA inhibitors to increase them (*Figure 3-2b* and *c*). These results suggest that miR-146a and -181b repress the transcription and expression of E-selectin, through inhibiting the NF-κB pathway.

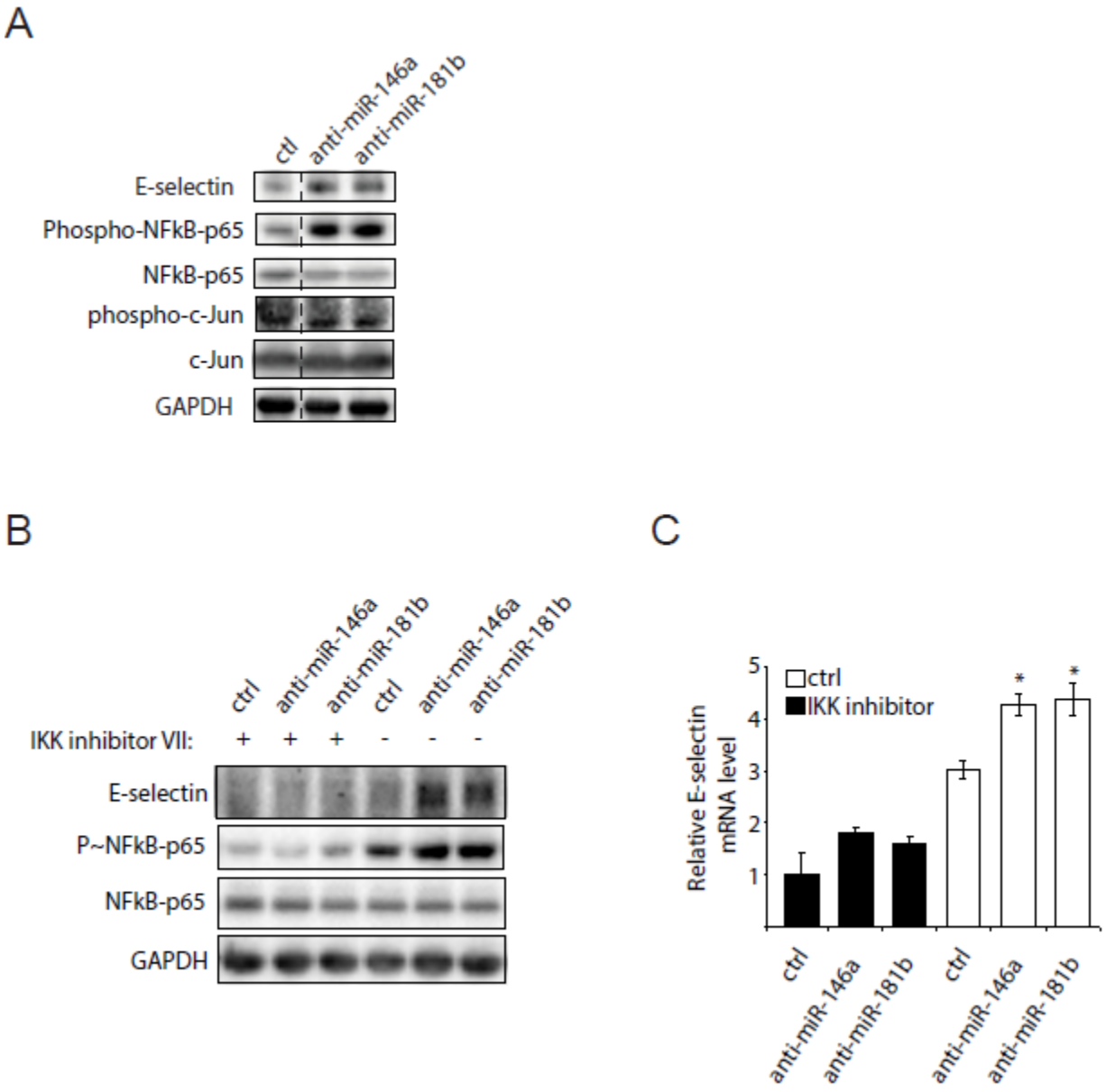


Figure 3-2. miR-146a and -181b repress the transcription of E-selectin by inhibiting NF-κB signaling. ***A.*** *Endothelial cells were treated as in Figure 3-1. The activities of NF-κB and c-jun signaling pathways were monitored by Western blotting using anti-phospho-NFkB-p65 (S^{536}) and anti-phospho-c-Jun (S^{63}) antibodies. GAPDH was used as loading control. The dashed lines indicate that unrelated lanes have been removed between samples. See full-length blots in Supplementary Fig. S9.* ***B. C.*** *Endothelial cells were transfected with 50nM of miRNA inhibitors or control, before being pretreated with 1μM IKK inhibitor VII, and being treated with IL-1β (20ng/ml) for four hours.* ***B.*** *The expression of E-selectin and the activity of NF-κB signaling*

pathway were monitored by Western blotting. GAPDH was used as loading control. The Western blot is representative of three independent experiments. ***C.*** *RT-qPCR monitored the level of E-selectin mRNA. GAPDH mRNA was used as loading control. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test. The p-values are calculated comparing to the "antagomir ctrl/DMSO/IL-1β" group (*p <0.05; **p<0.01).*

miR-146a and -181b modulate E-selectin-mediated adhesion to and migration through endothelial cells of colon cancer cells

Adhesion of colon cancer cells to E-selectin expressing endothelial cells is a prerequisite to their transendothelial migration (TEM) during metastatic dissemination(Auguste et al., 2007; Faryammanesh et al., 2014; Gout and Huot, 2008). To examine whether miR-146a and -181b could affect the adhesion of colon cancer cells to endothelial cells, adhesion assays of two metastatic colon cancer cells, HT29 and LoVo, were performed on HUVECs transfected with miRNA inhibitors or their control. The treatment with miRNA inhibitors could not increase the adhesion of colon cancer cells when these endothelial cells were not stimulated with IL-1β and thereby did not express E-selectin(Zhong et al., 2016) (*Figure 3-3a* and *b*, left panels). Moreover, treating endothelial cells with E-selectin neutralizing antibody significantly reduced the effect of miRNA inhibitors (*Figure 3-3a* and *b*, right panels), indicating that the increase in the adhesion of colon cancer cells mediated by inhibiting miR-146a and -181b was E-selectin-dependent. Overall, these results suggest that by regulating the expression of E-selectin, miR-146a and -181b are important modulators of E-selectin-dependent adhesion of metastatic colon cancer cells to endothelial cells.

The TEM of colon cancer cells is associated with their motile and survival potentials(Laferriere et al., 2001), which are enhanced by their binding to E-selectin(Gout et al., 2006). Since miR-146a and -181b are involved in modulating the expression of E-selectin and the adhesion of colon cancer cells to endothelial cells, we next studied whether they modulate the TEM of metastatic colon cancer cells by tuning their capacity to penetrate a Boyden chamber coated with a tight layer of HUVECs transfected with miRNA inhibitors or their control. We first verified whether the anti-miRs could affect by themselves the integrity of the endothelial layer. The permeability to FITC dextran of HUVECs transfected with the anti-miRs remained similar to the endothelial layer expressing the corresponding control (*Supplementary Figure S3-2a*). This indicates that the anti-miRs do not affect by themselves the integrity of the endothelial barrier. When we inhibited miR-146a and -181b with anti-miRs, we observed that the TEM of both HT29 and LoVo cancer cells was increased by about two-fold (*Figure 3-4a* and *b*, left panels; *Supplementary Figure S3-3* and *S3-4*) in the presence of IL-1β. Pre-treating E-selectin expressing HUVECs with anti-E-selectin antibody completely abolished the increase in TEM, supporting the essential role played by E-selectin in the process (*Figure 3-4a* and *b*, right panels).

Similar results of adhesion and TEM assays were obtained in HLSMECs when miR-146a was inhibited (*Supplementary Figure S3-5*). Taken all together, these results suggest that miR-146a and -181b are important modulators of the metastatic processes of colon cancer cells by regulating E-selectin expression.

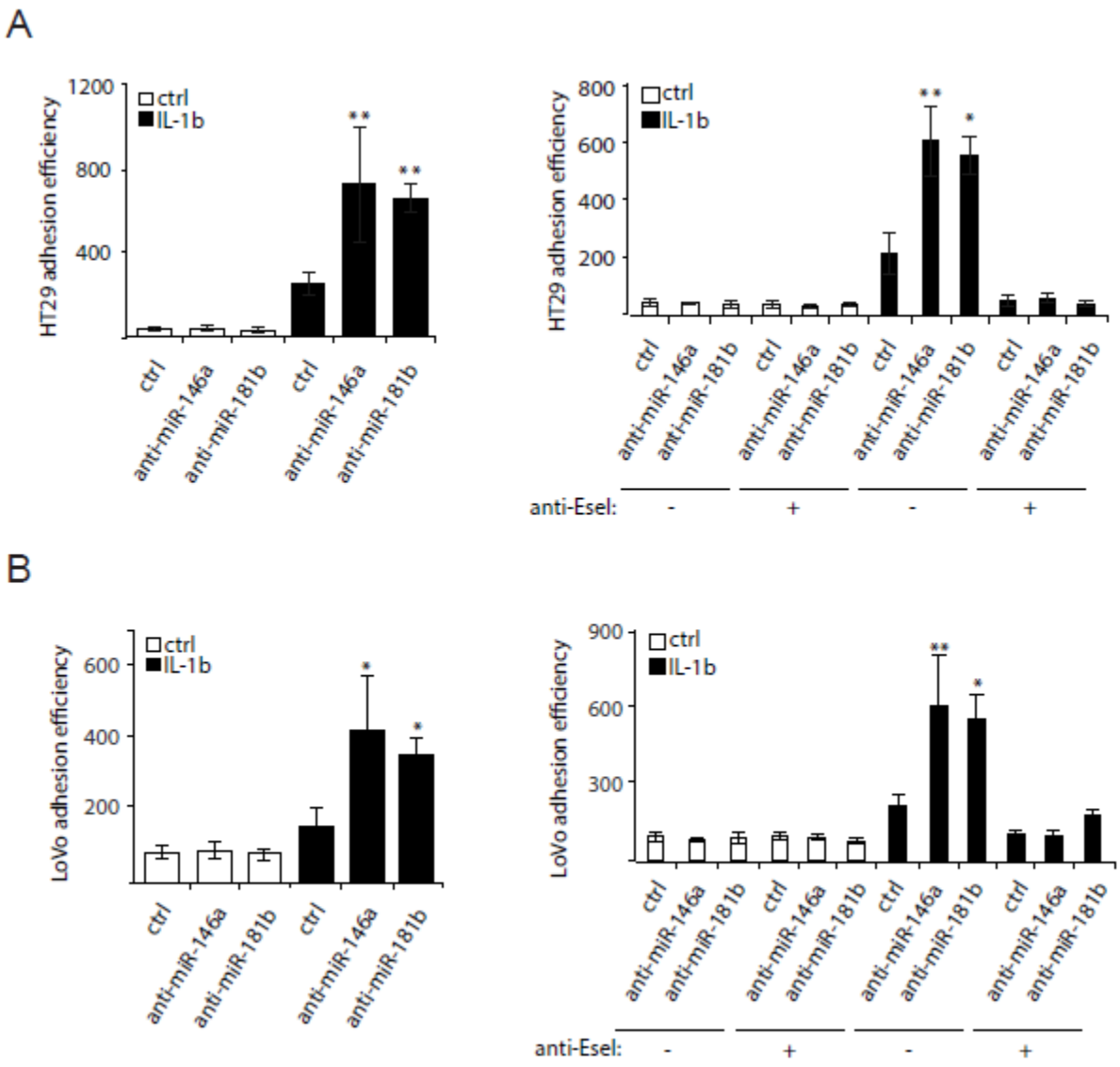


Figure 3-3. miR-146a and -181b modulate E-selectin-mediated adhesion of metastatic colon cancer cells to endothelial cells. A. *MiR-146a and -181b inhibit E-selectin-dependent adhesion of HT29 colon cancer cells to endothelial cells. Endothelial cells were transfected and stimulated as described in Figure 3-1. Calcein AM-stained HT29 metastatic colon cancer cells were added on the tight layer of endothelial cells and incubated for 30 minutes. Non-adhering cells were washed and cells adhering to endothelial cells were determined. To test the E-selectin dependence of the effect of both miRNAs on cancer cells adhesion, anti-E-selectin antibody or MOPC21antibody (1:40) was added to endothelial cells one hour before the addition of HT29 cells.* ***B.*** *MiR-146a and -181b inhibit E-selectin-dependent adhesion of LoVo colon cancer cells to endothelial cells. Experiments were carried out as described in a. Calcein AM-stained LoVo*

*metastatic colon cancer cells were used. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test. The p-values are calculated comparing to the "antagomir ctrl/IL-1β(/antibody ctrl)" group (*p <0.05; **p<0.01). Anti-Esel.: anti-E-selectin antibody.*

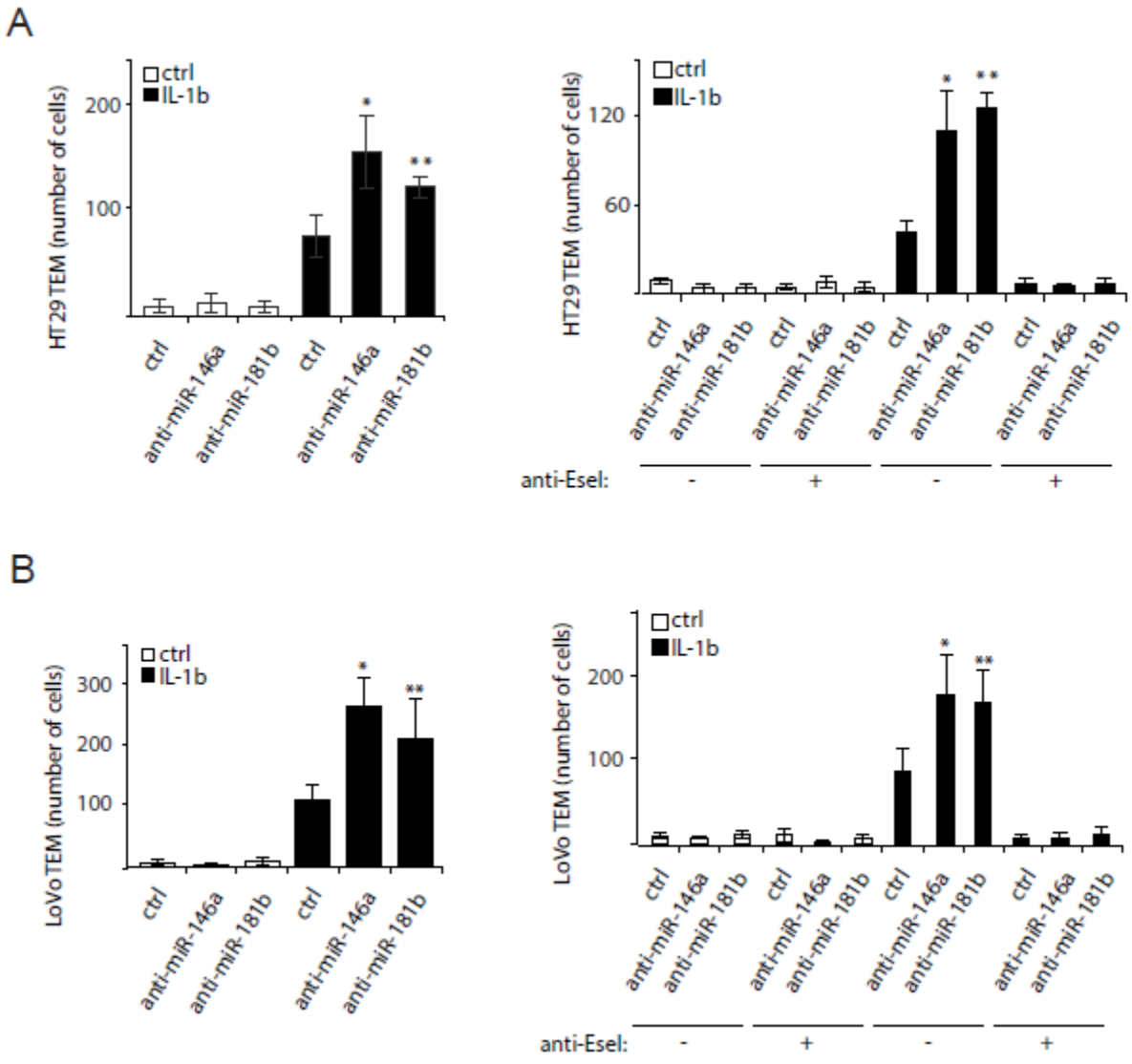


Figure 3-4. miR-146a and -181b modulate E-selectin-mediated migration of metastatic colon cancer cells through endothelial cells. A. *MiR-146a and -181b inhibit E-selectin-dependent TEM of HT29 colon cancer cells. In the Boyden Chambers, fluorescent HT29 cells that penetrated a tight layer of endothelial cells transfected with indicated miRNA inhibitors were counted. To test the E-selectin-dependence of the effect of both miRNAs on the TEM, anti-E-*

selectin antibody or MOPC21 antibody (1:40) was added to endothelial cells one hour before the addition of HT29 cells. Fluorescent cancer cells were counted with ImageJ with following parameters. Type: 8 bits, threshold: Otsu/dark background/12-255, analyze particle: 50-infinity. ***B.*** *MiR-146a and -181b inhibit E-selectin-dependent TEM of HT29 LoVo colon cancer cells. Experiments were carried out as described in a. Calcein AM-stained LoVo metastatic colon cancer cells were used. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test. The p-values are calculated comparing to the "antagomir ctrl/IL-1β(/antibody ctrl)" group (*p <0.05; **p<0.01). Anti-Esel.: anti-E-selectin antibody.*

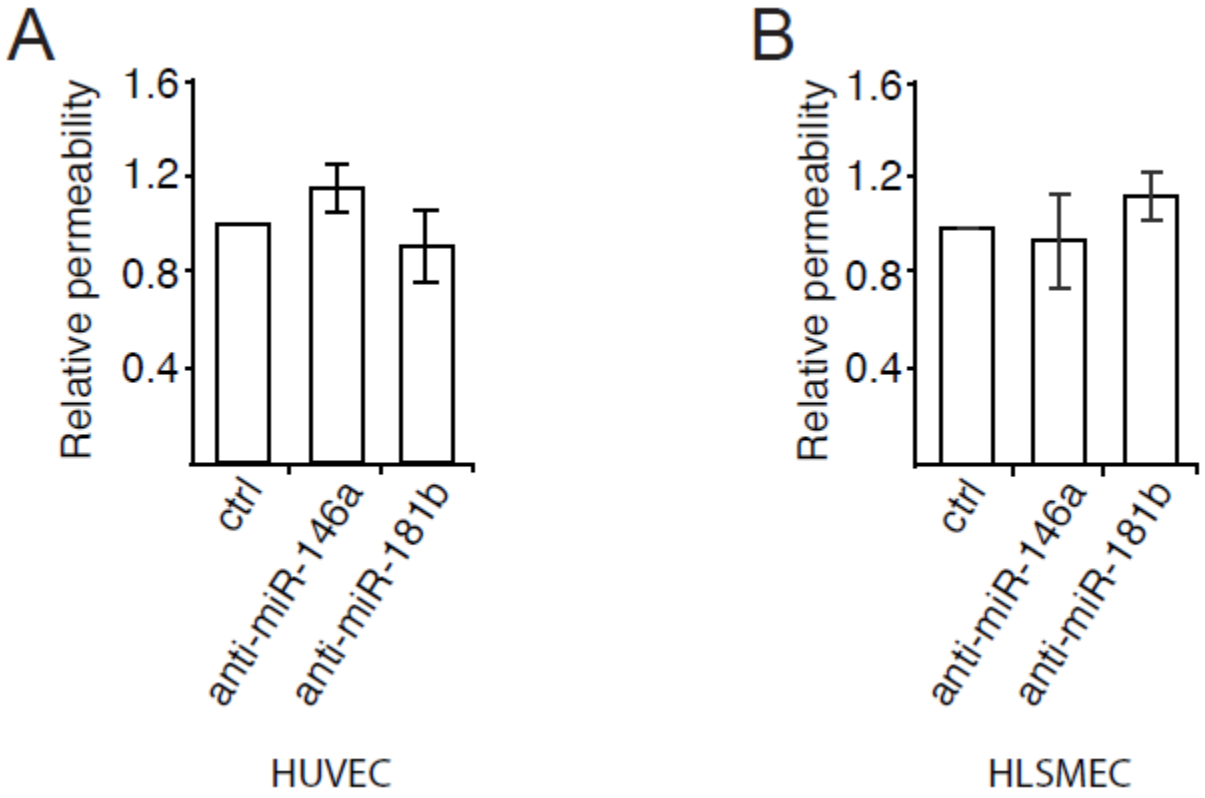


Figure S3-2. Inhibiting miR-146a and -181b does not change the integrity of HUVEC monolayer. A. *Images of endothelial cells transfected with miRNA inhibitors or control.* ***B.*** *The permeability of HUVECs transfected with miRNA inhibitors or control to FITC dextran. FITC dextran (1 mg/ml) was added to Boyden Chambers coated with indicated endothelial cells for 30 minutes. The quantifications are the mean values of three independent experiments, the error*

bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test.

