学　位　論　文

Studies on the roles of EMT-associated microRNAs in cervical cancer and aggressive endometrial cancer

（子宮頸癌及び高悪性度子宮体癌の上皮間葉転換における microRNA の役割とその作用メカニズムの解明に関する研究）

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List of Publications and Presentations

A part of this study was published in the following papers:

1. Dong, P., Xiong, Y., Yue, J., Xu, D., Ihira, K., Konno, Y., Kobayashi, N., Todo, Y., and Watari, H. Long noncoding RNA NEAT1 drives aggressive endometrial cancer progression via miR-361-regulated networks involving STAT3 and tumor microenvironment-related genes. Journal of Experimental & Clinical Cancer Research, 38, 295. 2019
2. Xu, D., Dong, P., Xiong, Y., Yue, J., Ihira, K., Konno, Y., Kobayashi, N., Todo, Y., and Watari, H. MicroRNA-361: A Multifaceted Player Regulating Tumor Aggressiveness and Tumor Microenvironment Formation. Cancers (Basel), 11, 1130. 2019
3. Xu, D., Dong, P., Xiong, Y., Yue, J., Konno, Y., Ihira, K., Kobayashi, N., Todo, Y., and Watari, H. MicroRNA-361-Mediated Inhibition of HSP90 Expression and EMT in Cervical Cancer Is Counteracted by Oncogenic lncRNA NEAT1. Cells, 9, 632. 2020
4. Dong, P., Xu, D., Xiong, Y., Yue, J., Ihira, K., Konno, Y., and Watari, H. The Expression, Functions and Mechanisms of Circular RNAs in Gynecological Cancers. Cancers (Basel), 12, 1472. 2020
5. Xu, D., Dong, P., Xiong, Y., Chen, R., Konno, Y., Ihira, K., Yue, J., and Watari, H. PD-L1 Is a Tumor Suppressor in Aggressive Endometrial Cancer Cells and Its Expression Is Regulated by miR-216a and lncRNA MEG3. Frontiers in Cell and Developmental Biology, 8, 598205. 2020
6. Dong, P., Xiong, Y., Konno, Y., Ihira, K., Xu, D., Kobayashi, N., Yue, J., and Watari, H. Critical Roles of PIWIL1 in Human Tumors: Expression, Functions, Mechanisms, and Potential Clinical Implications. Frontiers in

Cell and Developmental Biology, 9, 656993. 2021

A part of this study was presented in the following conferences:

1. Xu, D., Dong, P., Ihira, K., Konno, Y., and Watari, H. MicroRNA-361 directly targets HSP90 to repress EMT and invasion in cervical cancer cells. 72nd Annual Congress of the Japan Society of Obstetrics and Gynecology, April 23-28, 2020, Tokyo, Japan.
2. Dong, P., Xu, D., Ihira, K., Konno, Y., and Watari, H. Long non-coding RNA NEAT1 drives aggressive endometrial cancer progression via sponging microRNA-361. 72nd Annual Congress of the Japan Society of Obstetrics and Gynecology, April 23-28, 2020, Tokyo, Japan.
3. Xu, D., Dong, P., Ihira, K., Konno, Y., and Watari, H. PD-L1 is a tumor suppressor in aggressive endometrial cancer cells and its expression is regulated by miR-216a. 73rd Annual Congress of the Japan Society of Obstetrics and Gynecology, April 22-25, 2021, Niigata, Japan.

Summary

CHAPTER 1: Study on the role of NEAT1/miR-361/HSP90 signaling influencing epithelial-mesenchymal transition in cervical cancer

[Background and Purpose]

Cervical cancer (CC) is one of the most common gynecological malignancies. Cancer metastasis begins with the process of epithelial-mesenchymal transition (EMT), which converts well-polarized epithelial cells to non-polarized mesenchymal cells that acquire motility and invasion properties and exhibit cancer stem cell-like properties. The 90-kDa heat shock protein (HSP90) promotes EMT and tumor progression by protecting and stabilizing its client proteins. To date, the exact function of HSP90 in CC development is still unclear. MicroRNAs (miRNAs) function as post-transcriptional regulators of mRNAs by inhibiting the translation of their respective RNA targets or degrading their targets. The reduction in miR-361 expression in diverse tumor types and its tumor-suppressing function has been described. We previously reported that miR-361 represses the expression of target genes implicated in EMT and cancer stemness in endometrial cancer (EC). Long non-coding RNAs (lncRNAs) can act as molecular sponges of miRNAs, thereby affecting the expression of target genes of miRNAs. We demonstrated that lncRNA NEAT1 drives EC progression by sponging miR-361. Our meta-analysis indicated that the 3′-untranslated region (3′-UTR) of *HSP90* mRNA contains a sequence complementary to the seed regions of miR-361. We aimed to explore the role of miR-361 in cervical cancer and the mechanisms underlying its function in EMT. We speculated that miR-361 directly targets HSP90 to inhibit the invasion and EMT features, and NEAT1 functions as an oncogenic lncRNA that suppresses miR-361 expression and induces EMT and sphere formation in CC cells.