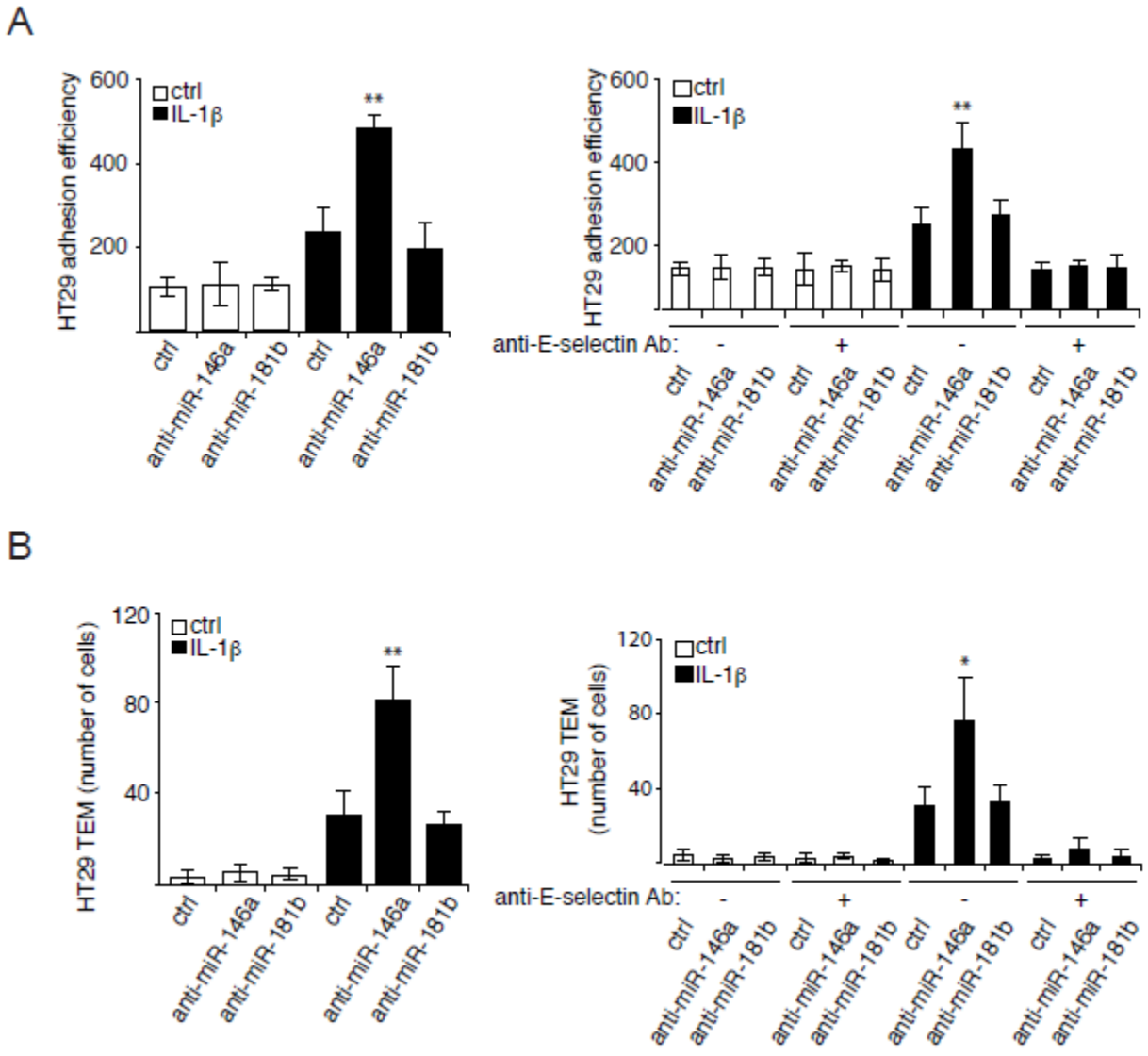


Figure S3-5. miR-146a modulate E-selectin-mediated adhesion and transendothelial migration of metastatic colon cancer cells to and through HLSMECs. ***A.*** *The permeability of HLSMECs transfected with miRNA inhibitors or control to FITC dextran. FITC dextran (1 mg/ml) was added to Boyden Chambers coated with indicated endothelial cells for 30 minutes.* ***B.*** *Mir-146a and miR-181b inhibit E-selectin-dependent adhesion of HT29 colon cancer cells to HLSMECs. The experiments were carried out as in Figure 3-3, only HLSMECs were used instead of HUVECs.* ***C.*** *Mir-146a and miR-181b inhibit E-selectin-dependent adhesion of HT29*

*colon cancer cells through HLSMECs. The experiments were carried out as in Figure 3-4, only HLSMECs were used instead of HUVECs. The quantifications are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test (*p <0.05; **p<0.01). Anti-Esel.: anti-E-selectin antibody.*

IL-1β induces the transcription of miR-146a via p38, JNK and ERK pathways

Since anti-inflammatory miRNAs are often subject to regulation by pro-inflammatory cytokines, we further studied whether these miRNAs are modulated by IL-1β. Among the tested miRNAs, only miR-146a was significantly induced upon exposure to IL-1β *(Figure 3-5a)*.

To investigate whether miR-146a is induced at the transcriptional level, pri-miR-146a was quantified with RT-quantitative PCR and we observed that its level was increased by IL-1β treatment, indicating that the transcription of miR-146a is induced (*Figure 3-5b*). To scrutinize which pathway is responsible for the induction, a panel of kinase inhibitors were applied, and the efficiency of inhibition was validated by Western blotting (*Supplementary Figure S3-7a*). Inhibiting p38, JNK and ERK reduced the level of pri-miR-146a by half four hours after IL-1β treatment, and the combination of the three inhibitors further diminished it, supporting the complementary role played by the three pathways (*Figure 3-5b*). Inhibiting PI3K mildly increased pri- and mature miR-146a (*Figure 3-5b*; *Supplementary Figure S3-8b*), while it significantly reduced E-selectin (*Supplementary Figure S3-7*). The latter observation most likely reflects the known involvement of PI3K in E-selectin transcription (Prasad et al., 2005) rather than its modest effect on miR-146a expression. These results indicate that IL-1β acts mainly through p38, JNK and ERK MAP kinases to activate the transcription of miR-146a.

We next studied which transcription factors are involved in the transcription of miR-146a. To this end, Chromatin ImmunoPrecipitation Sequencing (ChIP-seq) data of UCSC Genome Browser was used. Three pro-inflammatory transcription factors were found within the regulatory regions of miR-146a gene in un-stimulated basal endothelial cells, namely c-Jun, c-Fos and GATA2. C-Jun has been found to be activated by JNK, c-Fos has been found to be activated by ERK and p38, and GATA2 has been found to be activated by p38 (Baumgartner and Baccarini, 2014; Chen et al., 2006; Minden et al., 1994; Zarubin and Han, 2005). Accordingly, lentiviral vectors expressing shRNAs were employed to efficiently silence each of these transcription factors in endothelial cells (*Supplementary Figure S3-7b*). The knockdown of each of the three transcription factors significantly decreased the level of pri-miR-146a and thus, that of miR-146a (*Figure 3-5c; Supplementary Figure S3-8c*). Altogether, these data suggest that upon IL-1β treatment of endothelial cells, miR-146a transcription is induced through transcription factors c-Jun/c-Fos/GATA2, downstream of JNK/ERK/p38 pathways.

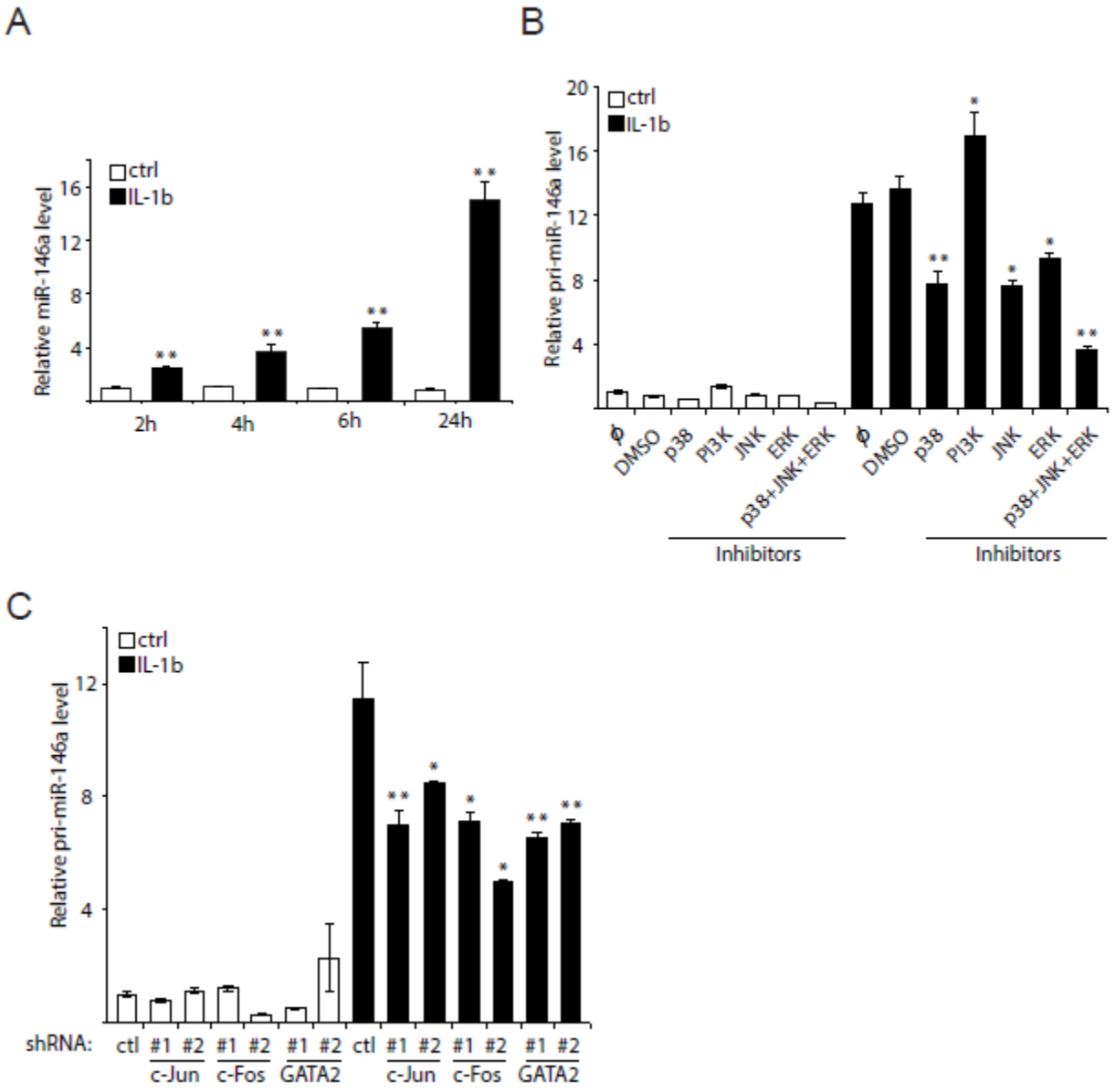


Figure 3-5. IL-1β induces the transcription of miR-146a via p38, JNK and ERK pathways. A. *Endothelial cells were treated with IL-1β (20ng/ml). The miR-146a level was measured by RT-qPCR and the snRNA U6 was used as the normalization control. The p-values are calculated comparing to the ctrl at each time point.* ***B.*** *Endothelial cells were pre-treated with 10μM of p38 inhibitor SB203580, 10μM of JNK inhibitor SP600125, 10μM of ERK inhibitor PD098059, or/and 10μM of PI3K inhibitor LY294002 for 1 hour before the addition of IL-1β (20ng/ml). Pri-miR-146a level was measured relative to GAPDH mRNA using RT-qPCR. The p-values are calculated comparing to the "DMSO/IL-1β" group.* ***C.*** *Upon knockdown with lentivirus expressing either control (scramble shRNA) or two different shRNAs targeting each*

*transcription factor (c-Jun, c-Fos and GATA2), pri-miR146a levels relative to GAPDH mRNA (control) were determined by RT-qPCR. The p-values are calculated comparing to the "shRNA ctrl/IL-1β" group. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test (*p <0.05; **p<0.01).*

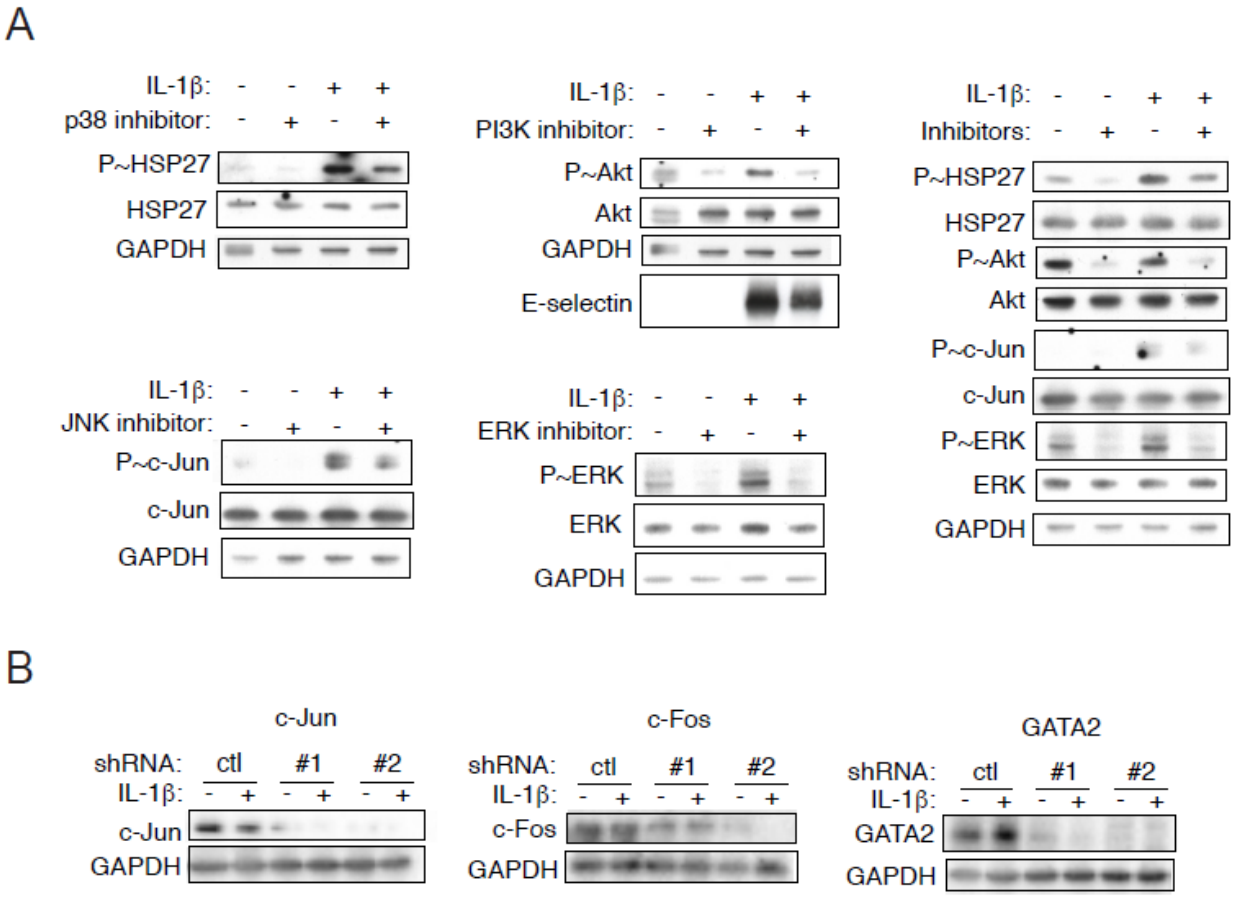


Figure S3-7. IL-1β induces the production of miR-146a via p38, JNK and ERK pathways. ***A.*** *The inhibition of p38, JNK, PI3K and ERK pathways were confirmed by Western blotting with antiphospho-HSP27 (S_{82}), anti-phospho-c-Jun (S_{63}), anti-phospho-Akt (S_{473}) and anti-phospho-ERK1/2 (T_{202}/Y_{204}) antibodies, respectively.* ***B.*** *The knockdowns of each transcription factor were validated by Western blotting. The Western blots represent three independent experiments.*

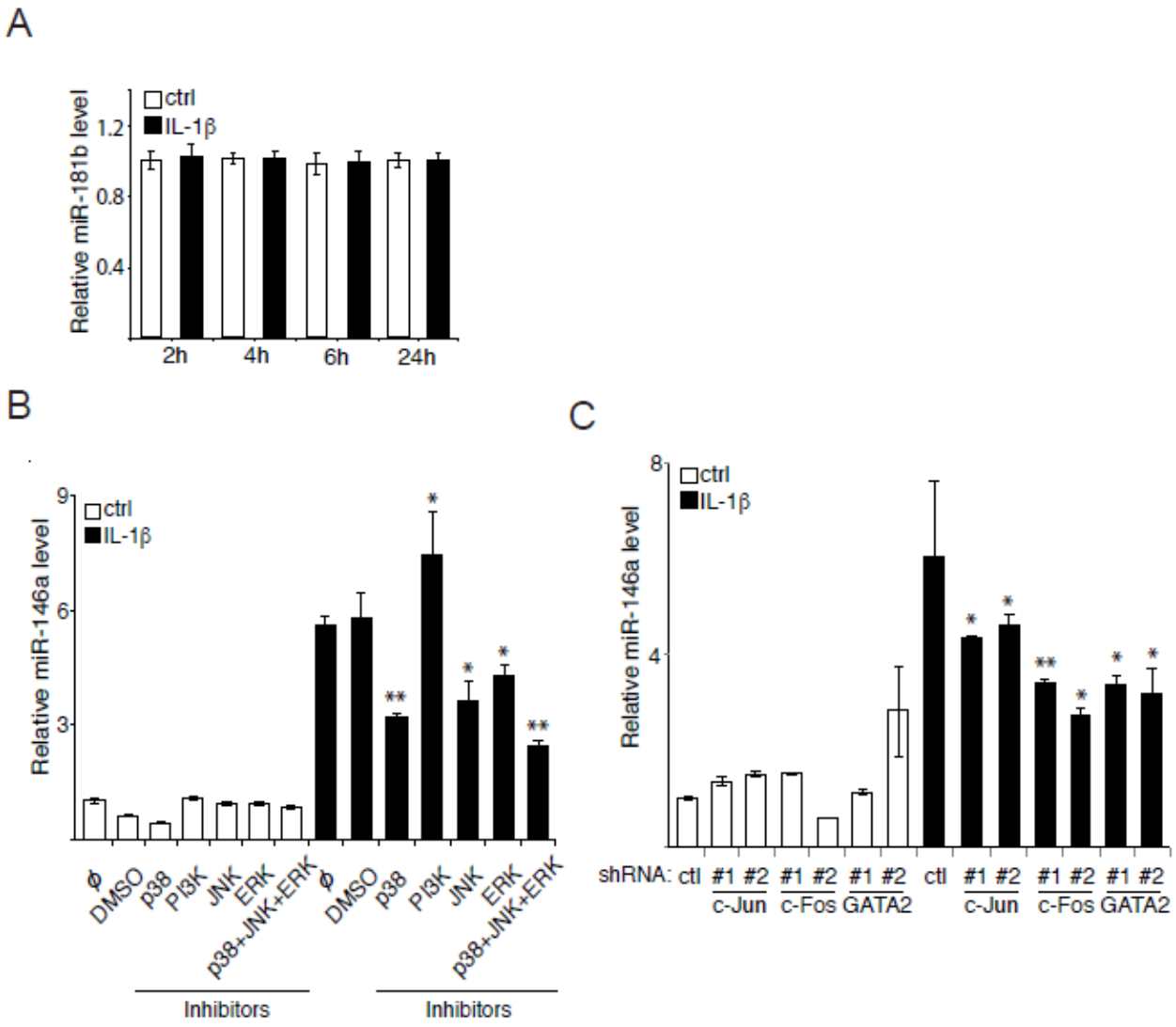


Figure S3-8. IL-1β induces the transcription of miR-146a via p38, JNK and ERK pathways. *miR-181b and -146a levels were measured by RT-qPCR and the snRNA U6 was used as the normalization control. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test (*p <0.05; **p<0.01).*

p38 MAP kinase downregulates the transcription and the translation of E-selectin by modulating miR-146a and -31

To reduce miRNAs hindering the expression of E-selectin and to de-repress the latter, endothelial cells were treated with different inhibitors of MAP kinases. Inhibiting p38 greatly increased the expression of E-selectin (*Figure 3-6a*). Interestingly, this inhibition also increased P~c-Jun and P~p65 (*Figure 3-6a*), two canonical pathways activating the transcription of E-selectin mRNA (Prasad et al., 2005; Read et al., 1997; Subramaniam et al., 1993; Zhong et al., 2016). As miR-146a depends on p38 for its expression and is a repressor of at least NF-κB (Cheng et al., 2013), one plausible hypothesis is that p38 acts through miR-146a to repress NF-κB and the transcription of E-selectin. To test this hypothesis, endothelial cells transfected with anti-miR-146a were treated with p38 inhibitor. When miR-146a was inhibited, inhibiting p38 could no longer increase P~p65, but still increased P~c-Jun (*Figure 3-6b*), suggesting that p38 acts through miR-146a to hinder NF-κB pathway activity, but not that of the JNK pathway. If p38 acts through miR-146a to inhibit NF-κB pathway thus impeding the expression of E-selectin, the transcription of E-selectin should be affected similarly. However, inhibiting p38 led to decreased E-selectin mRNA, even when miR-146a was blocked (*Figure 3-6c* and *d*). This observation is in line with the report that p38 mediates the transcription of E-selectin (Gout et al., 2008; Reymond et al., 2013; Vestweber and Blanks, 1999). Since inhibiting p38 increased E-selectin while decreasing its mRNA, p38 should repress E-selectin at the post-transcriptional level; an efficient way to promptly regulate protein level without the need of de novo transcription, mRNA maturation and export.

Similar to miR-146a, miR-31 also relies on p38 for its expression (Zhong et al., 2016). In addition, miR-31 inhibits the translation of E-selectin mRNA by targeting its 3' untranslated region (3'-UTR) (Zhong et al., 2016), making a p38 - miR-31 - E-selectin post-transcriptional regulation axis conceivable. To test this model, endothelial cells were transfected with anti-miR-31 to neutralize miR-31 and rule out its regulation of E-selectin. When miR-31 was blocked, inhibiting p38 diminished E-selectin, instead of raising it (*Figure 3-6e*), indicating that p38 acts through miR-31 to inhibit the expression of E-selectin post-transcriptionally.

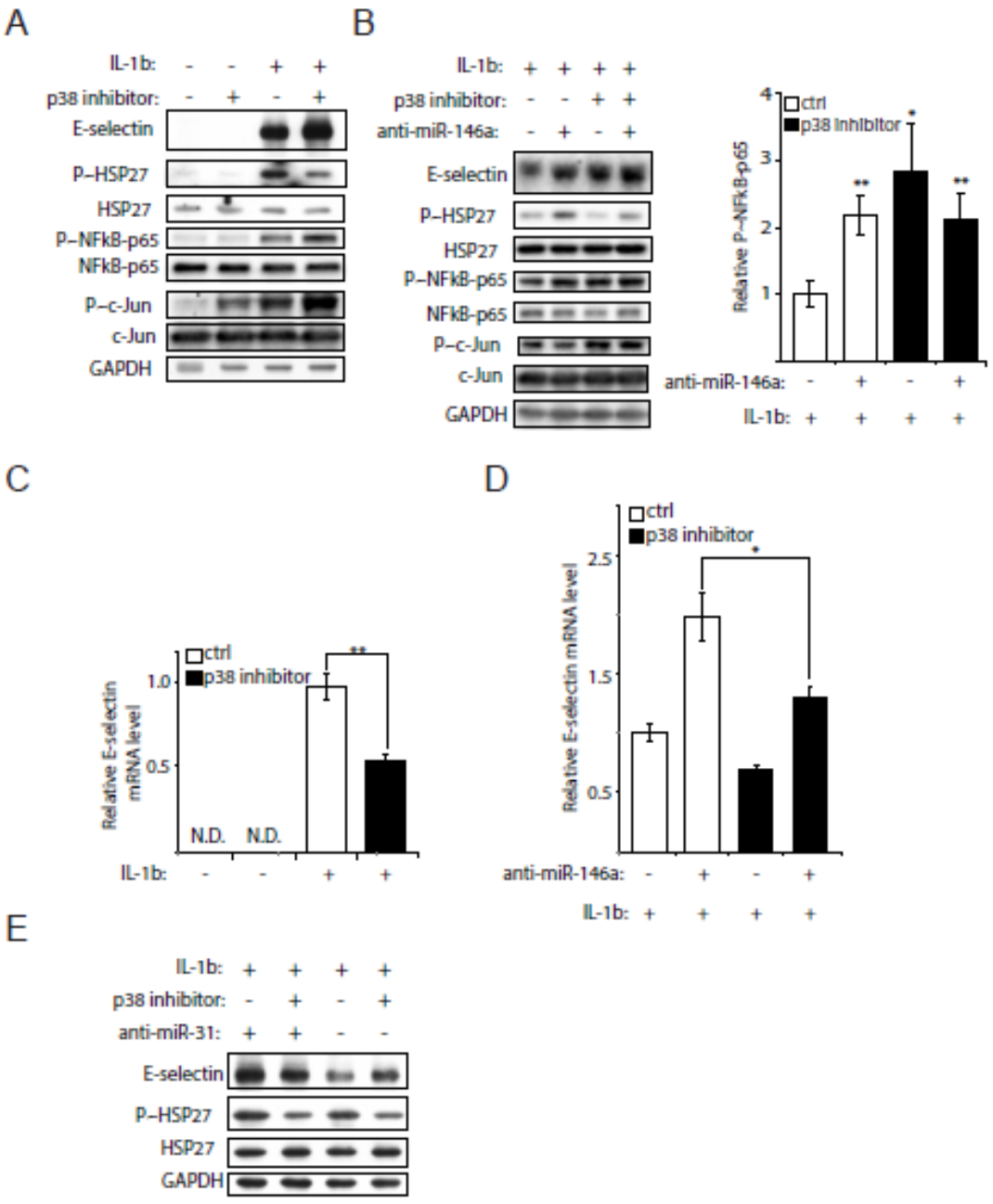


Figure 3-6. p38 MAP kinase downregulates the transcription and the translation of E-selectin by modulating miR-146a and miR-31. A. *Endothelial cells were pretreated with 10μM of p38 inhibitor SB203580, before being treated with IL-1β (20ng/ml) for four hours. Western blotting monitored the expression of E-selectin and the activities of p38 (anti-phospho-HSP27 (S^{82})), NF-κB (anti-phospho-p65 (S^{536})) and c-Jun (anti-phospho-c-Jun (S^{63})) signaling pathways. GAPDH was used as loading control.* ***B.*** *Endothelial cells were transfected with 50nM of miRNA inhibitors or control, before being pretreated with 10μM of p38 inhibitor SB203580, and treated*

*with IL-1β (20ng/ml) for four hours. Analysis as in **A**. The p-values are calculated comparing to the "antagomir ctrl/DMSO/IL-1β" group. **C.** Endothelial cells were treated as in **A**. RT-qPCR monitored the level of E-selectin mRNA relative to GAPDH mRNA. **D.** Endothelial cells were treated as in b., then analyzed as in **C**. **E.** Endothelial cells were treated as in **B**. Western blotting monitored the expression of E-selectin and the activities of p38 (anti-phospho-HSP27 (S^{82}) signaling pathway. GAPDH was used as loading control. The Western blots represent three independent experiments. The quantification are the mean values of three independent experiments, the error bars represent standard errors of three independent experiments, and the significance was analyzed using a Student's t-test (*p <0.05; **p<0.01). N.D.: non detectable.*

3.5 Discussion

Several lines of evidence indicate that cancer cells hijack the inflammatory system and interact with E-selectin to extravasate and form metastases. E-selectin interacts with colon cancer cells by binding to various counter-receptors constituted by a scaffold containing the Sialy Lewis a/x tetra-saccharide carbohydrate borne by signaling proteins including CD44v, CEA, PODXL, MUC16 and death receptor 3 (DR3) (Chen et al., 2012; Gout et al., 2008; Konstantopoulos and Thomas, 2009; Napier et al., 2007; Thomas et al., 2008, 2009). This interaction induces a forward signaling in endothelial cells and a reverse signaling in the cancer cells that both contribute to the TEM and extravasation of cancer cells (Corre et al., 2017; Gout et al., 2008). The mechanisms by which E-selectin activation triggers the TEM and extravasation of cancer cells is now well documented both in *in vitro* and *in vivo* models. Notably, the group of Lubor Borsig recently showed in mouse model that the transmigration of lung cancer cells occurs in mice expressing E-selectin via monocyte-mediated endothelial activation, which is associated with lung metastasis. In contrast, no metastasis occurs in E-selectin -/- mice. Moreover, depletion of monocytes prevents an increase in lung vascular permeability in E-selectin expressing mice.

They proposed that E-selectin-dependent TEM of cancer cells results from dissociation of VE-cadherin junctions and actin retraction, both as a result of E-selectin activation by CCL2 released by monocyte binding to E-selectin (Häuselmann et al., 2016). Interestingly, this confirms the results we obtained in earlier studies in which we show that the endothelial activation of E-selectin by clustering antibodies triggers TEM via dissociation of VE-cadherin/β catenin complex at adherent junctions in addition to p38-mediated actin retraction (Tremblay et al., 2006).

Despite the importance of E-selectin in metastatic progression, little is known about the mechanisms that downregulate its expression and stop the E-selectin-mediated adhesion process. Here, we show that miR-146a and -181b repress the expression of E-selectin by inhibiting the NF-κB pathway that controls the transcription of E-selectin. By doing so, miR-146a and -181b-mediated repression of E-selectin impairs the metastatic potentials of colon cancer cells by decreasing their adhesion to, and migration through, the endothelium.

MiR-146a is induced by IL-1β at the transcriptional level in a process involving p38, ERK and JNK MAP kinases and their downstream transcription factors c-Jun, c-Fos and GATA2. In addition, inhibiting p38 leads to decreased miR-146a, thus de-repressing the NF-κB pathway. However, due to the implication of transcription factors other than NF-κB in transcribing E-selectin downstream of p38, this de-repression is not transmitted into corresponding mRNA level change (Read et al., 1997). Inhibiting p38 de-represses the expression of E-selectin at the post-transcriptional level via miR-31, which also depends on p38 for its expression, and inhibits the translation of E-selectin mRNA (Zhong et al., 2016) (*Figure 3-7*).

MiR-146a has been reported to target the pro-inflammatory receptor adaptors Card10, TRAF6, IRAK1 and IRAK2. It thus has the potential to repress the pro-inflammatory NF-κB, ERK and JNK pathways (Cheng et al., 2013), among which NF-κB and JNK mediate the transcription of E-selectin (Prasad et al., 2005; Read et al., 1997). According to our results, miR-146a has detectable effect only on NF-κB pathway, but not on JNK pathway, suggesting the involvement of rather the former in miR-146a-mediated repression of E-selectin expression but not the latter. In addition, our observations are in line with the claim that miR-181b inhibits the activity of the NF-κB pathway by targeting Card10 and importin-α3 (Lin et al., 2016).

The conclusion that miR-146a regulates the expression of E-selectin is supported by three independent but complementary observations. First, IL-1β-induced expression of miR-146a is inversely correlated with that of E-selectin, being maximal at 24h, whereas E-selectin decreases to almost null level 24 hours after stimulation (Zhong et al., 2016). Second, inhibiting miR-146a is associated with an increase of E-selectin. Last, inhibiting miR-146a enhances the activity of NF-κB, upon which E-selectin mRNA is transcribed. Accordingly, inhibition of NF-κB totally abolished the ability of anti-miR-146a to increase E-selectin. MiR-181a, although cannot be induced by IL-1β, also exhibits the ability to hamper NF-κB-mediated E-selectin expression at least in HUVECs. On the contrary, our results indicate that inhibiting miR-10a, -30a or 92a is not associated with E-selectin expression.

One novelty of our study is the identification of p38, ERK and JNK as major pathways regulating the expression of miR-146a. This is supported by the observation that the three

pathways are induced by IL-1β and that their inhibition impairs the IL-1β-induced miR-146a. The regulation occurs at the transcriptional level given that: (1) IL-1β increased the level of pri-miR-146a; and (2) a decreased level of miR-146a in the presence of inhibitors is accompanied by a reduction of pri-miR-146a. ChIP-seq data from UCSC Genome Browser indicate that c-Jun, c-Fos and GATA2 are pro-inflammatory transcription factors bound to regulatory sites of the genome of miR-146a. They can act as transcription factors regulating miR-146a following IL-1β stimulation. Along these lines, the knockdown of c-Fos, c-Jun and GATA2 in endothelial cells reduced the IL-1β-induced transcription and production of miR-146a. Intriguingly, these transcription factors are all known to be activated by the three kinases, of which c-Jun can be activated by JNK, c-Fos can be activated by ERK and p38, and GATA2 can be activated by p38. It has been reported that AP1 (heterodimer of c-Jun and c-Fos) and GATA2 can act cooperatively to regulate transcription, which is in alignment with our data (Kawana et al., 1995).

Another important observation is that inhibiting p38 can de-repress NF-κB pathway by down-regulating miR-146a. The phosphorylation of c-Jun is also augmented by this inhibition, but as miR-146a does not affect phosphorylated c-Jun in our experiments, the mechanism still remains unknown. One plausible explanation is that, following stimulation by IL-1β, when one pathway such as p38 is inhibited, the activity of other pathways might be increased to compensate for the lost signal transduction. In any scenario, miR-146a plausibly acts as a bridge connecting NF-κB and p38 pathways to ensure signal transduction. Inhibiting p38 pathway also increased E-selectin without augmenting its mRNA, suggesting a post-transcriptional regulation. Further research revealed miR-31, another miRNA regulated by the p38 pathway that targets E-selectin mRNA (Zhong et al., 2016), as responsible for this de-repression of E-selectin. Since p38-ATF2 axis is

one of the three major pathways controlling the transcription of E-selectin mRNA (Read et al., 1997), increased c-Jun activity by a yet unknown mechanism and de-repressed NF-κB activity by reduced miR-146a do not play a major role in the de-repressed E-selectin expression upon inhibiting p38 MAP kinase. Hence, in addition to modulate the transcription of E-selectin directly, by mediating also the transcription of two miRNAs repressing E-selectin at two different levels (i.e. transcription and translation), p38 tightly controls the expression of this important cell adhesion molecule (*Figure 3-7*). Previous studies have shown that after 4-6 hours the expression of E-selectin at the cell surface decreases following its internalization and degradation in the lysosomes (Subramaniam et al., 1993). Our findings now highlight new mechanisms by which the expression of E-selectin is precisely down-regulated by miRNAs.

In accordance with our finding that miR-146a and -181b regulate the expression of E-selectin, inhibiting them is associated with increased adhesion and migration of metastatic colon cancer cells to, and through, endothelial monolayer, both of which are abolished by E-selectin neutralizing antibody. These findings support our argument that miR-146a and-181b have anti-metastatic properties against colon cancers. In the long run, miR-146a and -181b may be proved as key players in the metastatic process of not only colorectal carcinoma, but also breast, bladder, gastric, and pancreatic carcinoma, leukemia and lymphoma, which all depend on E-selectin for their extravasation (Gout et al., 2008; Reymond et al., 2013; Vestweber and Blanks, 1999). Our data are the first to report the miRNA modulation of E-selectin expression and functions in an *in vitro* model of TEM of cancer cells. Accordingly, we believe that our results are important and hope they will pave the road to *in vivo* studies. On the other hand, evidence is available for miRNA regulation of E-selectin during inflammatory diseases such as atherosclerosis (Corre et

al., 2017). Given that E-selectin is expressed in response to inflammation, we believe the results in support of miRNA/E-selectin as mediators of the inflammatory process *in vivo* should soon be translated into *in vivo* models of tumor initiation and progression associated with inflammation.

In conclusion, our study supports the critical role played by miRNAs in regulating E-selectin dynamics. Notably, it highlights the role of miR-146a and -181b as transcriptional modulators regulating E-selectin and E-selectin-dependent metastatic abilities of colon cancer. Furthermore, our findings raise the possibility that both miRNAs may be maintained at a low level in endothelial cells constantly expressing E-selectin, thus promoting the transendothelial migration of cancer cells during metastasis. In this context, increasing their expression in endothelial cells may be envisioned as an approach to reduce metastases of cancer cells which extravasate in an E-selectin-dependent manner into organs as various as liver, bone marrow, skin and lung (Gout et al., 2008; Reymond et al., 2013). In corollary, it might be expected that a low level of miR-146a and/or -181b in endothelial cells could serve as a biomarker of pro-metastatic states.

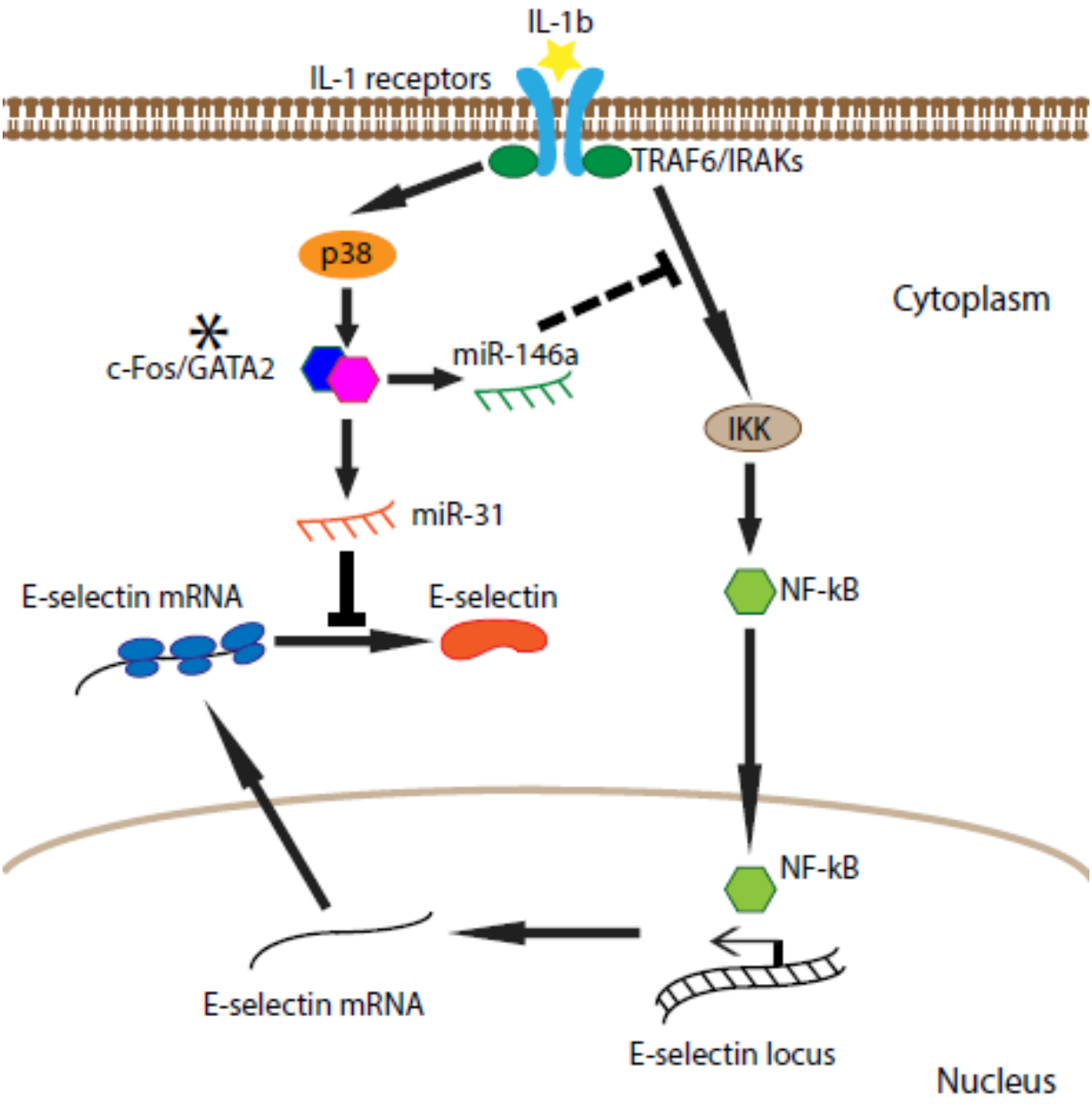


Figure 3-7. p38 mediates the transcription and the translation of E-selectin by modulating miR-146a and miR-31. *Following stimulation with IL-1β, the receptor-adaptor complex signals through NF-κB pathway to activate the transcription of E-selectin. In parallel, the complex also signals through p38 pathway to induce the production of miR-146a and miR-31. The former, in a feedback loop, targets adaptor proteins TRAF6 and IRAKs to repress NF-κB-dependent E-selectin expression at the transcriptional level. The latter targets E-selectin mRNA to inhibit its*

*translation. *: In order to simplify the figure, the transcription of miRNAs is presented in the cytoplasm.*

3.6 Materials and methods

Reagents

Chemical inhibitors PD098059, LY294002, SP600125 and SB203580 were obtained from Sigma (St Louis, MO). IKK Inhibitor VII was obtained from EMD Millipore (Billerica, MA). CHIR99021 was obtained from Thermo Fisher (Montreal, QC). Calcein-AM was obtained from Invitrogen Molecular Probes (Burlington, ON). Dimethylsulfoxyde was purchased from Thermo Fisher (Montreal, QC). IL-1β was obtained from R&D Systems (Minneapolis, MN).

Cells

Human umbilical vein endothelial cells (Endothelial cells) were isolated by collagenase digestion of umbilical veins from undamaged fresh cords, as described(Huot et al., 1997). This human material was obtained in accordance with the relevant guidelines and regulations. In that regard, the authorization of the human ethical committee (HEC) of the CRCHU de Québec-Université Laval was obtained to do the study and participating mothers signed informed consents. HUVECs at passages ≤4 were grown to form monolayer in EGM2 endothelial cell growth medium (Lonza, Allendale, NJ) in gelatin-coated tissue culture flasks. Human liver sinusoidal microvascular endothelial cells (HLSMECs) and its Complete Classic Medium With Serum and CultureBoost were purchased from Cell Systems (Kirkland, WA) and grown using the same protocol as for HUVECs. HT29 (ATCC) colorectal adenocarcinoma cells were cultivated in McCoy 5A medium (Sigma, St Louis, MO) supplemented with 10% foetal bovine serum (FBS). LoVo (ATCC) colorectal adenocarcinoma cells were cultivated in Ham's F12 nutrient mixture

(Life Technologies, Carlsbad, CA) supplemented with 10% FBS. All cells were cultivated at 37°C in 5% CO_2 humidified atmosphere.

Plasmids, shRNAs, miRNA mimics and inhibitors

MiRIDIAN miRNA hairpin inhibitors and negative control #1 were obtained from Dharmacon (Lafayette, CO, USA). These miRNA inhibitors bind to miRNAs by complementary sequences and thus block their capacity to silence mRNA target without affecting the level of miRNAs(Hutvágner et al., 2004). Lentiviral particles containing shRNAs against c-Jun (TRCN0000039589, TRCN0000039590), c-Fos (TRCN0000016004, TRCN000016007) and GATA2 (TRCN0000019264, TRCN0000019265) were obtained from Sigma (St Louis, MO).

Transfection and infection

Endothelial cells were transfected using X-tremeGene HP Transfection Reagent following manufacturers' protocol (Roche Life Science, Laval, QC). Endothelial cells were infected by lentivirus in the presence of 8 μg/mL hexadimethrine bromide (Sigma, St Louis, MO).

RNA extraction and quantification

Cells were lysed in TRIzol (Invitrogen, Carlsbad, CA) to extract total RNA following manufacturer's protocol. The quality of RNA was assessed on agarose gel and spectrometry. The reverse transcription was performed with TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using specific primers (Life Technologies, Carlsbad, CA) for miRNAs, or random primers for pri-miRNAs. Sybr Green was carried out with iScript reverse transcription supermix and SsoAdvanced universal Sybr Green supermix from Bio-Rad (Hercules, CA). The qPCR was carried out with Universal PCR Master Mix (Life Technologies,

Carlsbad, CA) and specific probes from the same company. For miR-181b, its reverse transcription was performed with TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA), and its qPCR was carried out with TaqMan Fast Advanced Master Mix from the same company.

Western blotting

Cells were lysed using SDS-PAGE loading buffer without reducing agents. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. Antibodies were applied according to their manufacturers' protocols. Blots were developed with SuperSignal West Pico Substrate (Thermo Fisher, Montreal, QC). Anti-phospho-ERK1/2 MAPK (T^{202}/Y^{204}) mouse antibody (1:1000), anti-ERK1/2 MAPK mouse antibody (1:1000), anti-phospho-Akt (S^{473}) rabbit antibody (1:1000), anti-Akt rabbit antibody (1:1000), anti-phospho-c-Jun (S^{63}) rabbit antibody (1:1000), anti-c-Jun rabbit antibody (1:1000), anti-phospho-p65 (S^{536}) rabbit antibody (1:1000), anti-p65 rabbit antibody (1:1000), anti-phospho-HSP27 (S^{82}) rabbit antibody (1:1000) and anti-HSP27 rabbit antibody (1:1000) were obtained from Cell Signaling Technology (Beverly, MA), Anti-E-selectin mouse monoclonal antibody (1:1000) was obtained from R&D Systems (Minneapolis, MN). Anti-METTL3 rabbit antibody (1:2000) was obtained from Abcam (Cambridge, UK). Anti-GAPDH mouse antibody (1:10000) was obtained from Novus Biologicals (Oakville, ON). Anti-mouse/rabbit-IgG-horseradish-peroxidase (HRP) goat antibodies (1:5000) were obtained from The Jackson Laboratory (Bar Harbor, ME).

Adhesion assay

Endothelial cells were plated on gelatin-coated wells and left to grow to confluence. HT29 and LoVo cells were labeled with calcein-AM for 30 min at 37 °C, then were added to endothelial

cells for 30min. The endothelial layer was washed twice with PBS and the attached cells were quantified by measuring the fluorescence emission with Fluoroskan Ascent™ Microplate Fluorometer (Thermo Scientific). To study the E-selectin-dependence of the adhesion, neutralizing anti-E-selectin mouse antibody (1:5000, Cedarlane Labs (Burlington, ON)) or the control MOPC21 antibody (1:40, Abcam (Cambridge, UK)) was introduced one hour before adding HT29 or LoVo cells.