[Materials and Methods]

We used an online database to examine the expression of miR-361 in human cervical cancer and normal cervical tissues. The levels of miR-361 in one immortalized but non-malignant human endometrial cell line (EM) and in two human CC cell lines were measured by real-time PCR analysis. The association between miR-361 expression and the overall survival outcomes in CC patients from the Cancer Genome Atlas cohort was analyzed using online databases. Cell functional assays were used to explore the effects of miR-361 overexpression/knockdown on the proliferation, invasion, EMT and sphere formation of CC cells. The possible target genes of miR-361 were predicted using miRNA-target prediction programs. Luciferase reporter assays were performed to verify the interaction between miR-361, HSP90, and lncRNA NEAT1.

[Results]

[1] The expression of miR-361 was downregulated in CC tissues. The levels of miR-361 in CC cell lines (HeLa and SiHa) were significantly lower than those in EM cells. Lower expression of miR-361 was significantly associated with a poorer prognosis in cervical cancer.

[2] Overexpression of miR-361 induced an epithelial phenotype and significantly decreased cell invasion and sphere formation. However, the knockdown of miR-361 promoted a mesenchymal phenotype and greatly increased cancer cell invasion and sphere formation. Upregulation of miR-361 increased the expression of E-cadherin and inhibited the expression of Vimentin in CC cells. Knockdown of miR-361 had opposite effects on these genes, suggesting that miR-361 inhibits EMT and sphere formation in CC cells.

[3] *HSP90* was predicted as a potential target of miR-361. HSP90 expression was significantly higher in CC tissues and CC cells. CC patients with higher *HSP90* expression had worse overall survival than those with lower *HSP90* expression. Ectopic expression of miR-361 decreased, while inhibition of miR-361 increased the expression of HSP90 in CC cells. The luciferase reporter assays demonstrated overexpression of miR-361 significantly decreases luciferase activity of the wild-type (WT) *HSP90*

3′-UTR, and the transfection with miR-361 inhibitor markedly induced the luciferase activity of the WT *HSP90* 3′-UTR in CC cells. However, transfection with miR-361 mimic or miR-361 inhibitor had no obvious influence on the luciferase activity of the reporter vector harboring the mutant *HSP90* 3′-UTR, suggesting that miR-361 directly targets and reduces HSP90 expression in CC cells.

[4] Knockdown of HSP90 reduced mesenchymal phenotype of SiHa cells, and overexpression of HSP90 in HeLa cells led to mesenchymal-like morphology and triggered cell scattering. Downregulation of HSP90 significantly inhibited the invasion and cancer stem cell properties of SiHa cells and overexpression of HSP90 promoted the invasion and cancer stem cell properties of HeLa cells. Silencing of HSP90 enhanced the expression of E-cadherin and reduced the protein expression of Vimentin. However, overexpression of HSP90 decreased E-cadherin expression and increased Vimentin expression, suggesting that HSP90 promotes EMT and sphere formation in CC cells.

[5] Our meta-analysis revealed a significantly higher level of NEAT1 in CC samples compared with normal samples. The expression of NEAT1 was significantly upregulated in CC cells compared with EM cells. The depletion of NEAT1 by siRNA significantly upregulated miR-361 levels in CC cells. The luciferase reporter assays verified the direct binding relationship between NEAT1 and miR-361 in CC cells. Silencing of NEAT1 significantly suppressed the expression of HSP90 and Vimentin but increased the levels of E-cadherin in CC cells. NEAT1 knockdown led to decreased cell invasion and sphere formation. Taken together, our results support the notion that NEAT1 promotes CC invasion and sphere formation through up-regulating HSP90 expression by binding with miR-361, a tumor suppressor that directly suppresses HSP90 expression.

[Discussion]

MiR-361 represses tumor progression by targeting multiple components of many essential

signaling pathways implicated in tumor growth, EMT, metastasis, drug resistance, angiogenesis and inflammation. HSP90 is a crucial molecular chaperone that forms a complex with co-chaperones to promote tumor progression by protecting and stabilizing numerous client proteins. Consistent with these previous reports, our findings supported the complexity of miR-361-regulated signaling pathways that determine the phenotypes of human tumor cells and demonstrated that the loss of miR-361 expression elevates HSP90 levels, leading to the acquisition of EMT and cancer stem cell-like phenotypes of CC cells. Additionally, HSP90 could be secreted by cancer cells, and extracellular HSP90 promotes EMT and cancer cell invasion and stimulates metastatic spread. Therefore, the impacts of miR-361 and NEAT1 expression on the secretion of HSP90 by CC cells should be studied using the enzyme-linked immunosorbent assay. Whether the secreted HSP90 acts as a pivotal regulator of CC progression and metastasis requires further investigation. Moreover, the overexpression of NEAT1 exerts its oncogenic functions in most human cancers by functioning as a molecular sponge for miRNAs. In this study, we demonstrated for the first time that, by competitively binding to miR-361 and suppressing its expression, NEAT1 upregulates the expression of HSP90 to promote EMT, invasion, and sphere formation of CC cells. Future investigation will be required to determine the mechanisms by which NEAT1 performs this function in cervical cancer.

[Conclusion]

MiR-361 directly targets HSP90 to inhibit the invasion and EMT features, and NEAT1 functions as an oncogenic lncRNA that suppresses miR-361 expression and induces EMT and sphere formation in CC cells. Our findings provided useful insights into the mechanisms of CC EMT and metastasis.

CHAPTER 2: Study on the role of MEG3/miR-216a/PD-L1 pathway implicated in epithelial-mesenchymal transition of aggressive endometrial cancer

[Background and Purpose]

In developed countries, endometrial cancer is the most frequent gynecologic malignancy. Unlike the majority of endometrial cancers, which are often linked with well-differentiated endometrioid histology, early-stage illness, and a better prognosis, poorly-differentiated endometrioid endometrial cancers and serous endometrial cancers are more aggressive, with a high risk of recurrence and metastasis. EC metastasis is closely associated with the EMT, in which epithelial cells lose their polarity and intracellular adherence while gaining migratory and invasive potential. The immune checkpoint ligand programmed death-ligand-1 (PD-L1) is expressed on the surface of tumor cells, and the binding of PD-L1 to its receptor programmed death-1 (PD-1) on activated T cells suppresses anti-tumor immunity. Despite this well-known function of PD-L1 in cancer immune evasion, new research showed that PD-L1 has a tumor cell-intrinsic role in triggering EMT and cancer metastasis. The EMT process is modulated by complex epigenetic regulatory mechanisms, such as miRNAs and lncRNAs. However, the functional role of tumor cell-intrinsic PD-L1 expression in aggressive EC cells and the epigenetic mechanisms that regulate PD-L1 expression remain unknown. In this study, we aimed to reveal the biological functions of PD-L1 and the miRNA-associated mechanisms that regulate PD-L1 expression in aggressive EC cells. We speculated that PD-L1 has a tumor cell-intrinsic role in aggressive EC cells and that novel miRNA and lncRNA work as key regulators to control the expression of PD-L1.

[Materials and Methods]

Cell functional experiments were used to explore the impact of PD-L1 expression on the proliferation, EMT, and invasion of aggressive EC cells. The luciferase reporter experiments were performed to investigate the miR-216a-dependent mechanisms influencing PD-L1

expression. The influence of lncRNA MEG3 on miR-216a and PD-L1 levels was investigated using RT-qPCR and western blotting assays.

[Results]

[1] Our meta-analysis suggested that the mRNA expression of *PD-L1* in primary EC tissues was lower than that in the normal samples. Also, we found that human EC tissues have lower PD-L1 protein expression compared with the adjacent normal tissues. Furthermore, we confirmed that higher expression of PD-L1 was associated with increased overall survival in EC patients.

[2] Our western blotting analysis demonstrated that PD-L1 protein was expressed at lower levels in aggressive EC cell lines compared to a normal endometrial cell line (EM).

[3] Cell functional screening showed that overexpression of PD-L1 in multiple aggressive EC cell lines significantly attenuated cell proliferation, migration, and invasion, while inducing cell apoptosis.