Endothelial permeability assay

The endothelial barrier integrity was evaluated by measuring the permeability across endothelial cell monolayers of FITC-Dextran in a gelatin-coated Transwell units (Boyden chambers) [6.5 mm diameter, 0.4 μm pore size polycarbonatefilter; Corning Costar; Pittston, PA, USA]. More precisely, endothelial permeability was determined by measuring the passage of FITC-labelled dextran (1 mg/ml of fluorescein isothiocyanate-dextran, molecular mass: 40 kDa; Sigma-Aldrich) through the endothelial monolayer during 30 minutes. 100 μl was collected from the lower compartment and fluorescence was evaluated using a Fluoroskan Ascent Microplate Fluorometer following manufacturer's protocol (Thermo Scientific, Mtl, Qc, Canada).

Transendothelial migration assay

Cell migration was investigated using a modified Boyden Chamber assay. Endothelial cells were grown to confluence on a 5.0μm-pore-sized gelatinized polycarbonate membrane separating the two compartments of a 6.5mm migration chamber (Transwell, Costar, MA). After IL-1β-mediated activation of endothelial cells for 4 hours, calcein-AM stained HT29 or LoVo cells suspended in migration buffer (medium199, 10mM HEPES pH7.4, 1.0mM $MgCl_2$, 0.5% BSA) were added to the monolayer of endothelial cells, previously washed with the same buffer. After

five hours, cells on the upper face of the membrane were scraped with a cotton swab. The number of HT29 or LoVo cells that have migrated to the lower face of the filter was counted using an inverted fluorescence microscope. To study the E-selectin-dependence of the migration, neutralizing anti-E-selectin mouse antibody (1:5000, Cedarlane Labs (Burlington, ON)) or the control MOPC21 antibody (1:40, Abcam (Cambridge, UK)) was introduced one hour before adding HT29 or LoVo cells.

Chapter 4. General discussion

4.1 E-selectin-mediated metastases

E-selectin has long been reported to interact with circulating cancer cells including colon cancer cells by binding to various counter-receptors constituted by a scaffold containing the Sialy Lewis a/x tetra-saccharide carbohydrate borne by signaling proteins including CD44v, CEA, PODXL, MUC16 and death receptor 3 (DR3). In this way, cancer cells hijack the inflammatory system to extravasate and form secondary tumors (Chen et al., 2012; Gout et al., 2008; Konstantopoulos and Thomas, 2009; Napier et al., 2007; Thomas et al., 2008, 2009). This interaction further contributes to the extravasation of cancer cells through inducing a forward signaling in endothelial cells and reverse signaling in the cancer cells (Corre et al., 2017; Gout et al., 2008). Recently the group of Lubor Borsig showed in mouse model that the transmigration of lung cancer cells occurs in mice expressing E-selectin via monocyte-mediated endothelial activation, which is associated with lung metastasis. They proposed that following E-selectin activation by CCL2 released by monocyte binding to E-selectin, VE-cadherin junctions dissociates and actin retracts, resulting in E-selectin-dependent TEM of cancer cells (Häuselmann et al., 2016), which is in correlation with our earlier observations that clustering antibodies activate E-selectin and trigger TEM via dissociation of VE-cadherin/β catenin complex at adherent junctions, in addition to p38-mediated actin retraction (Tremblay et al., 2006).

4.2 miRNAs repress the expression of E-selectin and the transendothelial migration of colon cancer cells through different mechanisms

Despite the importance of E-selectin in metastatic progression, little is known about the mechanisms that downregulate its expression and stop the E-selectin-mediated adhesion process. This project revealed three miRNAs capable of such task, namely miR-31, miR-146a and miR-181b.

Our data show that miR-31 and miR-146a are significantly induced by IL-1β and that their expression is inversely correlated with that of E-selectin and E-selectin-mediated metastatic potential of colon cancer cells. Three independent but complementary observations support our conclusion that miR-31 and miR-146a regulate the expression of E-selectin. 1) IL-1β-induced expression of miR-31 and miR-146a are inversely correlated with that of E-selectin. 2) Inhibiting both miRNAs are associated with an increase of E-selectin level in two types of endothelial cells. 3) Altering the predicted binding site of miR-31 in the 3'UTR of E-selectin mRNA hinders its repression, supporting a direct role of miR-31 in E-selectin targeting; as for miR-146a, inhibiting it enhances the activity of NF-κB, upon which E-selectin mRNA is transcribed. miR-181b, although cannot be induced by IL-1β, also represses NF-κB-mediated E-selectin expression according to our inhibitor assay. Our observations are in line with the claim that miR-146a targets pro-inflammatory receptor adaptors Card10, TRAF6, IRAK1 and IRAK2, and that miR-181b targets Card10 and importin-α3, to repress NF-κB activity (Lin et al., 2016).

In accordance with our finding that the three miRNAs inhibit the expression of E-selectin, inhibiting them is associated with increased adhesion and migration of colon cancer cells to and through endothelial layers, both of which are abolished by E-selectin neutralizing antibody. These findings support our argument that miR-31, miR-146a and miR-181b have anti-metastatic

properties against colon cancers. Our findings show for the first time that miRNAs can exert their anti-metastatic ability by repressing the expression of E-selectin, an adhesion molecule in endothelial cells. In the long run, miR-31, miR-146a and miR-181b may be proved as key players in the metastatic process of not only colorectal carcinoma, but also breast, bladder, gastric, and pancreatic carcinoma, leukemia and lymphoma, which all depend on E-selectin for their extravasation (Gout et al., 2008; Reymond et al., 2013; Vestweber and Blanks, 1999). We believe that our *in vitro* findings are important and hope they will pave the road to future *in vivo* studies.

On the contrary, our results indicate that inhibiting miR-10a, -30a or 92a is not associated with E-selectin expression.

4.3 The mechanisms through which IL-1β induces the expression of the miRNAs

We further report that the expression of miR-31 and miR-146a in response to IL-1β are stimulated at the transcriptional level in a process involving MAP kinase pathways and their downstream transcription factors c-Jun, c-Fos and GATA2. The identification of p38 and JNK as major pathways regulating the expression of miR-31 is supported by the observation that both pathways are induced by IL-1β and that their inhibition impairs the IL-1β-induced increase in miR-31. Similarly, p38, ERK and JNK were identified as major pathways regulating the expression of miR-146a. The regulation occurs at the transcriptional level given that: 1) IL-1β increased the level of pri-miRNAs and; 2) decreased miRNAs in the presence of inhibitors are accompanied by decreased pri-miRNAs (down to basal level without IL-1β). Chromatin ImmunoPrecipitations followed by sequencing (ChIP-seq) data from UCSC Genome Browser show that c-Jun, c-Fos and GATA2 are transcription factors bound to regulatory sites of the

genome of both miRNAs. They can act as transcription factors regulating their transcription following IL-1β stimulation. Along these lines, the knockdown of c-Fos, c-Jun and GATA2 in endothelial cells reduced the IL-1β-induced transcription and production of miR-31 and miR-146a. Intriguingly, these transcription factors are all known targets of MAP kinases, of which c-Jun can be activated by JNK, c-Fos can be activated by ERK and p38, and GATA2 can be activated by p38 (Baumgartner and Baccarini, 2014; Chen et al., 2006; Minden et al., 1994; Tanos et al., 2005; Zarubin and Han, 2005). It has been reported that AP1 (heterodimer of c-Jun and c-Fos) and GATA2 can act cooperatively to regulate transcription, which is in alignment with our data (Kawana et al., 1995).

4.4 p38 MAP kinase represses the transcription and the translation of E-selectin through miR-146a and miR-31, respectively

This project provides a glimpse into the intertwined relationships between miRNAs, MAP kinase pathways and E-selectin expression. Inhibiting p38 leads to decreased miR-146a, thus de-repressing the NF-κB pathway. However, due to the implication of transcription factors other than NF-κB in transcribing E-selectin downstream of p38, this de-repression is not transmitted into corresponding mRNA level change (Read et al., 1997). Inhibiting p38 de-represses the expression of E-selectin at the post-transcriptional level via miR-31, which also depends on p38 for its expression, and inhibits the translation of E-selectin mRNA (Zhong et al., 2016) (*Figure 4-2*). The phosphorylation of c-Jun is also increased by p38 inhibition, but as miR-146a does not affect phosphorylated c-Jun in our experiments, the mechanism remains unknown. One possibility is that, IL-1β-stimulated signal has to find another pathway to be propagated upon inhibiting one pathway. In other words, when one pathway such as p38 is inhibited, the activity

of other pathways might be increased to compensate for the lost signal transduction. In this sense, miR-146a plausibly acts as a bridge connecting NF-κB and p38 pathways to ensure signal transduction. Inhibiting p38 pathway also increases E-selectin without augmenting its mRNA, suggesting a post-transcriptional regulation. Further research revealed miR-31, which targets E-selectin post-transcriptionally (Zhong et al., 2016), as responsible for this de-repression of E-selectin. Hence, in addition to modulate the transcription of E-selectin directly, by mediating the transcription of two miRNAs repressing E-selectin at two different levels (i.e. transcription and translation), p38 tightly controls the expression of this important cell adhesion molecule (*Figure 3-7*).

Chapter 5. Conclusion and perspectives

5.1 Conclusion

In conclusion, our study supports the critical role played by miRNAs in regulating E-selectin dynamics. Notably, miR-31 targets E-selectin mRNA directly and represses E-selectin-dependent colon cancer metastasis, while miR-146a and miR-181b act as transcriptional modulators regulating E-selectin and E-selectin-dependent metastatic abilities of colon cancer by targeting the pro-inflammatory NF-κB pathway. The transcription and the expression of miR-31 and miR-146a are induced by the pro-inflammatory cytokine IL-1β through p38 pathway. In this sense p38 represses the metastatic abilities of colon cancer cells via modulating the expression of two miRNAs (*Figure 5-1*).

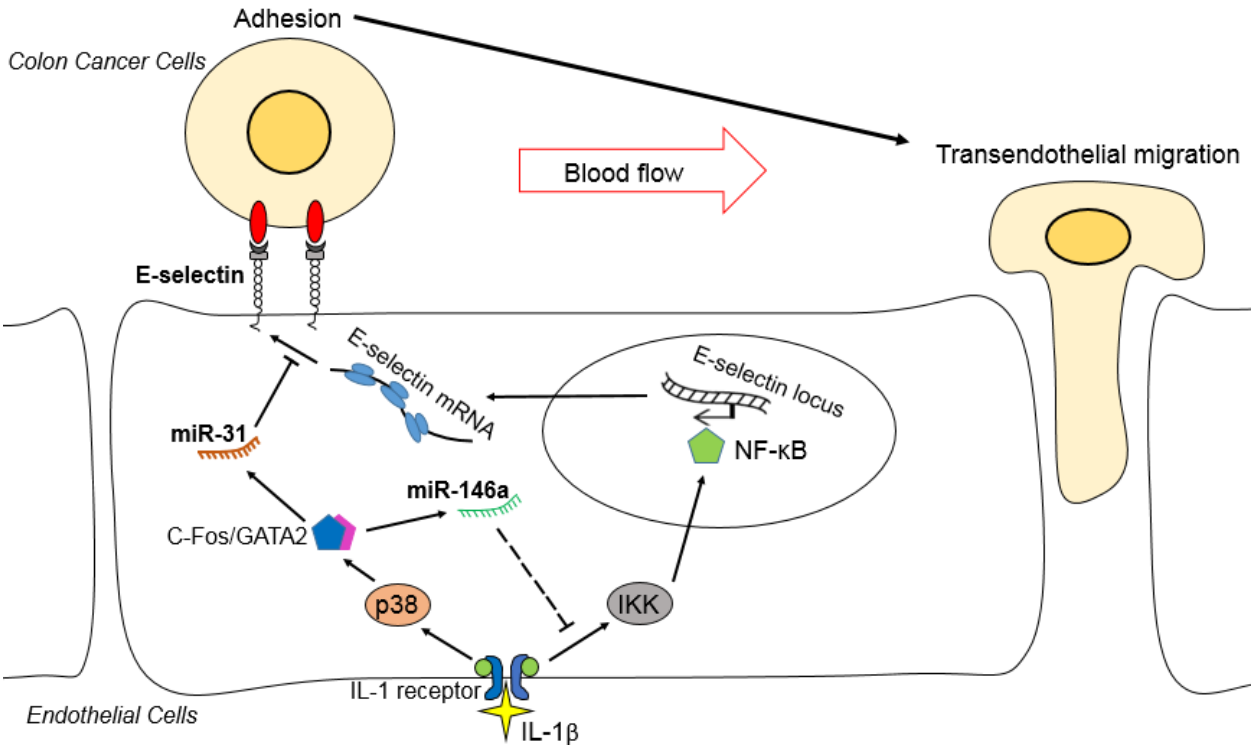


Figure 5-1. p38 MAP kinase represses the transcription and the translation of E-selectin by modulating miR-146a and miR-31. *miR-31 targets E-selectin mRNA directly, while miR-146a and miR-181b act as its transcriptional modulators by targeting NF-κB pathway. All three miRNAs repress E-selectin-dependent metastatic abilities of colon cancer cells. miR-31 and miR-146a are induced by IL-1β through the p38 pathway.*

Our findings further raise the possibility that these miRNAs may be maintained at a low level in endothelial cells constantly expressing E-selectin, thus promoting transendothelial migration of cancer cells during metastasis. In this context, increasing their expression in endothelial cells may be envisioned as an approach to reduce metastases of cancer cells which extravasate in an E-selectin-dependent manner into organs as various as liver, bone marrow, skin, and lung (Gout

et al., 2008; Reymond et al., 2013). In corollary, it might be expected that a low level of these miRNAs in endothelial cells could serve as a biomarker of pro-metastatic states.

5.2 Perspectives

The outcomes of this thesis open the paths to several potential projects.

Firstly, since p38 MAP kinase mediates the expression of miR-31 and miR-146a, two miRNAs repressing respectively the transcription and the translation of E-selectin, and the latter activity prevails over p38's involvement in transcribing E-selectin, it would be logical to try to specifically activate p38 MAP kinase using its dominant positive mutants to inhibit E-selectin expression and E-selectin-mediated transendothelial migration of colon cancer cells. However, the large spectrum of p38 targets, and the intertwining relationship between p38 and E-selectin may limit the specificity of the approach. The regulation of the metastatic process by E-selectin-p38 axis was firstly revealed by Laferrière and colleagues, where E-selectin activates p38 in colon cancer cells and modulates their transendothelial migration ability (Laferrière et al., 2002). Gout further revealed a new E-selectin counter-receptor, DR3, which is responsible for E-selectin-mediated p38 activation and metastatic advantage (Gout et al., 2006). ERK is also activated by the axis and confers a survival advantage to cancer cells. In endothelial cells, the binding of E-selectin to DR3 activates p38 and ERK, leading to F-actin-modulated cell retraction and intercellular VE-cadherin/β-catenin junction complex dissociation, respectively, further enhancing the diapedesis of colon cancer cells (Tremblay et al., 2008).

Secondly, the implication of miR-31, miR-146a, miR-181b, as well as p38 MAP kinase in mediating metastasis of circulating colon cancer cells should be further studied *in vivo*. The endothelium-specific manipulation of miRNAs can be approached in two ways. 1) miRNAs can be selectively carried to inflammatory endothelium using vectors targeting E-selectin. Ma et al. developed an E-selectin-targeting multistage vector (ESTA-MSV) to deliver miR-146a and miR-181b to inflamed endothelium in mice (Ma et al., 2016b). Similar systems are reviewed in

Chapter 3.9. 2) The recombinant adeno-associated virus (rAAV)-based genome engineering (Ding et al., 2016), combined with endothelial-specific VE-cadherin or E-selectin promoter (Gory et al., 1999) could prove useful for endothelial-specific overexpression or knockdown of miRNAs and/or p38 pathway factors. The promising engineering progresses on delivery vectors and genome engineering substantiate their use to further our research in vivo.

Thirdly, in the long run, miR-31, miR-146a and miR-181b, together with p38 MAP kinase may be proved as key players in the metastatic process of not only colorectal carcinoma, but also breast, bladder, gastric, and pancreatic carcinoma, leukemia and lymphoma, which all depend on E-selectin for their extravasation (Gout et al., 2008; Reymond et al., 2013; Vestweber and Blanks, 1999).

Finally, given the large spectrum of kinases activated by IL-1β treatment that are involved in miRNA maturation, such as microprocessing (Duan et al., 2016; Suzuki et al., 2017) and pre-miRNA export (See Introduction), the maturation of miRNA following its transcription induced by IL-1β merits further investigation.

Bibliography

Abe, J. i, Takahashi, M., Ishida, M., Lee, J.D., and Berk, B.C. (1997). c-Src is required for oxidative stress-mediated activation of big mitogen-activated protein kinase 1. J. Biol. Chem. *272*, 20389–20394.

Aigner, S., Ramos, C.L., Hafezi-Moghadam, A., Lawrence, M.B., Friederichs, J., Altevogt, P., and Ley, K. (1998). CD24 mediates rolling of breast carcinoma cells on P-selectin. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *12*, 1241–1251.

Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhal, S., and Wood, M.J.A. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat. Biotechnol. *29*, 341–345.

Ambros, V. (2004). The functions of animal microRNAs. Nature *431*, 350–355.

Ameres, S.L., and Zamore, P.D. (2013). Diversifying microRNA sequence and function. Nat. Rev. Mol. Cell Biol. *14*, 475–488.

Ameres, S.L., Horwich, M.D., Hung, J.-H., Xu, J., Ghildiyal, M., Weng, Z., and Zamore, P.D. (2010). Target RNA-directed trimming and tailing of small silencing RNAs. Science *328*, 1534–1539.

Antoniali, G., Serra, F., Lirussi, L., Tanaka, M., D'Ambrosio, C., Zhang, S., Radovic, S., Dalla, E., Ciani, Y., Scaloni, A., et al. (2017). Mammalian APE1 controls miRNA processing and its interactome is linked to cancer RNA metabolism. Nat. Commun. *8*, 797.

Araki, T., Miki, C., and Kusunoki, M. (2001). Biological implications of circulating soluble intercellular adhesion molecule-1 in colorectal cancer patients. Scand. J. Gastroenterol. *36*, 399–404.

Asgeirsdóttir, S.A., van Solingen, C., Kurniati, N.F., Zwiers, P.J., Heeringa, P., van Meurs, M., Satchell, S.C., Saleem, M.A., Mathieson, P.W., Banas, B., et al. (2012). MicroRNA-126 contributes to renal microvascular heterogeneity of VCAM-1 protein expression in acute inflammation. Am. J. Physiol. Renal Physiol. *302*, F1630-1639.

Auguste, P., Fallavollita, L., Wang, N., Burnier, J., Bikfalvi, A., and Brodt, P. (2007). The Host Inflammatory Response Promotes Liver Metastasis by Increasing Tumor Cell Arrest and Extravasation. Am. J. Pathol. *170*, 1781–1792.

Auyeung, V.C., Ulitsky, I., McGeary, S.E., and Bartel, D.P. (2013). Beyond secondary structure: primary-sequence determinants license pri-miRNA hairpins for processing. Cell *152*, 844–858.

Baccarini, A., Chauhan, H., Gardner, T.J., Jayaprakash, A.D., Sachidanandam, R., and Brown, B.D. (2011). Kinetic analysis reveals the fate of a microRNA following target regulation in mammalian cells. Curr. Biol. CB *21*, 369–376.

Banks, R.E., Gearing, A.J., Hemingway, I.K., Norfolk, D.R., Perren, T.J., and Selby, P.J. (1993). Circulating intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) in human malignancies. Br. J. Cancer *68*, 122–124.

Bao, H., Chen, H., Zhu, X., Zhang, M., Yao, G., Yu, Y., Qin, W., Zeng, C., Zen, K., and Liu, Z. (2014). MiR-223 downregulation promotes glomerular endothelial cell activation by upregulating importin α4 and α5 in IgA nephropathy. Kidney Int. *85*, 624–635.

Barber, B.A., and Rastegar, M. (2010). Epigenetic control of Hox genes during neurogenesis, development, and disease. Ann. Anat. Anat. Anz. Off. Organ Anat. Ges. *192*, 261–274.

Barišić-Jäger, E., Kręcioch, I., Hosiner, S., Antic, S., and Dorner, S. (2013). HPat a decapping activator interacting with the miRNA effector complex. PloS One *8*, e71860.

Barnes, D.E., and Lindahl, T. (2004). Repair and genetic consequences of endogenous DNA base damage in mammalian cells. Annu. Rev. Genet. *38*, 445–476.

Bartel, D.P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell *116*, 281–297.

Barthel, S.R., Gavino, J.D., Descheny, L., and Dimitroff, C.J. (2007). Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin. Ther. Targets *11*, 1473–1491.

Basu, A.K. (2018). DNA Damage, Mutagenesis and Cancer. Int. J. Mol. Sci. *19*.

Baumann, P., Cremers, N., Kroese, F., Orend, G., Chiquet-Ehrismann, R., Uede, T., Yagita, H., and Sleeman, J.P. (2005). CD24 expression causes the acquisition of multiple cellular properties associated with tumor growth and metastasis. Cancer Res. *65*, 10783–10793.

Baumgartner, C., and Baccarini, M. (2014). p38 links RAS to GATA2. EMBO Rep. *15*, 912–913.

Bayraktar, R., Van Roosbroeck, K., and Calin, G.A. (2017). Cell to cell communication: microRNAs as hormones. Mol. Oncol.

Benekli, M., Güllü, I.H., Tekuzman, G., Savaş, M.C., Hayran, M., Hasçelik, G., and Firat, D. (1998). Circulating intercellular adhesion molecule-1 and E-selectin levels in gastric cancer. Br. J. Cancer *78*, 267–271.

Berenblum, I., and Shubik, P. (1949). The Persistence of Latent Tumour Cells Induced in the Mouse's Skin by a Single Application of 9:10-Dimethyl-1:2-Benzanthracene. Br. J. Cancer *3*, 384–386.

Bernal-Mizrachi, L., Jy, W., Jimenez, J.J., Pastor, J., Mauro, L.M., Horstman, L.L., de Marchena, E., and Ahn, Y.S. (2003). High levels of circulating endothelial microparticles in patients with acute coronary syndromes. Am. Heart J. *145*, 962–970.

Bernstein, B.E., Meissner, A., and Lander, E.S. (2007). The Mammalian Epigenome. Cell *128*, 669–681.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Bird, N.C., Mangnall, D., and Majeed, A.W. (2006). Biology of colorectal liver metastases: A review. J. Surg. Oncol. *94*, 68–80.

Biswas, S., and Rao, C.M. (2017). Epigenetics in cancer: Fundamentals and Beyond. Pharmacol. Ther. *173*, 118–134.

Blann, A.D., Gurney, D., Wadley, M., Bareford, D., Stonelake, P., and Lip, G.Y. (2001). Increased soluble P-selectin in patients with haematological and breast cancer: a comparison with fibrinogen, plasminogen activator inhibitor and von Willebrand factor. Blood Coagul. Fibrinolysis Int. J. Haemost. Thromb. *12*, 43–50.