[4] Knocking down PD-L1 in aggressive EC cells enhanced the expression of MCL-1, Vimentin and Snail, and reduced the levels of ZO-1. However, overexpression of PD-L1 in aggressive EC cells led to the inhibition of EMT, featured with upregulation of ZO-1, and downregulation of MCL-1, Vimentin and Snail.

[5] The silencing of MCL-1 expression with MCL-1-specific siRNA largely reversed PD-L1 knockdown-induced mesenchymal cellular morphology and significantly inhibited the migratory and invasive ability that was enhanced by the knockdown of PD-L1.

[6] Using RT-qPCR assays, we validated that miR-216a was significantly upregulated in aggressive EC cells as compared to EM cells. Compared to the respective controls, the expression of PD-L1 decreased markedly when miR-216a was overexpressed in aggressive EC cells.

[7] The luciferase reporter assays confirmed that the overexpression of miR-216a significantly reduced the luciferase activities of WT *PD-L1* 3′-UTR, and the inhibition

of miR-216a increased the luciferase activities of WT *PD-L1* 3′-UTR in aggressive EC cells. The transfection with miR-216a mimic or miR-216a inhibitor into EC cells showed no significant effects on the luciferase activities of mutant *PD-L1* 3′-UTR.

[8] Furthermore, we found that overexpression of miR-216a significantly induced the migration and invasion of EC cells, whereas cell migration and invasion were significantly reduced in EC cells following knockdown of miR-216a.

[9] The mRNA expression of *MCL-1* and *Vimentin* was upregulated, whereas the levels of *ZO-1* were decreased when miR-216a was overexpressed in EC cells. In contrast, miR-216a-silenced EC cells showed decreased *MCL-1* and *Vimentin* and increased *ZO-1* expression.

[10] Using meta-analysis, the lncRNA MEG3 was identified as a possible regulator of miR-216a. MEG3 exhibited significantly lower expression in EC tissues as compared to normal tissues. High expression of MEG3 was associated with a favorable prognosis of EC patients.

[11] Using RT-qPCR analysis, we discovered that the levels of MEG3 were significantly downregulated in EC cells compared with EM cells.

[12] Knocking down MEG3 expression promoted, but the ectopic expression of MEG3 inhibited miR-216a expression in aggressive EC cells. The role of MEG3 in suppressing EC cell migration and invasion was confirmed by wound-healing and invasion assays. Consistent with these results, we found that knockdown of MEG3 inhibited the protein expression of PD-L1, while overexpression of MEG3 increased the expression of PD-L1 in EC cells.

[Discussion]

The incidence of aggressive endometrial cancer has been rapidly increasing in the US and Japan. The highly metastatic and often treatment-refractory nature of aggressive endometrial

cancer correlates with poorer patient survival. Therefore, a better understanding of the mechanisms behind the tumorigenesis and metastasis of aggressive endometrial cancer is urgently needed to improve the effective treatment of this cancer. Although it is known that EMT induction and immune evasion have been demonstrated to promote EC development, little is known about the functional significance of PD-L1 in aggressive EC cells. The expression pattern of PD-L1 and its predictive significance in endometrial cancer appear to be debatable in the literature. Here, we found that compared with normal endometrium samples as well as normal cells, the expression of PD-L1 was frequently lost in EC tissues and aggressive EC cells. PD-L1 loss can identify EC patients with a worse probability of survival. In previous studies, PD-L1 was shown to exhibit anti-tumor or pro-tumor effects via various mechanisms. Our findings showed that PD-L1 has tumor-suppressive effects in aggressive EC cells, at least via negative regulation of EMT. Thus, the loss of PD-L1 expression may represent the critical mechanism underlying the induction and maintenance of aggressive properties of EC cells. We validated that miR-216a directly targets and decreases PD-L1 levels, thereby promoting EMT and invasion of aggressive EC cells. Moreover, lncRNA MEG3 could increase PD-L1 expression via suppressing the levels of miR-216a. These results suggested that MEG3 and miR-216a are critical upstream regulators of PD-L1. Thus, our study provided a previously unreported mechanism responsible for PD-L1 dysregulation in aggressive EC cells.

[Conclusion]

In this study, we demonstrate a new tumor-suppressor role of PD-L1 in repressing the proliferation and EMT-associated migration and invasion in aggressive EC cells. Our results reveal that the downregulation of MEG3 and the induction of its downstream effector miR-216a is likely a novel mechanism underlying the downregulation of PD-L1 observed in EC tissues.

List of Abbreviations

3′-UTR	3′-untranslated region
ARF4	ADP ribosylation factor 4
BSG	basigin (Ok blood group)
CIN	cervical intraepithelial neoplasia
CC	cervical cancer
EC	endometrial cancer
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GO	Gene Ontology
HBMSC	human bone mesenchymal stem cell
HER2	erb-b2 receptor tyrosine kinase 2
HSP90	90-kDa heat shock protein
KEGG	Kyoto Encyclopedia of Genes and Genomes
LncRNA	long non-coding RNA
MCL-1	MCL1 apoptosis regulator, BCL2 family member
MDR-1	ATP binding cassette subfamily B member 1
MEG3	maternally expressed 3
MET	mesenchymal-epithelial transition
miRNA	microRNA
MUT	mutant
NEAT1	nuclear paraspeckle assembly transcript 1

OCT-4	POU class 5 homeobox 1
PD-1	programmed death-1
PD-L1	programmed death-ligand-1
pri-miRNA	primary miRNA
pre-miRNA	precursor miRNA
PTEN	phosphatase and tensin homolog
RT-qPCR	quantitative reverse transcription PCR
shRNA	short hairpin RNA
siRNA	small interfering RNA
SOX-2	SRY-box transcription factor 2
TCGA	The Cancer Genome Atlas
Twist1	Twist family BHLH transcription factor 1
VEGF-A	vascular endothelial growth factor A
WT	wild-type
ZO-1	zona occludens-1, tight junction protein 1

Introduction

Gynecological cancers, which mostly include ovarian cancer, cervical cancer (CC), and endometrial cancer (EC), have a significant impact on women's health globally and contribute significantly to global cancer incidence and mortality. Cervical cancer, the fourth most prevalent malignancy in women worldwide, is a major public health issue, particularly for women in underdeveloped nations. The cure rate of cervical cancer in early-stage disease (the International Federation of Gynecology and Obstetrics (FIGO) stage I–II) can approach 80% with early screening and effective therapies such as radical surgery or concurrent chemotherapy and radiation therapy. However, patients with distant metastatic illness, as well as those with unresectable recurrent disease and those who recur at a distance, have limited therapeutic options and a low survival rate. The sixth most prevalent disease in women is endometrial cancer, commonly known as uterine cancer. Elevated estrogen levels and advancing age are two well-known risk factors for endometrial cancer. Surgery with or without adjuvant radiotherapy and/or chemotherapy is the conventional treatment, which is determined by the risk of disease recurrence. The majority of EC patients with early-stage illnesses can be treated and have a good prognosis with surgery. However, advanced and aggressive endometrial cancer has a terrible prognosis.

Epigenetics is described as changes in gene expression that do not involve a change in DNA sequence. Recently, microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) are developing as promising regulators of cervical cancer and endometrial cancer. MiRNAs play an increasingly important role in the post-transcriptional regulation of oncogenes and tumor suppressor genes, influencing tumor cell biological characteristics such as invasion, metastasis, proliferation, and apoptosis. LncRNAs can work as sponges to bind miRNAs, thereby

regulating the expression of genes.

Epithelial-mesenchymal transition (EMT) has been associated with increased cell motility, invasion, and metastasis, as well as increased cancer aggressiveness. It is known that lncRNAs and miRNAs can interact, and these interactions are important in cancer metastasis because they regulate essential events such as EMT.

In our research, we are aiming to establish a network consisting of lncRNA–miRNA–mRNA in the EMT process of cervical cancer and aggressive endometrial cancer, which could lead to more insights into the molecular mechanisms of these two cancer types and might provide potential prognostic biomarkers and therapeutic targets.

CHAPTER 1

Study on the role of NEAT1/miR-361/HSP90 signaling influencing epithelial-mesenchymal transition in cervical cancer

CHAPTER 1: Introduction

Cervical cancer is a malignant disease that develops from the cells in the lining of the cervix. In 2020, over 604127 women were diagnosed with cervical cancer around the world. Over 341831 women died of this disease (Sung et al., 2021). In Japan, approximately 10908 women will be diagnosed with cervical cancer each year, and cervical cancer results in around 3000 deaths in Japan (Yamagami et al., 2017). The incidence of cervical cancer has increased in Japanese women (especially among the younger generation (Yamagami et al., 2017)). When diagnosed at an early stage, the five-year survival rate of cervical cancer will exceed 90% (Rotman et al., 2006). However, the 5-year survival rate of advanced cervical cancer is still poor, ranging from 17% to 30% (Li et al., 2016; Wright et al., 2015). Understanding the biological mechanisms of metastatic CC cells would contribute to the development of effective therapeutic approaches that improve the survival rates of patients with cervical cancer.