Boele, J., Persson, H., Shin, J.W., Ishizu, Y., Newie, I.S., Søkilde, R., Hawkins, S.M., Coarfa, C., Ikeda, K., Takayama, K., et al. (2014). PAPD5-mediated 3' adenylation and subsequent degradation of miR-21 is disrupted in proliferative disease. Proc. Natl. Acad. Sci. U. S. A. *111*, 11467–11472.

Bohnsack, M.T., Czaplinski, K., and Gorlich, D. (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA N. Y. N *10*, 185–191.

Bossé, G.D., Rüegger, S., Ow, M.C., Vasquez-Rifo, A., Rondeau, E.L., Ambros, V.R., Grosshans, H., and Simard, M.J. (2013). The decapping scavenger enzyme DCS-1 controls microRNA levels in Caenorhabditis elegans. Mol. Cell *50*, 281–287.

Bostick, M., Kim, J.K., Estève, P.-O., Clark, A., Pradhan, S., and Jacobsen, S.E. (2007). UHRF1 plays a role in maintaining DNA methylation in mammalian cells. Science *317*, 1760–1764.

Boveri, T. (2008). Concerning the Origin of Malignant Tumours by Theodor Boveri. Translated and annotated by Henry Harris. J. Cell Sci. *121*, 1–84.

Boyland, E., and Wolf, G. (1950). Metabolism of polycyclic compounds. 6. Conversion of phenanthrene into dihydroxydihydrophenanthrenes. Biochem. J. *47*, 64–69.

Breitwieser, W., Lyons, S., Flenniken, A.M., Ashton, G., Bruder, G., Willington, M., Lacaud, G., Kouskoff, V., and Jones, N. (2007). Feedback regulation of p38 activity via ATF2 is essential for survival of embryonic liver cells. Genes Dev. *21*, 2069–2082.

Bridge, K.S., Shah, K.M., Li, Y., Foxler, D.E., Wong, S.C.K., Miller, D.C., Davidson, K.M., Foster, J.G., Rose, R., Hodgkinson, M.R., et al. (2017). Argonaute Utilization for miRNA Silencing Is Determined by Phosphorylation-Dependent Recruitment of LIM-Domain-Containing Proteins. Cell Rep. *20*, 173–187.

Brodt, P., Fallavollita, L., Bresalier, R.S., Meterissian, S., Norton, C.R., and Wolitzky, B.A. (1997). Liver endothelial E-selectin mediates carcinoma cell adhesion and promotes liver metastasis. Int. J. Cancer *71*, 612–619.

Bulut, D., Tüns, H., and Mügge, A. (2009). CD31+/Annexin V+ microparticles in healthy offsprings of patients with coronary artery disease. Eur. J. Clin. Invest. *39*, 17–22.

Burke, J.M., Kincaid, R.P., Nottingham, R.M., Lambowitz, A.M., and Sullivan, C.S. (2016). DUSP11 activity on triphosphorylated transcripts promotes Argonaute association with noncanonical viral microRNAs and regulates steady-state levels of cellular noncoding RNAs. Genes Dev. *30*, 2076–2092.

Burroughs, A.M., Ando, Y., de Hoon, M.J.L., Tomaru, Y., Nishibu, T., Ukekawa, R., Funakoshi, T., Kurokawa, T., Suzuki, H., Hayashizaki, Y., et al. (2010). A comprehensive survey of 3' animal miRNA

modification events and a possible role for 3' adenylation in modulating miRNA targeting effectiveness. Genome Res. *20*, 1398–1410.

Byrne, G.J., Ghellal, A., Iddon, J., Blann, A.D., Venizelos, V., Kumar, S., Howell, A., and Bundred, N.J. (2000). Serum soluble vascular cell adhesion molecule-1: role as a surrogate marker of angiogenesis. J. Natl. Cancer Inst. *92*, 1329–1336.

Cai, X., Hagedorn, C.H., and Cullen, B.R. (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA N. Y. N *10*, 1957–1966.

Ceppi, M., Pereira, P.M., Dunand-Sauthier, I., Barras, E., Reith, W., Santos, M.A., and Pierre, P. (2009). MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc. Natl. Acad. Sci. U. S. A. *106*, 2735–2740.

Cerutti, C., Soblechero-Martin, P., Wu, D., Lopez-Ramirez, M.A., Vries, H. de, Sharrack, B., Male, D.K., and Romero, I.A. (2016). MicroRNA-155 contributes to shear-resistant leukocyte adhesion to human brain endothelium in vitro. Fluids Barriers CNS *13*.

Cerutti, C., Edwards, L.J., de Vries, H.E., Sharrack, B., Male, D.K., and Romero, I.A. (2017). MiR-126 and miR-126* regulate shear-resistant firm leukocyte adhesion to human brain endothelium. Sci. Rep. *7*, 45284.

Chamorro-Jorganes, A., Lee, M.Y., Araldi, E., Landskroner-Eiger, S., Fernández-Fuertes, M., Sahraei, M., Quiles Del Rey, M., van Solingen, C., Yu, J., Fernández-Hernando, C., et al. (2016). VEGF-Induced Expression of miR-17-92 Cluster in Endothelial Cells Is Mediated by ERK/ELK1 Activation and Regulates Angiogenesis. Circ. Res. *118*, 38–47.

Chang, H.-M., Triboulet, R., Thornton, J.E., and Gregory, R.I. (2013). A role for the Perlman syndrome exonuclease Dis3l2 in the Lin28-let-7 pathway. Nature *497*, 244–248.

Chatterjee, S., and Grosshans, H. (2009). Active turnover modulates mature microRNA activity in Caenorhabditis elegans. Nature *461*, 546–549.

Chatterjee, S., Fasler, M., Büssing, I., and Grosshans, H. (2011). Target-mediated protection of endogenous microRNAs in C. elegans. Dev. Cell *20*, 388–396.

Chen, Q.W., Zhu, X.Y., Li, Y.Y., and Meng, Z.Q. (2014). Epigenetic regulation and cancer (Review). Oncol. Rep. *31*, 523–532.

Chen, S., Qiong, Y., and Gardner, D.G. (2006). A role for p38 mitogen-activated protein kinase and c-myc in endothelin-dependent rat aortic smooth muscle cell proliferation. Hypertens. Dallas Tex 1979 *47*, 252–258.

Chen, S.-H., Dallas, M.R., Balzer, E.M., and Konstantopoulos, K. (2012). Mucin 16 is a functional selectin ligand on pancreatic cancer cells. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *26*, 1349–1359.

Chen, Y., Banda, M., Speyer, C.L., Smith, J.S., Rabson, A.B., and Gorski, D.H. (2010). Regulation of the expression and activity of the antiangiogenic homeobox gene GAX/MEOX2 by ZEB2 and microRNA-221. Mol. Cell. Biol. *30*, 3902–3913.

Cheng, H.S., Sivachandran, N., Lau, A., Boudreau, E., Zhao, J.L., Baltimore, D., Delgado-Olguin, P., Cybulsky, M.I., and Fish, J.E. (2013). MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. EMBO Mol. Med. *5*, 1017–1034.

Cheng, H.S., Njock, M.-S., Khyzha, N., Dang, L.T., and Fish, J.E. (2014). Noncoding RNAs regulate NF-κB signaling to modulate blood vessel inflammation. Front. Genet. *5*.

Cho, M., Uemura, H., Kim, S.-C., Kawada, Y., Yoshida, K., Hirao, Y., Konishi, N., Saga, S., and Yoshikawa, K. (2001). Hypomethylation of the MN/CA9 promoter and upregulated MN/CA9 expression in human renal cell carcinoma. Br. J. Cancer *85*, 563–567.

Chong, M.M.W., Zhang, G., Cheloufi, S., Neubert, T.A., Hannon, G.J., and Littman, D.R. (2010). Canonical and alternate functions of the microRNA biogenesis machinery. Genes Dev. *24*, 1951–1960.

Chu, M., Qin, S., Wu, R., Zhou, X., Tang, X., Zhang, S., Zhao, Q., Wang, H., Liu, Y., Han, X., et al. (2016). Role of MiR-126a-3p in Endothelial Injury in Endotoxic Mice. Crit. Care Med. *44*, e639–e650.

Codagnone, M., Recchiuti, A., Lanuti, P., Pierdomenico, A.M., Cianci, E., Patruno, S., Mari, V.C., Simiele, F., Di Tomo, P., Pandolfi, A., et al. (2017). Lipoxin A4 stimulates endothelial miR-126-5p expression and its transfer via microvesicles. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *31*, 1856–1866.

Corre, I., Paris, F., and Huot, J. (2017). The p38 pathway, a major pleiotropic cascade that transduces stress and metastatic signals in endothelial cells. Oncotarget *8*, 55684–55714.

Coskun, U., Sancak, B., Sen, I., Bukan, N., Tufan, M.A., Gülbahar, O., and Sozen, S. (2006). Serum P-selectin, soluble vascular cell adhesion molecule-I (s-VCAM-I) and soluble intercellular adhesion molecule-I (s-ICAM-I) levels in bladder carcinoma patients with different stages. Int. Immunopharmacol. *6*, 672–677.

Cowan, C., Muraleedharan, C.K., O'Donnell, J.J., Singh, P.K., Lum, H., Kumar, A., and Xu, S. (2014). MicroRNA-146 inhibits thrombin-induced NF-κB activation and subsequent inflammatory responses in human retinal endothelial cells. Invest. Ophthalmol. Vis. Sci. *55*, 4944–4951.

Csiszar, A., Wang, M., Lakatta, E.G., and Ungvari, Z. (2008). Inflammation and endothelial dysfunction during aging: role of NF-κB. J. Appl. Physiol. *105*, 1333–1341.

Cuenda, A., and Rousseau, S. (2007). p38 MAP-kinases pathway regulation, function and role in human diseases. Biochim. Biophys. Acta *1773*, 1358–1375.

Dahlman, J.E., Barnes, C., Khan, O., Thiriot, A., Jhunjunwala, S., Shaw, T.E., Xing, Y., Sager, H.B., Sahay, G., Speciner, L., et al. (2014). In vivo endothelial siRNA delivery using polymeric nanoparticles with low molecular weight. Nat. Nanotechnol. *9*, 648–655.

Daou, G.B., and Srivastava, A.K. (2004). Reactive oxygen species mediate Endothelin-1-induced activation of ERK1/2, PKB, and Pyk2 signaling, as well as protein synthesis, in vascular smooth muscle cells. Free Radic. Biol. Med. *37*, 208–215.

Das, S.K., Sokhi, U.K., Bhutia, S.K., Azab, B., Su, Z.-Z., Sarkar, D., and Fisher, P.B. (2010). Human polynucleotide phosphorylase selectively and preferentially degrades microRNA-221 in human melanoma cells. Proc. Natl. Acad. Sci. U. S. A. *107*, 11948–11953.

Davis, B.N., Hilyard, A.C., Lagna, G., and Hata, A. (2008). SMAD proteins control DROSHA-mediated microRNA maturation. Nature *454*, 56–61.

Davis, B.N., Hilyard, A.C., Nguyen, P.H., Lagna, G., and Hata, A. (2010). Smad proteins bind a conserved RNA sequence to promote microRNA maturation by Drosha. Mol. Cell *39*, 373–384.

Demolli, S., Doebele, C., Doddaballapur, A., Lang, V., Fisslthaler, B., Chavakis, E., Vinciguerra, M., Sciacca, S., Henschler, R., Hecker, M., et al. (2015). MicroRNA-30 mediates anti-inflammatory effects of shear stress and KLF2 via repression of angiopoietin 2. J. Mol. Cell. Cardiol. *88*, 111–119.

DeNardo, D.G., Andreu, P., and Coussens, L.M. (2010). Interactions between lymphocytes and myeloid cells regulate pro- versus anti-tumor immunity. Cancer Metastasis Rev. *29*, 309–316.

Deng, H., Song, Z., Xu, H., Deng, X., Zhang, Q., Chen, H., Wang, Y., Qin, Y., and Li, Y. (2017a). MicroRNA-1185 Promotes Arterial Stiffness though Modulating VCAM-1 and E-Selectin Expression. Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. *41*, 2183–2193.

Deng, H.-T., Liu, H.-L., Zhai, B.-B., Zhang, K., Xu, G.-C., Peng, X.-M., Zhang, Q.-Z., and Li, L.-Y. (2017b). Vascular endothelial growth factor suppresses TNFSF15 production in endothelial cells by stimulating miR-31 and miR-20a expression via activation of Akt and Erk signals. FEBS Open Bio *7*, 108–117.

Dentelli, P., Rosso, A., Orso, F., Olgasi, C., Taverna, D., and Brizzi, M.F. (2010). microRNA-222 controls neovascularization by regulating signal transducer and activator of transcription 5A expression. Arterioscler. Thromb. Vasc. Biol. *30*, 1562–1568.

Dhillon, A.S., Hagan, S., Rath, O., and Kolch, W. (2007). MAP kinase signalling pathways in cancer. Oncogene *26*, 3279.

Di Carlo, V., Grossi, E., Laneve, P., Morlando, M., Dini Modigliani, S., Ballarino, M., Bozzoni, I., and Caffarelli, E. (2013). TDP-43 regulates the microprocessor complex activity during in vitro neuronal differentiation. Mol. Neurobiol. *48*, 952–963.

Ding, J., Lin, Z.-Q., Jiang, J.-M., Seidman, C.E., Seidman, J.G., Pu, W.T., and Wang, D.-Z. (2016). Preparation of rAAV9 to Overexpress or Knockdown Genes in Mouse Hearts. J. Vis. Exp. JoVE.

Doebele, C., Bonauer, A., Fischer, A., Scholz, A., Reiss, Y., Urbich, C., Hofmann, W.-K., Zeiher, A.M., and Dimmeler, S. (2010). Members of the microRNA-17-92 cluster exhibit a cell-intrinsic antiangiogenic function in endothelial cells. Blood *115*, 4944–4950.

Donners, M.M.P.C., Wolfs, I.M.J., Stöger, L.J., van der Vorst, E.P.C., Pöttgens, C.C.H., Heymans, S., Schroen, B., Gijbels, M.J.J., and de Winther, M.P.J. (2012). Hematopoietic miR155 deficiency enhances atherosclerosis and decreases plaque stability in hyperlipidemic mice. PloS One *7*, e35877.

Drake, M., Furuta, T., Suen, K.M., Gonzalez, G., Liu, B., Kalia, A., Ladbury, J.E., Fire, A.Z., Skeath, J.B., and Arur, S. (2014). A requirement for ERK-dependent Dicer phosphorylation in coordinating oocyte-to-embryo transition in C. elegans. Dev. Cell *31*, 614–628.

Drew, B.A., Burow, M.E., and Beckman, B.S. (2012). MEK5/ERK5 pathway: the first fifteen years. Biochim. Biophys. Acta *1825*, 37–48.

Duan, M., Yao, H., Hu, G., Chen, X., Lund, A.K., and Buch, S. (2013). HIV Tat induces expression of ICAM-1 in HUVECs: implications for miR-221/-222 in HIV-associated cardiomyopathy. PloS One *8*, e60170.

Duan, Q., Mao, X., Xiao, Y., Liu, Z., Wang, Y., Zhou, H., Zhou, Z., Cai, J., Xia, K., Zhu, Q., et al. (2016). Super enhancers at the miR-146a and miR-155 genes contribute to self-regulation of inflammation. Biochim. Biophys. Acta *1859*, 564–571.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. *315*, 1650–1659.

Echavarria, R., Mayaki, D., Neel, J.-C., Harel, S., Sanchez, V., and Hussain, S.N.A. (2015). Angiopoietin-1 inhibits toll-like receptor 4 signalling in cultured endothelial cells: role of miR-146b-5p. Cardiovasc. Res. *106*, 465–477.

Egger, G., Liang, G., Aparicio, A., and Jones, P.A. (2004). Epigenetics in human disease and prospects for epigenetic therapy. Nature *429*, 457–463.

Elkayam, E., Kuhn, C.-D., Tocilj, A., Haase, A.D., Greene, E.M., Hannon, G.J., and Joshua-Tor, L. (2012). The structure of human argonaute-2 in complex with miR-20a. Cell *150*, 100–110.

Ellinger, J., Kahl, P., von der Gathen, J., Rogenhofer, S., Heukamp, L.C., Gütgemann, I., Walter, B., Hofstädter, F., Büttner, R., Müller, S.C., et al. (2010). Global levels of histone modifications predict prostate cancer recurrence. The Prostate *70*, 61–69.

Eritja, N., Chen, B.-J., Rodríguez-Barrueco, R., Santacana, M., Gatius, S., Vidal, A., Martí, M.D., Ponce, J., Bergadà, L., Yeramian, A., et al. (2017). Autophagy orchestrates adaptive responses to targeted therapy in endometrial cancer. Autophagy *13*, 608–624.

Fan, W., Fang, R., Wu, X., Liu, J., Feng, M., Dai, G., Chen, G., and Wu, G. (2015). Shear-sensitive microRNA-34a modulates flow-dependent regulation of endothelial inflammation. J. Cell Sci. *128*, 70–80.

Fan, Y., Ma, X., Li, H., Gao, Y., Huang, Q., Zhang, Y., Bao, X., Du, Q., Luo, G., Liu, K., et al. (2018). miR-122 promotes metastasis of clear-cell renal cell carcinoma by downregulating Dicer. Int. J. Cancer *142*, 547–560.

Fang, Y., and Davies, P.F. (2012). Site-specific microRNA-92a regulation of Kruppel-like factors 4 and 2 in atherosusceptible endothelium. Arterioscler. Thromb. Vasc. Biol. *32*, 979–987.

Fang, Y., Shi, C., Manduchi, E., Civelek, M., and Davies, P.F. (2010). MicroRNA-10a regulation of proinflammatory phenotype in athero-susceptible endothelium in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A. *107*, 13450–13455.

Faryammanesh, R., Lange, T., Magbanua, E., Haas, S., Meyer, C., Wicklein, D., Schumacher, U., and Hahn, U. (2014). SDA, a DNA aptamer inhibiting E- and P-selectin mediated adhesion of cancer and leukemia cells, the first and pivotal step in transendothelial migration during metastasis formation. PloS One *9*, e93173.

Fearon, E.R., and Vogelstein, B. (1990). A genetic model for colorectal tumorigenesis. Cell *61*, 759–767.

Feinberg, A.P., and Vogelstein, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature *301*, 89–92.

Feinberg, A.P., Gehrke, C.W., Kuo, K.C., and Ehrlich, M. (1988). Reduced genomic 5-methylcytosine content in human colonic neoplasia. Cancer Res. *48*, 1159–1161.

Feng, Y., Ma, X., Deng, L., Yao, B., Xiong, Y., Wu, Y., Wang, L., Ma, Q., and Ma, F. (2017). Role of selectins and their ligands in human implantation stage. Glycobiology *27*, 385–391.

Ferroni, P., Roselli, M., Martini, F., D'Alessandro, R., Mariotti, S., Basili, S., Spila, A., Aloe, S., Palmirotta, R., Maggini, A., et al. (2004). Prognostic value of soluble P-selectin levels in colorectal cancer. Int. J. Cancer *111*, 404–408.

Fichtlscherer, S., De Rosa, S., Fox, H., Schwietz, T., Fischer, A., Liebetrau, C., Weber, M., Hamm, C.W., Röxe, T., Müller-Ardogan, M., et al. (2010). Circulating microRNAs in patients with coronary artery disease. Circ. Res. *107*, 677–684.

Fiedler, U., Reiss, Y., Scharpfenecker, M., Grunow, V., Koidl, S., Thurston, G., Gale, N.W., Witzenrath, M., Rosseau, S., Suttorp, N., et al. (2006). Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat. Med. *12*, 235–239.

Finzel, A.H., Reininger, A.J., Bode, P.A., and Wurzinger, L.J. (2004). ICAM-1 supports adhesion of human small-cell lung carcinoma to endothelial cells. Clin. Exp. Metastasis *21*, 185–189.

Forman, J.J., Legesse-Miller, A., and Coller, H.A. (2008). A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc. Natl. Acad. Sci. U. S. A. *105*, 14879–14884.

Fox, S.B., Turner, G.D., Gatter, K.C., and Harris, A.L. (1995). The increased expression of adhesion molecules ICAM-3, E- and P-selectins on breast cancer endothelium. J. Pathol. *177*, 369–376.

Fraga, M.F., Ballestar, E., Villar-Garea, A., Boix-Chornet, M., Espada, J., Schotta, G., Bonaldi, T., Haydon, C., Ropero, S., Petrie, K., et al. (2005). Loss of acetylation at Lys16 and trimethylation at Lys20 of histone H4 is a common hallmark of human cancer. Nat. Genet. *37*, 391–400.

Friederichs, J., Zeller, Y., Hafezi-Moghadam, A., Gröne, H.J., Ley, K., and Altevogt, P. (2000). The CD24/P-selectin binding pathway initiates lung arrest of human A125 adenocarcinoma cells. Cancer Res. *60*, 6714–6722.

Frixa, T., Sacconi, A., Cioce, M., Roscilli, G., Ferrara, F.F., Aurisicchio, L., Pulito, C., Telera, S., Carosi, M.A., Muti, P., et al. (2017). MicroRNA-128-3p-mediated depletion of Drosha promotes lung cancer cell migration. Carcinogenesis.

Gangopadhyay, A., Lazure, D.A., and Thomas, P. (1998). Adhesion of colorectal carcinoma cells to the endothelium is mediated by cytokines from CEA stimulated Kupffer cells. Clin. Exp. Metastasis *16*, 703–712.

Gaudet, F., Hodgson, J.G., Eden, A., Jackson-Grusby, L., Dausman, J., Gray, J.W., Leonhardt, H., and Jaenisch, R. (2003). Induction of tumors in mice by genomic hypomethylation. Science *300*, 489–492.

Gidlöf, O., van der Brug, M., Ohman, J., Gilje, P., Olde, B., Wahlestedt, C., and Erlinge, D. (2013). Platelets activated during myocardial infarction release functional miRNA, which can be taken up by endothelial cells and regulate ICAM1 expression. Blood *121*, 3908–3917, S1-26.

Gidlöf, O., Sathanoori, R., Magistri, M., Faghihi, M.A., Wahlestedt, C., Olde, B., and Erlinge, D. (2015). Extracellular Uridine Triphosphate and Adenosine Triphosphate Attenuate Endothelial Inflammation through miR-22-Mediated ICAM-1 Inhibition. J. Vasc. Res. *52*, 71–80.

Girard, N., Bazille, C., Lhuissier, E., Benateau, H., Llombart-Bosch, A., Boumediene, K., and Bauge, C. (2014). 3-Deazaneplanocin A (DZNep), an inhibitor of the histone methyltransferase EZH2, induces apoptosis and reduces cell migration in chondrosarcoma cells. PloS One *9*, e98176.

Golden, R.J., Chen, B., Li, T., Braun, J., Manjunath, H., Chen, X., Wu, J., Schmid, V., Chang, T.-C., Kopp, F., et al. (2017). An Argonaute phosphorylation cycle promotes microRNA-mediated silencing. Nature *542*, 197–202.

Gorelik, E., Galili, U., and Raz, A. (2001). On the role of cell surface carbohydrates and their binding proteins (lectins) in tumor metastasis. Cancer Metastasis Rev. *20*, 245–277.

Gory, S., Vernet, M., Laurent, M., Dejana, E., Dalmon, J., and Huber, P. (1999). The vascular endothelial-cadherin promoter directs endothelial-specific expression in transgenic mice. Blood *93*, 184–192.

Gout, S., and Huot, J. (2008). Role of Cancer Microenvironment in Metastasis: Focus on Colon Cancer. Cancer Microenviron. *1*, 69–83.

Gout, S., Morin, C., Houle, F., and Huot, J. (2006). Death receptor-3, a new E-Selectin counter-receptor that confers migration and survival advantages to colon carcinoma cells by triggering p38 and ERK MAPK activation. Cancer Res. *66*, 9117–9124.

Gout, S., Tremblay, P.-L., and Huot, J. (2008). Selectins and selectin ligands in extravasation of cancer cells and organ selectivity of metastasis. Clin. Exp. Metastasis *25*, 335–344.

Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. *54*, 4855–4878.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G.L., Hunter, C., Bignell, G., Davies, H., Teague, J., Butler, A., Stevens, C., et al. (2007). Patterns of somatic mutation in human cancer genomes. Nature *446*, 153–158.

Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G., and Mello, C.C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23–34.

Guil, S., and Cáceres, J.F. (2007). The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. Nat. Struct. Mol. Biol. *14*, 591–596.

Ha, M., and Kim, V.N. (2014). Regulation of microRNA biogenesis. Nat. Rev. Mol. Cell Biol. *15*, 509–524.

Haery, L., Lugo-Picó, J.G., Henry, R.A., Andrews, A.J., and Gilmore, T.D. (2014). Histone acetyltransferase-deficient p300 mutants in diffuse large B cell lymphoma have altered transcriptional regulatory activities and are required for optimal cell growth. Mol. Cancer *13*, 29.

Hagan, J.P., Piskounova, E., and Gregory, R.I. (2009). Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. Nat. Struct. Mol. Biol. *16*, 1021–1025.

Hamik, A., Lin, Z., Kumar, A., Balcells, M., Sinha, S., Katz, J., Feinberg, M.W., Gerszten, R.E., Edelman, E.R., and Jain, M.K. (2007). Kruppel-like Factor 4 Regulates Endothelial Inflammation. J. Biol. Chem. *282*, 13769–13779.

Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R., and Hannon, G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146–1150.

Han, J., Pedersen, J.S., Kwon, S.C., Belair, C.D., Kim, Y.-K., Yeom, K.-H., Yang, W.-Y., Haussler, D., Blelloch, R., and Kim, V.N. (2009). Posttranscriptional crossregulation between Drosha and DGCR8. Cell *136*, 75–84.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of Cancer: The Next Generation. Cell *144*, 646–674.

Harris, T.A., Yamakuchi, M., Ferlito, M., Mendell, J.T., and Lowenstein, C.J. (2008). MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. Proc. Natl. Acad. Sci. U. S. A. *105*, 1516–1521.

Harris, T.A., Yamakuchi, M., Kondo, M., Oettgen, P., and Lowenstein, C.J. (2010). Ets-1 and Ets-2 regulate the expression of microRNA-126 in endothelial cells. Arterioscler. Thromb. Vasc. Biol. *30*, 1990–1997.

Hasler, D., Lehmann, G., Murakawa, Y., Klironomos, F., Jakob, L., Grässer, F.A., Rajewsky, N., Landthaler, M., and Meister, G. (2016). The Lupus Autoantigen La Prevents Mis-channeling of tRNA Fragments into the Human MicroRNA Pathway. Mol. Cell *63*, 110–124.

Häuselmann, I., Roblek, M., Protsyuk, D., Huck, V., Knopfova, L., Grässle, S., Bauer, A.T., Schneider, S.W., and Borsig, L. (2016). Monocyte Induction of E-Selectin-Mediated Endothelial Activation Releases VE-Cadherin Junctions to Promote Tumor Cell Extravasation in the Metastasis Cascade. Cancer Res. *76*, 5302–5312.

Hayashi, M., and Lee, J.-D. (2004). Role of the BMK1/ERK5 signaling pathway: lessons from knockout mice. J. Mol. Med. Berl. Ger. *82*, 800–808.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-κB. Genes Dev. *18*, 2195–2224.

He, J., Li, Y., Yang, X., He, X., Zhang, H., He, J., and Zhang, L. (2012). The feedback regulation of PI3K-miR-19a, and MAPK-miR-23b/27b in endothelial cells under shear stress. Mol. Basel Switz. *18*, 1–13.

He, L.Z., Tolentino, T., Grayson, P., Zhong, S., Warrell, R.P., Rifkind, R.A., Marks, P.A., Richon, V.M., and Pandolfi, P.P. (2001). Histone deacetylase inhibitors induce remission in transgenic models of therapy-resistant acute promyelocytic leukemia. J. Clin. Invest. *108*, 1321–1330.

Heo, I., Joo, C., Cho, J., Ha, M., Han, J., and Kim, V.N. (2008). Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol. Cell *32*, 276–284.

Heo, I., Joo, C., Kim, Y.-K., Ha, M., Yoon, M.-J., Cho, J., Yeom, K.-H., Han, J., and Kim, V.N. (2009). TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell *138*, 696–708.

Heo, I., Ha, M., Lim, J., Yoon, M.-J., Park, J.-E., Kwon, S.C., Chang, H., and Kim, V.N. (2012). Mono-uridylation of pre-microRNA as a key step in the biogenesis of group II let-7 microRNAs. Cell *151*, 521–532.

Herbert, K.M., Pimienta, G., DeGregorio, S.J., Alexandrov, A., and Steitz, J.A. (2013). Phosphorylation of DGCR8 increases its intracellular stability and induces a progrowth miRNA profile. Cell Rep. *5*, 1070–1081.

Hergenreider, E., Heydt, S., Tréguer, K., Boettger, T., Horrevoets, A.J.G., Zeiher, A.M., Scheffer, M.P., Frangakis, A.S., Yin, X., Mayr, M., et al. (2012). Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. Nat. Cell Biol. *14*, 249–256.

Hommes, D.W., Peppelenbosch, M.P., and Deventer, S.J.H. van (2003). Mitogen activated protein (MAP) kinase signal transduction pathways and novel anti-inflammatory targets. Gut *52*, 144–151.

Horman, S.R., Janas, M.M., Litterst, C., Wang, B., MacRae, I.J., Sever, M.J., Morrissey, D.V., Graves, P., Luo, B., Umesalma, S., et al. (2013). Akt-mediated phosphorylation of argonaute 2 downregulates cleavage and upregulates translational repression of MicroRNA targets. Mol. Cell *50*, 356–367.

Hou, J., Wang, P., Lin, L., Liu, X., Ma, F., An, H., Wang, Z., and Cao, X. (2009). MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J. Immunol. Baltim. Md 1950 *183*, 2150–2158.

Howard, G., Eiges, R., Gaudet, F., Jaenisch, R., and Eden, A. (2008). Activation and transposition of endogenous retroviral elements in hypomethylation induced tumors in mice. Oncogene *27*, 404–408.

Hu, R., and O'Connell, R.M. (2012). MiR-23b is a safeguard against autoimmunity. Nat. Med. *18*, 1009–1010.

Hu, J., Zeng, L., Huang, J., Wang, G., and Lu, H. (2015). miR-126 promotes angiogenesis and attenuates inflammation after contusion spinal cord injury in rats. Brain Res. *1608*, 191–202.

Hu, J., Adebali, O., Adar, S., and Sancar, A. (2017). Dynamic maps of UV damage formation and repair for the human genome. Proc. Natl. Acad. Sci. U. S. A. *114*, 6758–6763.

Huang, R., Hu, G., Lin, B., Lin, Z., and Sun, C. (2010). MicroRNA-155 silencing enhances inflammatory response and lipid uptake in oxidized low-density lipoprotein-stimulated human THP-1 macrophages. J. Investig. Med. Off. Publ. Am. Fed. Clin. Res. *58*, 961–967.

Hubbard, A.K., and Rothlein, R. (2000). Intercellular adhesion molecule-1 (ICAM-1) expression and cell signaling cascades. Free Radic. Biol. Med. *28*, 1379–1386.

Huntzinger, E., and Izaurralde, E. (2011). Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat. Rev. Genet. *12*, 99.

Huot, J., Houle, F., Marceau, F., and Landry, J. (1997). Oxidative Stress-Induced Actin Reorganization Mediated by the p38 Mitogen-Activated Protein Kinase/Heat Shock Protein 27 Pathway in Vascular Endothelial Cells. Circ. Res. *80*, 383–392.

Hutvágner, G., Simard, M.J., Mello, C.C., and Zamore, P.D. (2004). Sequence-Specific Inhibition of Small RNA Function. PLOS Biol. *2*, e98.

Iademarco, M.F., McQuillan, J.J., and Dean, D.C. (1993). Vascular cell adhesion molecule 1: contrasting transcriptional control mechanisms in muscle and endothelium. Proc. Natl. Acad. Sci. U. S. A. *90*, 3943–3947.

Illingworth, R., Kerr, A., Desousa, D., Jørgensen, H., Ellis, P., Stalker, J., Jackson, D., Clee, C., Plumb, R., Rogers, J., et al. (2008). A novel CpG island set identifies tissue-specific methylation at developmental gene loci. PLoS Biol. *6*, e22.

Issa, J.-P. (2004). CpG island methylator phenotype in cancer. Nat. Rev. Cancer *4*, 988–993.

Iwasaki, Y.W., Kiga, K., Kayo, H., Fukuda-Yuzawa, Y., Weise, J., Inada, T., Tomita, M., Ishihama, Y., and Fukao, T. (2013). Global microRNA elevation by inducible Exportin 5 regulates cell cycle entry. RNA N. Y. N *19*, 490–497.

Iyer, R.R., Pluciennik, A., Burdett, V., and Modrich, P.L. (2006). DNA mismatch repair: functions and mechanisms. Chem. Rev. *106*, 302–323.

Jansen, F., Yang, X., Baumann, K., Przybilla, D., Schmitz, T., Flender, A., Paul, K., Alhusseiny, A., Nickenig, G., and Werner, N. (2015). Endothelial microparticles reduce ICAM-1 expression in a microRNA-222-dependent mechanism. J. Cell. Mol. Med. *19*, 2202–2214.

Jeanmougin, F., Wurtz, J.M., Le Douarin, B., Chambon, P., and Losson, R. (1997). The bromodomain revisited. Trends Biochem. Sci. *22*, 151–153.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074–1080.

Jiang, L., Shao, C., Wu, Q.-J., Chen, G., Zhou, J., Yang, B., Li, H., Gou, L.-T., Zhang, Y., Wang, Y., et al. (2017). NEAT1 scaffolds RNA-binding proteins and the Microprocessor to globally enhance pri-miRNA processing. Nat. Struct. Mol. Biol. *24*, 816–824.

Jo, S.Y., Granowicz, E.M., Maillard, I., Thomas, D., and Hess, J.L. (2011). Requirement for Dot1l in murine postnatal hematopoiesis and leukemogenesis by MLL translocation. Blood *117*, 4759–4768.

Jones, M.R., Quinton, L.J., Blahna, M.T., Neilson, J.R., Fu, S., Ivanov, A.R., Wolf, D.A., and Mizgerd, J.P. (2009). Zcchc11-dependent uridylation of microRNA directs cytokine expression. Nat. Cell Biol. *11*, 1157–1163.

Jubeli, E., Moine, L., Vergnaud-Gauduchon, J., and Barratt, G. (2012). E-selectin as a target for drug delivery and molecular imaging. J. Control. Release Off. J. Control. Release Soc. *158*, 194–206.

Kadener, S., Rodriguez, J., Abruzzi, K.C., Khodor, Y.L., Sugino, K., Marr, M.T., Nelson, S., and Rosbash, M. (2009). Genome-wide identification of targets of the drosha-pasha/DGCR8 complex. RNA N. Y. N *15*, 537–545.

Kane, M.F., Loda, M., Gaida, G.M., Lipman, J., Mishra, R., Goldman, H., Jessup, J.M., and Kolodner, R. (1997). Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res. *57*, 808–811.

Kannagi, R., Izawa, M., Koike, T., Miyazaki, K., and Kimura, N. (2004). Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. Cancer Sci. *95*, 377–384.

Karnoub, A.E., and Weinberg, R.A. (2006). Chemokine networks and breast cancer metastasis. Breast Dis. *26*, 75–85.

Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S.-I., Baba, T., and Suzuki, T. (2009). Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. Genes Dev. *23*, 433–438.

Kawahara, Y., and Mieda-Sato, A. (2012). TDP-43 promotes microRNA biogenesis as a component of the Drosha and Dicer complexes. Proc. Natl. Acad. Sci. U. S. A. *109*, 3347–3352.

Kawahara, Y., Zinshteyn, B., Chendrimada, T.P., Shiekhattar, R., and Nishikura, K. (2007). RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. EMBO Rep. *8*, 763–769.

Kawana, M., Lee, M.E., Quertermous, E.E., and Quertermous, T. (1995). Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. Mol. Cell. Biol. *15*, 4225–4231.

Khatib, A.-M., Auguste, P., Fallavollita, L., Wang, N., Samani, A., Kontogiannea, M., Meterissian, S., and Brodt, P. (2005). Characterization of the host proinflammatory response to tumor cells during the initial stages of liver metastasis. Am. J. Pathol. *167*, 749–759.

Kheirolomoom, A., Kim, C.W., Seo, J.W., Kumar, S., Son, D.J., Gagnon, M.K.J., Ingham, E.S., Ferrara, K.W., and Jo, H. (2015). Multifunctional Nanoparticles Facilitate Molecular Targeting and miRNA Delivery to Inhibit Atherosclerosis in ApoE(-/-) Mice. ACS Nano *9*, 8885–8897.

Kikuchi, T., Toyota, M., Itoh, F., Suzuki, H., Obata, T., Yamamoto, H., Kakiuchi, H., Kusano, M., Issa, J.-P.J., Tokino, T., et al. (2002). Inactivation of p57KIP2 by regional promoter hypermethylation and histone deacetylation in human tumors. Oncogene *21*, 2741–2749.

Kim, E.K., and Choi, E.-J. (2010). Pathological roles of MAPK signaling pathways in human diseases. Biochim. Biophys. Acta *1802*, 396–405.

Kim, K.H., and Roberts, C.W.M. (2016). Targeting EZH2 in cancer. Nat. Med. *22*, 128–134.

Kim, V.N., Han, J., and Siomi, M.C. (2009). Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. *10*, 126–139.

Kim, Y.J., Borsig, L., Varki, N.M., and Varki, A. (1998). P-selectin deficiency attenuates tumor growth and metastasis. Proc. Natl. Acad. Sci. U. S. A. *95*, 9325–9330.

Knudson, A.G. (1996). Hereditary cancer: Two hits revisited. J. Cancer Res. Clin. Oncol. *122*, 135–140.

Konstantopoulos, K., and Thomas, S.N. (2009). Cancer cells in transit: the vascular interactions of tumor cells. Annu. Rev. Biomed. Eng. *11*, 177–202.

Koppers-Lalic, D., Hackenberg, M., Bijnsdorp, I.V., van Eijndhoven, M.A.J., Sadek, P., Sie, D., Zini, N., Middeldorp, J.M., Ylstra, B., de Menezes, R.X., et al. (2014). Nontemplated nucleotide additions distinguish the small RNA composition in cells from exosomes. Cell Rep. *8*, 1649–1658.

Kuo, C.-H., Leon, L., Chung, E.J., Huang, R.-T., Sontag, T.J., Reardon, C.A., Getz, G.S., Tirrell, M., and Fang, Y. (2014). Inhibition of atherosclerosis-promoting microRNAs via targeted polyelectrolyte complex micelles. J. Mater. Chem. B Mater. Biol. Med. *2*, 8142–8153.

Kuo, T.-C., Chen, C.-K., Hua, K.-T., Yu, P., Lee, W.-J., Chen, M.-W., Jeng, Y.-M., Chien, M.-H., Kuo, K.-T., Hsiao, M., et al. (2016). Glutaminase 2 stabilizes Dicer to repress Snail and metastasis in hepatocellular carcinoma cells. Cancer Lett. *383*, 282–294.

Laferriere, J., Houle, F., Taher, M.M., Valerie, K., and Huot, J. (2001). Transendothelial migration of colon carcinoma cells requires expression of E-selectin by endothelial cells and activation of stress-activated protein kinase-2 (SAPK2/p38) in the tumor cells. J. Biol. Chem. *276*, 33762–33772.

Laferriere, J., Houle, F., and Huot, J. (2002). Regulation of the metastatic process by E-selectin and stress-activated protein kinase-2/p38. Ann. N. Y. Acad. Sci. *973*, 562–572.

Laferrière, J., Houle, F., and Huot, J. (2004). Adhesion of HT-29 colon carcinoma cells to endothelial cells requires sequential events involving E-selectin and integrin beta4. Clin. Exp. Metastasis *21*, 257–264.

Lagos, D., Pollara, G., Henderson, S., Gratrix, F., Fabani, M., Milne, R.S.B., Gotch, F., and Boshoff, C. (2010). miR-132 regulates antiviral innate immunity through suppression of the p300 transcriptional co-activator. Nat. Cell Biol. *12*, 513–519.

Lahtz, C., and Pfeifer, G.P. (2011). Epigenetic changes of DNA repair genes in cancer. J. Mol. Cell Biol. *3*, 51–58.

Lai, H.-H., Li, J.-N., Wang, M.-Y., Huang, H.-Y., Croce, C.M., Sun, H.-L., Lyu, Y.-J., Kang, J.-W., Chiu, C.-F., Hung, M.-C., et al. (2017). HIF-1α promotes autophagic proteolysis of Dicer and enhances tumor metastasis. J. Clin. Invest.

Lamalice, L., Houle, F., Jourdan, G., and Huot, J. (2004). Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. Oncogene *23*, 434–445.

Langley, R.R., and Fidler, I.J. (2011). The seed and soil hypothesis revisited - the role of tumor-stroma interactions in metastasis to different organs. Int. J. Cancer J. Int. Cancer *128*, 2527–2535.

Läubli, H., and Borsig, L. (2010). Selectins promote tumor metastasis. Semin. Cancer Biol. *20*, 169–177.

Lee, D.-Y., Lin, T.-E., Lee, C.-I., Zhou, J., Huang, Y.-H., Lee, P.-L., Shih, Y.-T., Chien, S., and Chiu, J.-J. (2017). MicroRNA-10a is crucial for endothelial response to different flow patterns via interaction of retinoid acid receptors and histone deacetylases. Proc. Natl. Acad. Sci. U. S. A. *114*, 2072–2077.

Lee, Y., Jeon, K., Lee, J.-T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. *21*, 4663–4670.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S., et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Leonov, G., Shah, K., Yee, D., Timmis, J., Sharp, T.V., and Lagos, D. (2015). Suppression of AGO2 by miR-132 as a determinant of miRNA-mediated silencing in human primary endothelial cells. Int. J. Biochem. Cell Biol. *69*, 75–84.

Leung, A.K.L., Vyas, S., Rood, J.E., Bhutkar, A., Sharp, P.A., and Chang, P. (2011). Poly(ADP-ribose) regulates stress responses and microRNA activity in the cytoplasm. Mol. Cell *42*, 489–499.

Li, C.C.Y., Eaton, S.A., Young, P.E., Lee, M., Shuttleworth, R., Humphreys, D.T., Grau, G.E., Combes, V., Bebawy, M., Gong, J., et al. (2013). Glioma microvesicles carry selectively packaged coding and non-coding RNAs which alter gene expression in recipient cells. RNA Biol. *10*, 1333–1344.

Li, H., Liu, G., Pan, K., Miao, X., and Xie, Y. (2017a). Methylation-induced downregulation and tumor suppressive role of microRNA-29b in gastric cancer through targeting LASP1. Oncotarget *8*, 95880–95895.

Li, J., Tan, M., Xiang, Q., Zhou, Z., and Yan, H. (2017b). Thrombin-activated platelet-derived exosomes regulate endothelial cell expression of ICAM-1 via microRNA-223 during the thrombosis-inflammation response. Thromb. Res. *154*, 96–105.

Li, P., Wei, J., Li, X., Cheng, Y., Chen, W., Cui, Y., Simoncini, T., Gu, Z., Yang, J., and Fu, X. (2017c). 17β-Estradiol Enhances Vascular Endothelial Ets-1/miR-126-3p Expression: The Possible Mechanism for Attenuation of Atherosclerosis. J. Clin. Endocrinol. Metab. *102*, 594–603.

Li, W., Adebali, O., Yang, Y., Selby, C.P., and Sancar, A. (2018). Single-nucleotide resolution dynamic repair maps of UV damage inSaccharomyces cerevisiaegenome. Proc. Natl. Acad. Sci. U. S. A.

Lin, J., He, S., Sun, X., Franck, G., Deng, Y., Yang, D., Haemmig, S., Wara, A.K.M., Icli, B., Li, D., et al. (2016). MicroRNA-181b inhibits thrombin-mediated endothelial activation and arterial thrombosis by targeting caspase recruitment domain family member 10. FASEB J. fj.201500163R.

Lin, P.-C., Chiu, Y.-L., Banerjee, S., Park, K., Mosquera, J.M., Giannopoulou, E., Alves, P., Tewari, A.K., Gerstein, M.B., Beltran, H., et al. (2013). Epigenetic repression of miR-31 disrupts androgen receptor homeostasis and contributes to prostate cancer progression. Cancer Res. *73*, 1232–1244.

Lin, Z., Kumar, A., SenBanerjee, S., Staniszewski, K., Parmar, K., Vaughan, D.E., Gimbrone, M.A., Balasubramanian, V., García-Cardeña, G., and Jain, M.K. (2005). Kruppel-like factor 2 (KLF2) regulates endothelial thrombotic function. Circ. Res. *96*, e48-57.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709–715.

Lindahl, T., and Wood, R.D. (1999). Quality control by DNA repair. Science *286*, 1897–1905.

Liu, D.Z., Jickling, G.C., Ander, B.P., Hull, H., Zhan, X., Cox, C., Shroff, N., Dykstra-Aiello, C., Stamova, B., and Sharp, F.R. (2016). Elevating microRNA-122 in blood improves outcomes after temporary middle cerebral artery occlusion in rats. J. Cereb. Blood Flow Metab. Off. J. Int. Soc. Cereb. Blood Flow Metab. *36*, 1374–1383.