To date, the hallmarks of human cancer (including sustained proliferative signaling, evasion of growth suppressors, evasion of cell death, limitless replicative potential, induction of angiogenesis, cancer cell invasion and metastasis, increased tendency of genome instability and mutations, deregulated cellular energetics, and tumor inflammatory microenvironment) have been reported (Hanahan and Weinberg, 2011) (Figure 1). Human cancer cells acquire these malignant capabilities during the process of cancer progression (Hanahan and Weinberg, 2011).

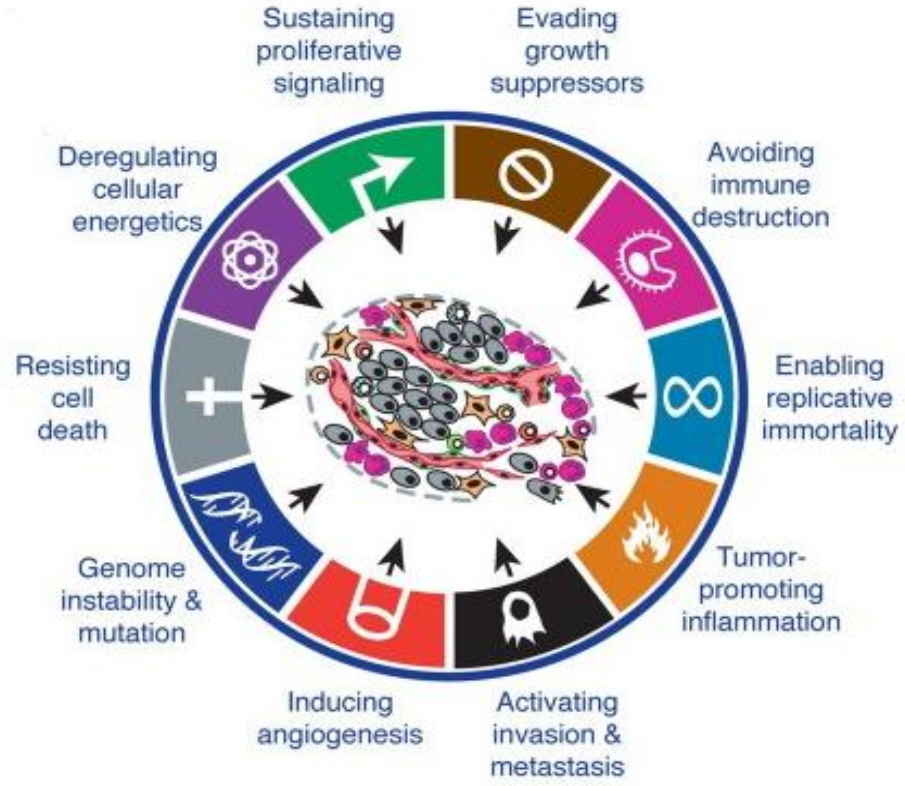


Figure 1: Human cancer hallmarks (Hanahan D, et al. Cell. 2011).

Cancer's hallmarks were initially six biological capacities gained throughout the multistep evolution of human malignancies: maintaining proliferative signals, avoiding growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. This concept has been revised, with the addition of new hallmarks, such as cancer cells' capacity to change energy metabolism, evasion of immune response, as well as two enabling factors: genomic instability and tumor-promoting inflammation.

Cancer metastasis refers to a complex and multi-step event encompassing (I) local infiltration of tumor cells into the adjacent normal tissue, (II) invasion into blood vessels, (III) survival in the bloodstream, (IV) attaching to endothelial cells that facilitate their extravasation, and (V) subsequent proliferation in distant sites, leading to the establishment of cancer metastasis (Fares et al., 2020) (Figure 2). Metastasis is the major cause of cancer-related deaths.

Exploring the detailed mechanisms of cancer metastasis would help identify valuable targets for molecular therapies and possibly improve the survival of patients with cancer.