Liu, R.R., Li, J., Gong, J.Y., Kuang, F., Liu, J.Y., Zhang, Y.S., Ma, Q.L., Song, C.J., Truax, A.D., Gao, F., et al. (2015). MicroRNA-141 regulates the expression level of ICAM-1 on endothelium to decrease myocardial ischemia-reperfusion injury. Am. J. Physiol. Heart Circ. Physiol. *309*, H1303-1313.

Loughlin, F.E., Gebert, L.F.R., Towbin, H., Brunschweiger, A., Hall, J., and Allain, F.H.-T. (2011). Structural basis of pre-let-7 miRNA recognition by the zinc knuckles of pluripotency factor Lin28. Nat. Struct. Mol. Biol. *19*, 84–89.

Loyer, X., Potteaux, S., Vion, A.-C., Guérin, C.L., Boulkroun, S., Rautou, P.-E., Ramkhelawon, B., Esposito, B., Dalloz, M., Paul, J.-L., et al. (2014). Inhibition of microRNA-92a prevents endothelial dysfunction and atherosclerosis in mice. Circ. Res. *114*, 434–443.

Ludwig, R.J., Alban, S., Bistrian, R., Boehncke, W.-H., Kaufmann, R., Henschler, R., and Gille, J. (2006). The ability of different forms of heparins to suppress P-selectin function in vitro correlates to their inhibitory capacity on bloodborne metastasis in vivo. Thromb. Haemost. *95*, 535–540.

Luger, K., Mäder, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature *389*, 251–260.

Lund, E., Güttinger, S., Calado, A., Dahlberg, J.E., and Kutay, U. (2004). Nuclear export of microRNA precursors. Science *303*, 95–98.

Ma, H., Wang, X., Ha, T., Gao, M., Liu, L., Wang, R., Yu, K., Kalbfleisch, J.H., Kao, R.L., Williams, D.L., et al. (2016a). MicroRNA-125b Prevents Cardiac Dysfunction in Polymicrobial Sepsis by Targeting TRAF6-Mediated Nuclear Factor κB Activation and p53-Mediated Apoptotic Signaling. J. Infect. Dis. *214*, 1773–1783.

Ma, S., Tian, X.Y., Zhang, Y., Mu, C., Shen, H., Bismuth, J., Pownall, H.J., Huang, Y., and Wong, W.T. (2016b). E-selectin-targeting delivery of microRNAs by microparticles ameliorates endothelial inflammation and atherosclerosis. Sci. Rep. *6*, 22910.

Mallat, Z., Benamer, H., Hugel, B., Benessiano, J., Steg, P.G., Freyssinet, J.M., and Tedgui, A. (2000). Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. Circulation *101*, 841–843.

Manuyakorn, A., Paulus, R., Farrell, J., Dawson, N.A., Tze, S., Cheung-Lau, G., Hines, O.J., Reber, H., Seligson, D.B., Horvath, S., et al. (2010). Cellular histone modification patterns predict prognosis and treatment response in resectable pancreatic adenocarcinoma: results from RTOG 9704. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. *28*, 1358–1365.

Marquez, J., Fernandez-Piñeiro, I., Araúzo-Bravo, M.J., Poschmann, G., Stühler, K., Khatib, A.-M., Sanchez, A., Unda, F., Ibarretxe, G., Bernales, I., et al. (2018). Targeting liver sinusoidal endothelial cells with miR-20a-loaded nanoparticles reduces murine colon cancer metastasis to the liver. Int. J. Cancer.

Martineau, D., Lemberger, K., Dallaire, A., Labelle, P., Lipscomb, T.P., Michel, P., and Mikaelian, I. (2002). Cancer in wildlife, a case study: beluga from the St. Lawrence estuary, Québec, Canada. Environ. Health Perspect. *110*, 285–292.

Martinez, N.J., and Walhout, A.J.M. (2009). The interplay between transcription factors and microRNAs in genome-scale regulatory networks. BioEssays News Rev. Mol. Cell. Dev. Biol. *31*, 435–445.

Martinez, I., Hayes, K.E., Barr, J.A., Harold, A.D., Xie, M., Bukhari, S.I.A., Vasudevan, S., Steitz, J.A., and DiMaio, D. (2017). An Exportin-1-dependent microRNA biogenesis pathway during human cell quiescence. Proc. Natl. Acad. Sci. U. S. A. *114*, E4961–E4970.

Mayer, B., Spatz, H., Funke, I., Johnson, J.P., and Schildberg, F.W. (1998). De novo expression of the cell adhesion molecule E-selectin on gastric cancer endothelium. Langenbecks Arch. Surg. *383*, 81–86.

Mazumder, A., Bose, M., Chakraborty, A., Chakrabarti, S., and Bhattacharyya, S.N. (2013). A transient reversal of miRNA-mediated repression controls macrophage activation. EMBO Rep. *14*, 1008–1016.

McCarty, O.J., Mousa, S.A., Bray, P.F., and Konstantopoulos, K. (2000). Immobilized platelets support human colon carcinoma cell tethering, rolling, and firm adhesion under dynamic flow conditions. Blood *96*, 1789–1797.

McKenzie, A.J., Hoshino, D., Hong, N.H., Cha, D.J., Franklin, J.L., Coffey, R.J., Patton, J.G., and Weaver, A.M. (2016). KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. Cell Rep. *15*, 978–987.

Melo, S.A., Moutinho, C., Ropero, S., Calin, G.A., Rossi, S., Spizzo, R., Fernandez, A.F., Davalos, V., Villanueva, A., Montoya, G., et al. (2010). A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. Cancer Cell *18*, 303–315.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B., and Sidransky, D. (1995). 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. Nat. Med. *1*, 686–692.

Meziane, O., Piquet, S., Bossé, G.D., Gagné, D., Paquet, E., Robert, C., Tones, M.A., and Simard, M.J. (2015). The human decapping scavenger enzyme DcpS modulates microRNA turnover. Sci. Rep. *5*, 16688.

Michlewski, G., Guil, S., Semple, C.A., and Cáceres, J.F. (2008). Posttranscriptional regulation of miRNAs harboring conserved terminal loops. Mol. Cell *32*, 383–393.

Miki, T.S., Rüegger, S., Gaidatzis, D., Stadler, M.B., and Großhans, H. (2014). Engineering of a conditional allele reveals multiple roles of XRN2 in Caenorhabditis elegans development and substrate specificity in microRNA turnover. Nucleic Acids Res. *42*, 4056–4067.

Minden, A., Lin, A., Smeal, T., Dérijard, B., Cobb, M., Davis, R., and Karin, M. (1994). c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol. Cell. Biol. *14*, 6683–6688.

Mondadori dos Santos, A., Metzinger, L., Haddad, O., M'baya-Moutoula, E., Taïbi, F., Charnaux, N., Massy, Z.A., Hlawaty, H., and Metzinger-Le Meuth, V. (2015). miR-126 Is Involved in Vascular Remodeling under Laminar Shear Stress. BioMed Res. Int. *2015*, 497280.

Morgan, M.J., Kim, Y.-S., and Liu, Z. (2008). TNFα and reactive oxygen species in necrotic cell death. Cell Res. *18*, 343–349.

Mori, M., Triboulet, R., Mohseni, M., Schlegelmilch, K., Shrestha, K., Camargo, F.D., and Gregory, R.I. (2014). Hippo signaling regulates microprocessor and links cell-density-dependent miRNA biogenesis to cancer. Cell *156*, 893–906.

Moss, M.A., Zimmer, S., and Anderson, K.W. (2000). Role of metastatic potential in the adhesion of human breast cancer cells to endothelial monolayers. Anticancer Res. *20*, 1425–1433.

Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes Dev. *16*, 720–728.

Murphy, L.O., and Blenis, J. (2006). MAPK signal specificity: the right place at the right time. Trends Biochem. Sci. *31*, 268–275.

Nam, Y., Chen, C., Gregory, R.I., Chou, J.J., and Sliz, P. (2011). Molecular basis for interaction of let-7 microRNAs with Lin28. Cell *147*, 1080–1091.

Nan, X., Ng, H.H., Johnson, C.A., Laherty, C.D., Turner, B.M., Eisenman, R.N., and Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature *393*, 386–389.

Napier, S.L., Healy, Z.R., Schnaar, R.L., and Konstantopoulos, K. (2007). Selectin ligand expression regulates the initial vascular interactions of colon carcinoma cells: the roles of CD44v and alternative sialofucosylated selectin ligands. J. Biol. Chem. *282*, 3433–3441.

Nazari-Jahantigh, M., Wei, Y., Noels, H., Akhtar, S., Zhou, Z., Koenen, R.R., Heyll, K., Gremse, F., Kiessling, F., Grommes, J., et al. (2012). MicroRNA-155 promotes atherosclerosis by repressing Bcl6 in macrophages. J. Clin. Invest. *122*, 4190–4202.

Ni, C.-W., Qiu, H., and Jo, H. (2011). MicroRNA-663 upregulated by oscillatory shear stress plays a role in inflammatory response of endothelial cells. Am. J. Physiol. Heart Circ. Physiol. *300*, H1762-1769.

Nicoli, S., Standley, C., Walker, P., Hurlstone, A., Fogarty, K.E., and Lawson, N.D. (2010). MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. Nature *464*, 1196–1200.

Nishigaki, M., Aoyagi, K., Danjoh, I., Fukaya, M., Yanagihara, K., Sakamoto, H., Yoshida, T., and Sasaki, H. (2005). Discovery of aberrant expression of R-RAS by cancer-linked DNA hypomethylation in gastric cancer using microarrays. Cancer Res. *65*, 2115–2124.

Nooijen, P.T., Westphal, J.R., Eggermont, A.M., Schalkwijk, C., Max, R., de Waal, R.M., and Ruiter, D.J. (1998). Endothelial P-selectin expression is reduced in advanced primary melanoma and melanoma metastasis. Am. J. Pathol. *152*, 679–682.

Nozaki, T., Sugiyama, S., Koga, H., Sugamura, K., Ohba, K., Matsuzawa, Y., Sumida, H., Matsui, K., Jinnouchi, H., and Ogawa, H. (2009). Significance of a multiple biomarkers strategy including endothelial dysfunction to improve risk stratification for cardiovascular events in patients at high risk for coronary heart disease. J. Am. Coll. Cardiol. *54*, 601–608.

Ohtani-Fujita, N., Fujita, T., Aoike, A., Osifchin, N.E., Robbins, P.D., and Sakai, T. (1993). CpG methylation inactivates the promoter activity of the human retinoblastoma tumor-suppressor gene. Oncogene *8*, 1063–1067.

Oliveira, P.A., Colaço, A., Chaves, R., Guedes-Pinto, H., De-La-Cruz P, L.F., and Lopes, C. (2007). Chemical carcinogenesis. An. Acad. Bras. Cienc. *79*, 593–616.

Ott, C.A., Linck, L., Kremmer, E., Meister, G., and Bosserhoff, A.K. (2016). Induction of exportin-5 expression during melanoma development supports the cellular behavior of human malignant melanoma cells. Oncotarget *7*, 62292–62304.

Ozsolak, F., Poling, L.L., Wang, Z., Liu, H., Liu, X.S., Roeder, R.G., Zhang, X., Song, J.S., and Fisher, D.E. (2008). Chromatin structure analyses identify miRNA promoters. Genes Dev. *22*, 3172–3183.

Pagès, F., Galon, J., Dieu-Nosjean, M.-C., Tartour, E., Sautès-Fridman, C., and Fridman, W.-H. (2010). Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene *29*, 1093–1102.

Palmieri, D., Capponi, S., Geroldi, A., Mura, M., Mandich, P., and Palombo, D. (2014). TNFα induces the expression of genes associated with endothelial dysfunction through p38MAPK-mediated down-regulation of miR-149. Biochem. Biophys. Res. Commun. *443*, 246–251.

Pan, Q., Liao, X., Liu, H., Wang, Y., Chen, Y., Zhao, B., Lazartigues, E., Yang, Y., and Ma, X. (2017). MicroRNA-125a-5p alleviates the deleterious effects of ox-LDL on multiple functions of human brain microvessel endothelial cells. Am. J. Physiol. Cell Physiol. *312*, C119–C130.

Park, J.-E., Heo, I., Tian, Y., Simanshu, D.K., Chang, H., Jee, D., Patel, D.J., and Kim, V.N. (2011). Dicer recognizes the 5’ end of RNA for efficient and accurate processing. Nature *475*, 201–205.

Partin, A.C., Ngo, T.D., Herrell, E., Jeong, B.-C., Hon, G., and Nam, Y. (2017). Heme enables proper positioning of Drosha and DGCR8 on primary microRNAs. Nat. Commun. *8*, 1737.

Pasquinelli, A.E., Reinhart, B.J., Slack, F., Martindale, M.Q., Kuroda, M.I., Maller, B., Hayward, D.C., Ball, E.E., Degnan, B., Müller, P., et al. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86–89.

Pearson, G., Robinson, F., Gibson, T.B., Xu, B., Karandikar, M., Berman, K., and Cobb, M.H. (2011). Mitogen-Activated Protein (MAP) Kinase Pathways: Regulation and Physiological Functions1. Endocr. Rev.

Peeters, C.F.J.M., Ruers, T.J.M., Westphal, J.R., and de Waal, R.M.W. (2005). Progressive loss of endothelial P-selectin expression with increasing malignancy in colorectal cancer. Lab. Investig. J. Tech. Methods Pathol. *85*, 248–256.

Pfeifer, G.P. (2006). Mutagenesis at Methylated CpG Sequences. In DNA Methylation: Basic Mechanisms, (Springer, Berlin, Heidelberg), pp. 259–281.

Pigati, L., Yaddanapudi, S.C.S., Iyengar, R., Kim, D.-J., Hearn, S.A., Danforth, D., Hastings, M.L., and Duelli, D.M. (2010). Selective release of microRNA species from normal and malignant mammary epithelial cells. PloS One *5*, e13515.

Pin, A.-L., Houle, F., Guillonneau, M., Paquet, E.R., Simard, M.J., and Huot, J. (2012). miR-20a represses endothelial cell migration by targeting MKK3 and inhibiting p38 MAP kinase activation in response to VEGF. Angiogenesis *15*, 593–608.

Poliseno, L., Tuccoli, A., Mariani, L., Evangelista, M., Citti, L., Woods, K., Mercatanti, A., Hammond, S., and Rainaldi, G. (2006). MicroRNAs modulate the angiogenic properties of HUVECs. Blood *108*, 3068–3071.

Pourhoseini, S., Seth, R.K., Das, S., Dattaroy, D., Kadiiska, M.B., Xie, G., Michelotti, G.A., Nagarkatti, M., Diehl, A.M., and Chatterjee, S. (2015). Upregulation of miR21 and repression of Grhl3 by leptin mediates sinusoidal endothelial injury in experimental nonalcoholic steatohepatitis. PloS One *10*, e0116780.

Prasad, R., Giri, S., Nath, N., Singh, I., and Singh, A.K. (2005). Inhibition of phosphoinositide 3 kinase-Akt (protein kinase B)-nuclear factor-kappa B pathway by lovastatin limits endothelial-monocyte cell interaction. J. Neurochem. *94*, 204–214.

Qi, H.H., Ongusaha, P.P., Myllyharju, J., Cheng, D., Pakkanen, O., Shi, Y., Lee, S.W., Peng, J., and Shi, Y. (2008). Prolyl 4-hydroxylation regulates Argonaute 2 stability. Nature *455*, 421–424.

Qian, B.-Z., and Pollard, J.W. (2010). Macrophage diversity enhances tumor progression and metastasis. Cell *141*, 39–51.

Quévillon Huberdeau, M., Zeitler, D.M., Hauptmann, J., Bruckmann, A., Fressigné, L., Danner, J., Piquet, S., Strieder, N., Engelmann, J.C., Jannot, G., et al. (2017). Phosphorylation of Argonaute proteins affects mRNA binding and is essential for microRNA-guided gene silencing in vivo. EMBO J. *36*, 2088–2106.

Rasheed, S.A.K., Teo, C.R., Beillard, E.J., Voorhoeve, P.M., Zhou, W., Ghosh, S., and Casey, P.J. (2015). MicroRNA-31 controls G protein alpha-13 (GNA13) expression and cell invasion in breast cancer cells. Mol. Cancer *14*, 67.

Rau, C.-S., Yang, J.C.-S., Chen, Y.-C., Wu, C.-J., Lu, T.-H., Tzeng, S.-L., Wu, Y.-C., and Hsieh, C.-H. (2014). Lipopolysaccharide-induced microRNA-146a targets CARD10 and regulates angiogenesis in human umbilical vein endothelial cells. Toxicol. Sci. Off. J. Soc. Toxicol. *140*, 315–326.

Read, M.A., Whitley, M.Z., Gupta, S., Pierce, J.W., Best, J., Davis, R.J., and Collins, T. (1997). Tumor necrosis factor alpha-induced E-selectin expression is activated by the nuclear factor-kappaB and c-JUN N-terminal kinase/p38 mitogen-activated protein kinase pathways. J. Biol. Chem. *272*, 2753–2761.

Regidor, P.A., Callies, R., Regidor, M., and Schindler, A.E. (1998). Expression of the cell adhesion molecules ICAM-1 and VCAM-1 in the cytosol of breast cancer tissue, benign breast tissue and corresponding sera. Eur. J. Gynaecol. Oncol. *19*, 377–383.

Reymond, N., d'Água, B.B., and Ridley, A.J. (2013). Crossing the endothelial barrier during metastasis. Nat. Rev. Cancer *13*, 858–870.

Rijnkels, M., Kabotyanski, E., Montazer-Torbati, M.B., Hue Beauvais, C., Vassetzky, Y., Rosen, J.M., and Devinoy, E. (2010). The epigenetic landscape of mammary gland development and functional differentiation. J. Mammary Gland Biol. Neoplasia *15*, 85–100.

Rissland, O.S., Mikulasova, A., and Norbury, C.J. (2007). Efficient RNA polyuridylation by noncanonical poly(A) polymerases. Mol. Cell. Biol. *27*, 3612–3624.

Rohde, J.-H., Weigand, J.E., Suess, B., and Dimmeler, S. (2015). A Universal Aptamer Chimera for the Delivery of Functional microRNA-126. Nucleic Acid Ther. *25*, 141–151.

Rose, B.A., Force, T., and Wang, Y. (2010). Mitogen-activated protein kinase signaling in the heart: angels versus demons in a heart-breaking tale. Physiol. Rev. *90*, 1507–1546.

Roselli, M., Mineo, T.C., Martini, F., Mariotti, S., Ambrogi, V., Spila, A., D'Alessandro, R., Basili, S., Guadagni, F., and Ferroni, P. (2002). Soluble selectin levels in patients with lung cancer. Int. J. Biol. Markers *17*, 56–62.

Rouleau, S.G., Garant, J.-M., Bolduc, F., Bisaillon, M., and Perreault, J.-P. (2017). G-Quadruplexes influence pri-microRNA processing. RNA Biol. 1–9.

Rousseau, S., Houle, F., Kotanides, H., Witte, L., Waltenberger, J., Landry, J., and Huot, J. (2000). Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. J. Biol. Chem. *275*, 10661–10672.

Rüdel, S., Wang, Y., Lenobel, R., Körner, R., Hsiao, H.-H., Urlaub, H., Patel, D., and Meister, G. (2011). Phosphorylation of human Argonaute proteins affects small RNA binding. Nucleic Acids Res. *39*, 2330–2343.

Ruoming, W., Zhen, Y., Tengteng, Z., and Jisheng, H. (2015). Tumor suppressor microRNA-31 inhibits gastric carcinogenesis by targeting Smad4 and SGPP2. Cancer Gene Ther. *22*, 564–572.

Rybak, A., Fuchs, H., Hadian, K., Smirnova, L., Wulczyn, E.A., Michel, G., Nitsch, R., Krappmann, D., and Wulczyn, F.G. (2009). The let-7 target gene mouse lin-41 is a stem cell specific E3 ubiquitin ligase for the miRNA pathway protein Ago2. Nat. Cell Biol. *11*, 1411–1420.

Sahoo, M.R., Gaikwad, S., Khuperkar, D., Ashok, M., Helen, M., Yadav, S.K., Singh, A., Magre, I., Deshmukh, P., Dhanvijay, S., et al. (2017). Nup358 binds to AGO proteins through its SUMO-interacting motifs and promotes the association of target mRNA with miRISC. EMBO Rep. *18*, 241–263.

Sato, N., Maitra, A., Fukushima, N., van Heek, N.T., Matsubayashi, H., Iacobuzio-Donahue, C.A., Rosty, C., and Goggins, M. (2003). Frequent hypomethylation of multiple genes overexpressed in pancreatic ductal adenocarcinoma. Cancer Res. *63*, 4158–4166.

Sawada, R., Tsuboi, S., and Fukuda, M. (1994). Differential E-selectin-dependent adhesion efficiency in sublines of a human colon cancer exhibiting distinct metastatic potentials. J. Biol. Chem. *269*, 1425–1431.

Schadendorf, D., Heidel, J., Gawlik, C., Suter, L., and Czarnetzki, B.M. (1995). Association with clinical outcome of expression of VLA-4 in primary cutaneous malignant melanoma as well as P-selectin and E-selectin on intratumoral vessels. J. Natl. Cancer Inst. *87*, 366–371.

Schirle, N.T., and MacRae, I.J. (2012). The crystal structure of human Argonaute2. Science *336*, 1037–1040.

Schmiedel, J.M., Klemm, S.L., Zheng, Y., Sahay, A., Blüthgen, N., Marks, D.S., and van Oudenaarden, A. (2015). Gene expression. MicroRNA control of protein expression noise. Science *348*, 128–132.

SenBanerjee, S., Lin, Z., Atkins, G.B., Greif, D.M., Rao, R.M., Kumar, A., Feinberg, M.W., Chen, Z., Simon, D.I., Luscinskas, F.W., et al. (2004). KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. J. Exp. Med. *199*, 1305–1315.

Seo, G.J., Kincaid, R.P., Phanaksri, T., Burke, J.M., Pare, J.M., Cox, J.E., Hsiang, T.-Y., Krug, R.M., and Sullivan, C.S. (2013). Reciprocal inhibition between intracellular antiviral signaling and the RNAi machinery in mammalian cells. Cell Host Microbe *14*, 435–445.

Sharma, S., Kelly, T.K., and Jones, P.A. (2010). Epigenetics in cancer. Carcinogenesis *31*, 27–36.

Shen, J., Xia, W., Khotskaya, Y.B., Huo, L., Nakanishi, K., Lim, S.-O., Du, Y., Wang, Y., Chang, W.-C., Chen, C.-H., et al. (2013). EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature *497*, 383–387.

Slattery, M.J., and Dong, C. (2003). Neutrophils influence melanoma adhesion and migration under flow conditions. Int. J. Cancer *106*, 713–722.

Song, J., Song, J., Mo, B., and Chen, X. (2015). Uridylation and adenylation of RNAs. Sci. China Life Sci. *58*, 1057–1066.

Sossey-Alaoui, K., Downs-Kelly, E., Das, M., Izem, L., Tubbs, R., and Plow, E.F. (2011). WAVE3, an actin remodeling protein, is regulated by the metastasis suppressor microRNA, miR-31, during the invasion-metastasis cascade. Int. J. Cancer *129*, 1331–1343.

Sporn, M.B. (1996). The war on cancer. Lancet Lond. Engl. *347*, 1377–1381.

St Hill, C.A. (2011). Interactions between endothelial selectins and cancer cells regulate metastasis. Front. Biosci. Landmark Ed. *16*, 3233–3251.

Stroka, K.M., and Konstantopoulos, K. (2014). Physical Biology in Cancer. 4. Physical cues guide tumor cell adhesion and migration. Am. J. Physiol. - Cell Physiol. *306*, C98–C109.

Suárez, Y., Fernández-Hernando, C., Pober, J.S., and Sessa, W.C. (2007). Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. Circ. Res. *100*, 1164–1173.

Suárez, Y., Wang, C., Manes, T.D., and Pober, J.S. (2010). Cutting edge: TNF-induced microRNAs regulate TNF-induced expression of E-selectin and intercellular adhesion molecule-1 on human endothelial cells: feedback control of inflammation. J. Immunol. Baltim. Md 1950 *184*, 21–25.

Subramaniam, M., Koedam, J.A., and Wagner, D.D. (1993). Divergent fates of P- and E-selectins after their expression on the plasma membrane. Mol. Biol. Cell *4*, 791–801.

Suh, M.-R., Lee, Y., Kim, J.Y., Kim, S.-K., Moon, S.-H., Lee, J.Y., Cha, K.-Y., Chung, H.M., Yoon, H.S., Moon, S.Y., et al. (2004). Human embryonic stem cells express a unique set of microRNAs. Dev. Biol. *270*, 488–498.

Sun, H.-L., Cui, R., Zhou, J., Teng, K.-Y., Hsiao, Y.-H., Nakanishi, K., Fassan, M., Luo, Z., Shi, G., Tili, E., et al. (2016). ERK Activation Globally Downregulates miRNAs through Phosphorylating Exportin-5. Cancer Cell *30*, 723–736.

Sun, J.J., Zhou, X.D., Liu, Y.K., Tang, Z.Y., Feng, J.X., Zhou, G., Xue, Q., and Chen, J. (1999). Invasion and metastasis of liver cancer: expression of intercellular adhesion molecule 1. J. Cancer Res. Clin. Oncol. *125*, 28–34.

Sun, X., Icli, B., Wara, A.K., Belkin, N., He, S., Kobzik, L., Hunninghake, G.M., Vera, M.P., MICU Registry, Blackwell, T.S., et al. (2012). MicroRNA-181b regulates NF-κB-mediated vascular inflammation. J. Clin. Invest. *122*, 1973–1990.

Sun, X., He, S., Wara, A.K.M., Icli, B., Shvartz, E., Tesmenitsky, Y., Belkin, N., Li, D., Blackwell, T.S., Sukhova, G.K., et al. (2014). Systemic delivery of microRNA-181b inhibits nuclear factor-κB activation, vascular inflammation, and atherosclerosis in apolipoprotein E-deficient mice. Circ. Res. *114*, 32–40.

Sun, Y., Liu, W.-Z., Liu, T., Feng, X., Yang, N., and Zhou, H.-F. (2015). Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. J. Recept. Signal Transduct. Res. *35*, 600–604.

Suzuki, H.I., Yamagata, K., Sugimoto, K., Iwamoto, T., Kato, S., and Miyazono, K. (2009). Modulation of microRNA processing by p53. Nature *460*, 529–533.

Suzuki, H.I., Arase, M., Matsuyama, H., Choi, Y.L., Ueno, T., Mano, H., Sugimoto, K., and Miyazono, K. (2011). MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. Mol. Cell *44*, 424–436.

Suzuki, H.I., Young, R.A., and Sharp, P.A. (2017). Super-Enhancer-Mediated RNA Processing Revealed by Integrative MicroRNA Network Analysis. Cell *168*, 1000-1014.e15.

Syrigos, K.N., Salgami, E., Karayiannakis, A.J., Katirtzoglou, N., Sekara, E., and Roussou, P. (2004). Prognostic significance of soluble adhesion molecules in Hodgkin's disease. Anticancer Res. *24*, 1243–1247.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., and Mello, C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. Cell *99*, 123–132.

Tabet, F., Vickers, K.C., Cuesta Torres, L.F., Wiese, C.B., Shoucri, B.M., Lambert, G., Catherinet, C., Prado-Lourenco, L., Levin, M.G., Thacker, S., et al. (2014). HDL-transferred microRNA-223 regulates ICAM-1 expression in endothelial cells. Nat. Commun. *5*, 3292.

Taganov, K.D., Boldin, M.P., Chang, K.-J., and Baltimore, D. (2006). NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc. Natl. Acad. Sci. U. S. A. *103*, 12481–12486.

Taguchi, O., Gabazza, E.C., Kobayashi, T., Yoshida, M., Yasui, H., and Kobayashi, H. (1997). Circulating intercellular adhesion molecule-1 in patients with lung cancer. Intern. Med. Tokyo Jpn. *36*, 14–18.

Takahashi, T., Yamaguchi, S., Chida, K., and Shibuya, M. (2001). A single autophosphorylation site on KDR/Flk-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. *20*, 2768–2778.

Tang, X., Zhang, Y., Tucker, L., and Ramratnam, B. (2010). Phosphorylation of the RNase III enzyme Drosha at Serine300 or Serine302 is required for its nuclear localization. Nucleic Acids Res. *38*, 6610–6619.

Tang, X., Li, M., Tucker, L., and Ramratnam, B. (2011). Glycogen synthase kinase 3 beta (GSK3β) phosphorylates the RNAase III enzyme Drosha at S300 and S302. PloS One *6*, e20391.

Tang, X., Wen, S., Zheng, D., Tucker, L., Cao, L., Pantazatos, D., Moss, S.F., and Ramratnam, B. (2013). Acetylation of drosha on the N-terminus inhibits its degradation by ubiquitination. PloS One *8*, e72503.

Tanigawa, N., Hagiwara, M., Tada, H., Komatsu, T., Sugiura, S., Kobayashi, K., Kato, Y., Ishida, N., Nishida, K., Ninomiya, M., et al. (2013). Acacetin inhibits expression of E-selectin on endothelial cells through regulation of the MAP kinase signaling pathway and activation of NF-κB. Immunopharmacol. Immunotoxicol. *35*, 471–477.

Tanos, T., Marinissen, M.J., Leskow, F.C., Hochbaum, D., Martinetto, H., Gutkind, J.S., and Coso, O.A. (2005). Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. J. Biol. Chem. *280*, 18842–18852.

Thomas, S.N., Zhu, F., Schnaar, R.L., Alves, C.S., and Konstantopoulos, K. (2008). Carcinoembryonic antigen and CD44 variant isoforms cooperate to mediate colon carcinoma cell adhesion to E- and L-selectin in shear flow. J. Biol. Chem. *283*, 15647–15655.

Thomas, S.N., Schnaar, R.L., and Konstantopoulos, K. (2009). Podocalyxin-like protein is an E-/L-selectin ligand on colon carcinoma cells: comparative biochemical properties of selectin ligands in host and tumor cells. Am. J. Physiol. Cell Physiol. *296*, C505-513.

Thornton, J.E., Du, P., Jing, L., Sjekloca, L., Lin, S., Grossi, E., Sliz, P., Zon, L.I., and Gregory, R.I. (2014). Selective microRNA uridylation by Zcchc6 (TUT7) and Zcchc11 (TUT4). Nucleic Acids Res. *42*, 11777–11791.

To, S.K.Y., Mak, A.S.C., Eva Fung, Y.M., Che, C.-M., Li, S.-S., Deng, W., Ru, B., Zhang, J., and Wong, A.S.T. (2017). β-catenin downregulates Dicer to promote ovarian cancer metastasis. Oncogene *36*, 5927–5938.

Tokumaru, S., Suzuki, M., Yamada, H., Nagino, M., and Takahashi, T. (2008). let-7 regulates Dicer expression and constitutes a negative feedback loop. Carcinogenesis *29*, 2073–2077.

Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A.D., Filipowicz, W., Ramos, A., Gherzi, R., and Rosenfeld, M.G. (2009). The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature *459*, 1010–1014.

Tremblay, P.-L., Auger, F.A., and Huot, J. (2006). Regulation of transendothelial migration of colon cancer cells by E-selectin-mediated activation of p38 and ERK MAP kinases. Oncogene *25*, 6563–6573.

Tremblay, P.-L., Huot, J., and Auger, F.A. (2008). Mechanisms by which E-selectin regulates diapedesis of colon cancer cells under flow conditions. Cancer Res. *68*, 5167–5176.

Upton, J.-P., Wang, L., Han, D., Wang, E.S., Huskey, N.E., Lim, L., Truitt, M., McManus, M.T., Ruggero, D., Goga, A., et al. (2012). IRE1α cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. Science *338*, 818–822.

Ushijima, T. (2005). Detection and interpretation of altered methylation patterns in cancer cells. Nat. Rev. Cancer *5*, 223–231.

Ustianenko, D., Hrossova, D., Potesil, D., Chalupnikova, K., Hrazdilova, K., Pachernik, J., Cetkovska, K., Uldrijan, S., Zdrahal, Z., and Vanacova, S. (2013). Mammalian DIS3L2 exoribonuclease targets the uridylated precursors of let-7 miRNAs. RNA N. Y. N *19*, 1632–1638.

Vestweber, D., and Blanks, J.E. (1999). Mechanisms that regulate the function of the selectins and their ligands. Physiol. Rev. *79*, 181–213.

Viswanathan, S.R., Daley, G.Q., and Gregory, R.I. (2008). Selective blockade of microRNA processing by Lin28. Science *320*, 97–100.

Vogelstein, B., Fearon, E.R., Hamilton, S.R., Kern, S.E., Preisinger, A.C., Leppert, M., Nakamura, Y., White, R., Smits, A.M., and Bos, J.L. (1988). Genetic alterations during colorectal-tumor development. N. Engl. J. Med. *319*, 525–532.

Wada, T., Kikuchi, J., and Furukawa, Y. (2012). Histone deacetylase 1 enhances microRNA processing via deacetylation of DGCR8. EMBO Rep. *13*, 142–149.

Waelchli, R., Bollbuck, B., Bruns, C., Buhl, T., Eder, J., Feifel, R., Hersperger, R., Janser, P., Revesz, L., Zerwes, H.-G., et al. (2006). Design and preparation of 2-benzamido-pyrimidines as inhibitors of IKK. Bioorg. Med. Chem. Lett. *16*, 108–112.

Walzog, B., and Gaehtgens, P. (2000). Adhesion Molecules: The Path to a New Understanding of Acute Inflammation. News Physiol. Sci. Int. J. Physiol. Prod. Jointly Int. Union Physiol. Sci. Am. Physiol. Soc. *15*, 107–113.

Wan, G., Zhang, X., Langley, R.R., Liu, Y., Hu, X., Han, C., Peng, G., Ellis, L.M., Jones, S.N., and Lu, X. (2013). DNA-damage-induced nuclear export of precursor microRNAs is regulated by the ATM-AKT pathway. Cell Rep. *3*, 2100–2112.

Wang, D., Sun, X., Wei, Y., Liang, H., Yuan, M., Jin, F., Chen, X., Liu, Y., Zhang, C.-Y., Li, L., et al. (2017). Nuclear miR-122 directly regulates the biogenesis of cell survival oncomiR miR-21 at the posttranscriptional level. Nucleic Acids Res.

Wang, L., Zhang, H., Rodriguez, S., Cao, L., Parish, J., Mumaw, C., Zollman, A., Kamoka, M.M., Mu, J., Chen, D.Z., et al. (2014). Notch-dependent repression of miR-155 in the bone marrow niche regulates hematopoiesis in an NF-κB-dependent manner. Cell Stem Cell *15*, 51–65.

Weber, M., Hellmann, I., Stadler, M.B., Ramos, L., Pääbo, S., Rebhan, M., and Schübeler, D. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat. Genet. *39*, 457–466.

Wei, C., Salichos, L., Wittgrove, C.M., Rokas, A., and Patton, J.G. (2012). Transcriptome-wide analysis of small RNA expression in early zebrafish development. RNA N. Y. N *18*, 915–929.

Whyte, W.A., Orlando, D.A., Hnisz, D., Abraham, B.J., Lin, C.Y., Kagey, M.H., Rahl, P.B., Lee, T.I., and Young, R.A. (2013). Master Transcription Factors and Mediator Establish Super-Enhancers at Key Cell Identity Genes. Cell *153*, 307–319.

Wienholds, E., Kloosterman, W.P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H.R., Kauppinen, S., and Plasterk, R.H.A. (2005). MicroRNA expression in zebrafish embryonic development. Science *309*, 310–311.

Witkowski, M., Weithauser, A., Tabaraie, T., Steffens, D., Kränkel, N., Witkowski, M., Stratmann, B., Tschoepe, D., Landmesser, U., and Rauch-Kroehnert, U. (2016). Micro-RNA-126 Reduces the Blood Thrombogenicity in Diabetes Mellitus via Targeting of Tissue Factor. Arterioscler. Thromb. Vasc. Biol. *36*, 1263–1271.

Wogan, G.N., Hecht, S.S., Felton, J.S., Conney, A.H., and Loeb, L.A. (2004). Environmental and chemical carcinogenesis. Semin. Cancer Biol. *14*, 473–486.

Wu, C., So, J., Davis-Dusenbery, B.N., Qi, H.H., Bloch, D.B., Shi, Y., Lagna, G., and Hata, A. (2011a). Hypoxia potentiates microRNA-mediated gene silencing through posttranslational modification of Argonaute2. Mol. Cell. Biol. *31*, 4760–4774.

Wu, M., Gu, J.-T., Yi, B., Tang, Z.-Z., and Tao, G.-C. (2015). microRNA-23b regulates the expression of inflammatory factors in vascular endothelial cells during sepsis. Exp. Ther. Med. *9*, 1125–1132.

Wu, W., Xiao, H., Laguna-Fernandez, A., Villarreal, G., Wang, K.-C., Geary, G.G., Zhang, Y., Wang, W.-C., Huang, H.-D., Zhou, J., et al. (2011b). Flow-Dependent Regulation of Kruppel-Like Factor 2 Is Mediated by MicroRNA-92a. Circulation *124*, 633–641.

Wu, X.-Y., Fan, W.-D., Fang, R., and Wu, G.-F. (2014). Regulation of microRNA-155 in endothelial inflammation by targeting nuclear factor (NF)-κB P65. J. Cell. Biochem. *115*, 1928–1936.

Xhemalce, B., Robson, S.C., and Kouzarides, T. (2012). Human RNA methyltransferase BCDIN3D regulates microRNA processing. Cell *151*, 278–288.

Yamashita, S., Hosoya, K., Gyobu, K., Takeshima, H., and Ushijima, T. (2009). Development of a novel output value for quantitative assessment in methylated DNA immunoprecipitation-CpG island microarray analysis. DNA Res. Int. J. Rapid Publ. Rep. Genes Genomes *16*, 275–286.

Yang, Q., Li, W., She, H., Dou, J., Duong, D.M., Du, Y., Yang, S.-H., Seyfried, N.T., Fu, H., Gao, G., et al. (2015). Stress Induces p38 MAPK-mediated Phosphorylation and Inhibition of Drosha-dependent Cell Survival. Mol. Cell *57*, 721.

Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg, P.H., Shiekhattar, R., and Nishikura, K. (2006). Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat. Struct. Mol. Biol. *13*, 13–21.

Yeom, K.-H., Lee, Y., Han, J., Suh, M.R., and Kim, V.N. (2006). Characterization of DGCR8/Pasha, the essential cofactor for Drosha in primary miRNA processing. Nucleic Acids Res. *34*, 4622–4629.

Yi, R., Qin, Y., Macara, I.G., and Cullen, B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. *17*, 3011–3016.

Yin, Y., Li, F., Shi, J., Li, S., Cai, J., and Jiang, Y. (2016). MiR-146a Regulates Inflammatory Infiltration by Macrophages in Polymyositis/Dermatomyositis by Targeting TRAF6 and Affecting IL-17/ICAM-1 Pathway. Cell. Physiol. Biochem. Int. J. Exp. Cell. Physiol. Biochem. Pharmacol. *40*, 486–498.

Yoshizaki, K., Wakita, H., Takeda, K., and Takahashi, K. (2008). Conditional expression of microRNA against E-selectin inhibits leukocyte-endothelial adhesive interaction under inflammatory condition. Biochem. Biophys. Res. Commun. *371*, 747–751.

Yuan, H., Deng, R., Zhao, X., Chen, R., Hou, G., Zhang, H., Wang, Y., Xu, M., Jiang, B., and Yu, J. (2017). SUMO1 modification of KHSRP regulates tumorigenesis by preventing the TL-G-Rich miRNA biogenesis. Mol. Cancer *16*, 157.

Zangari, J., Ilie, M., Rouaud, F., Signetti, L., Ohanna, M., Didier, R., Roméo, B., Goldoni, D., Nottet, N., Staedel, C., et al. (2017). Rapid decay of engulfed extracellular miRNA by XRN1 exonuclease promotes transient epithelial-mesenchymal transition. Nucleic Acids Res. *45*, 4131–4141.

Zarubin, T., and Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. Cell Res. *15*, 11–18.

Zeng, Y., Sankala, H., Zhang, X., and Graves, P.R. (2008). Phosphorylation of Argonaute 2 at serine-387 facilitates its localization to processing bodies. Biochem. J. *413*, 429–436.

Zernecke, A., Bidzhekov, K., Noels, H., Shagdarsuren, E., Gan, L., Denecke, B., Hristov, M., Köppel, T., Jahantigh, M.N., Lutgens, E., et al. (2009). Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. Sci. Signal. *2*, ra81.

Zhang, H., Lu, Y., Chen, E., Li, X., Lv, B., Vikis, H.G., and Liu, P. (2017). XRN2 promotes EMT and metastasis through regulating maturation of miR-10a. Oncogene *36*, 3925–3933.

Zhang, K., Cai, H.-X., Gao, S., Yang, G.-L., Deng, H.-T., Xu, G.-C., Han, J., Zhang, Q.-Z., and Li, L.-Y. (2016a). TNFSF15 suppresses VEGF production in endothelial cells by stimulating miR-29b expression via activation of JNK-GATA3 signals. Oncotarget *7*, 69436–69449.

Zhang, L., Huang, D., Wang, Q., Shen, D., Wang, Y., Chen, B., Zhang, J., and Gai, L. (2014a). MiR-132 inhibits expression of SIRT1 and induces pro-inflammatory processes of vascular endothelial inflammation through blockade of the SREBP-1c metabolic pathway. Cardiovasc. Drugs Ther. *28*, 303–311.

Zhang, Q., Padi, S.K.R., Tindall, D.J., and Guo, B. (2014b). Polycomb protein EZH2 suppresses apoptosis by silencing the proapoptotic miR-31. Cell Death Dis. *5*, e1486.

Zhang, X.-B., Song, L., Wen, H.-J., Bai, X.-X., Li, Z.-J., and Ma, L.-J. (2016b). Upregulation of microRNA-31 targeting integrin α5 suppresses tumor cell invasion and metastasis by indirectly regulating PI3K/AKT pathway in human gastric cancer SGC7901 cells. Tumour Biol. J. Int. Soc. Oncodevelopmental Biol. Med. *37*, 8317–8325.

Zhang, Y., Yang, P., Sun, T., Li, D., Xu, X., Rui, Y., Li, C., Chong, M., Ibrahim, T., Mercatali, L., et al. (2013). miR-126 and miR-126* repress recruitment of mesenchymal stem cells and inflammatory monocytes to inhibit breast cancer metastasis. Nat. Cell Biol. *15*, 284–294.

Zheng, Q., Yang, H.-J., and Yuan, Y.A. (2017). Autoantigen La Regulates MicroRNA Processing from Stem-Loop Precursors by Association with DGCR8. Biochemistry (Mosc.) *56*, 6098–6110.

Zhong, L., Simoneau, B., Tremblay, P.-L., Gout, S., Simard, M.J., and Huot, J. (2014). E-Selectin-Mediated Adhesion and Extravasation in Cancer. 1–7.

Zhong, L., Simoneau, B., Huot, J., and Simard, M.J. (2016). p38 and JNK pathways control E-selectin-dependent extravasation of colon cancer cells by modulating miR-31 transcription. Oncotarget *8*, 1678–1687.

Zhong, L., Simard, M.J., and Huot, J. (2018). Endothelial microRNAs regulating the NF-κB pathway and cell adhesion molecules during inflammation. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. fj201701536R.

Zhou, G., Hamik, A., Nayak, L., Tian, H., Shi, H., Lu, Y., Sharma, N., Liao, X., Hale, A., Boerboom, L., et al. (2012). Endothelial Kruppel-like factor 4 protects against atherothrombosis in mice. J. Clin. Invest. *122*, 4727–4731.

Zhou, J., Wang, K.-C., Wu, W., Subramaniam, S., Shyy, J.Y.-J., Chiu, J.-J., Li, J.Y.-S., and Chien, S. (2011). MicroRNA-21 targets peroxisome proliferators-activated receptor-alpha in an autoregulatory loop to modulate flow-induced endothelial inflammation. Proc. Natl. Acad. Sci. U. S. A. *108*, 10355–10360.

Zhu, C., Chen, C., Huang, J., Zhang, H., Zhao, X., Deng, R., Dou, J., Jin, H., Chen, R., Xu, M., et al. (2015). SUMOylation at K707 of DGCR8 controls direct function of primary microRNA. Nucleic Acids Res. *43*, 7945–7960.

Zhu, C., Chen, C., Chen, R., Deng, R., Zhao, X., Zhang, H., Duo, J., Chen, Q., Jin, H., Wang, Y., et al. (2016). K259-SUMOylation of DGCR8 promoted by p14ARF exerts a tumor-suppressive function. J. Mol. Cell Biol.

Zhu, H.-Q., li, Q., Dong, L.-Y., Zhou, Q., Wang, H., and Wang, Y. (2014). MicroRNA-29b promotes high-fat diet-stimulated endothelial permeability and apoptosis in apoE knock-out mice by down-regulating MT1 expression. Int. J. Cardiol. *176*, 764–770.

Zhu, N., Zhang, D., Chen, S., Liu, X., Lin, L., Huang, X., Guo, Z., Liu, J., Wang, Y., Yuan, W., et al. (2011a). Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. Atherosclerosis *215*, 286–293.

Zhu, N., Zhang, D., Xie, H., Zhou, Z., Chen, H., Hu, T., Bai, Y., Shen, Y., Yuan, W., Jing, Q., et al. (2011b). Endothelial-specific intron-derived miR-126 is down-regulated in human breast cancer and targets both VEGFA and PIK3R2. Mol. Cell. Biochem. *351*, 157–164.