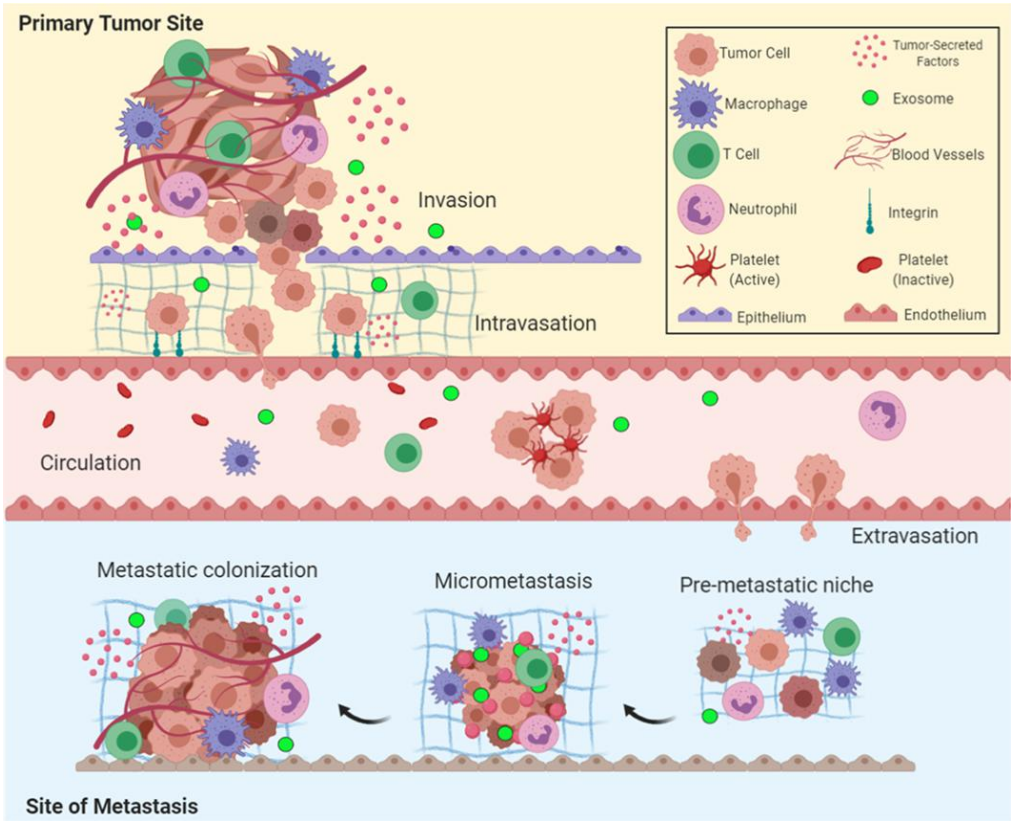


Figure 2: Metastatic cascade (Fares J, et al. Signal Transduct Target Ther. 2020).

Cancer is characterized by invasion and metastasis. The metastatic cascade is a multistep process. To colonize a metastatic site, cancer cells must detach from the primary tumor using extracellular matrix-degrading proteases, invade into the lymphatic and/or blood vessels, survive in the circulatory system, evade the immune response, extravasate out of the lymphatic and/or blood vessels, and reach the target tissue, where metastatic foci are established.

A key component of cancer cell invasion and metastasis is an EMT, which converts well-polarized epithelial cells to non-polarized mesenchymal cells that acquire motility and invasion properties and exhibit cancer stem cell-like properties (Valastyan and Weinberg, 2011;

Cao et al., 2017) (Figure 3). EMT can be induced in human cancer cells upon the activation of specific transcription factors (including Snail, Twist, and ZEB families) (Valastyan and Weinberg, 2011; Cao et al., 2017). The overexpression of these transcription factors could down-regulate the expression of epithelial markers (such as E-cadherin and ZO-1), and up-regulate the expression of mesenchymal markers (such as N-cadherin and Vimentin) (Valastyan and Weinberg, 2011; Cao et al., 2017). In addition to these transcription factors, several oncogenes (including *MCL-1*, *OCT-4*, and *SOX-2*) have been shown to promote EMT and contribute to the metastatic potential of tumor cells (Konno et al., 2014; Lee et al., 2015; Dai et al., 2013; Liu et al., 2018). However, the molecular mechanisms underlying the regulation of EMT in CC cells are still largely unknown.

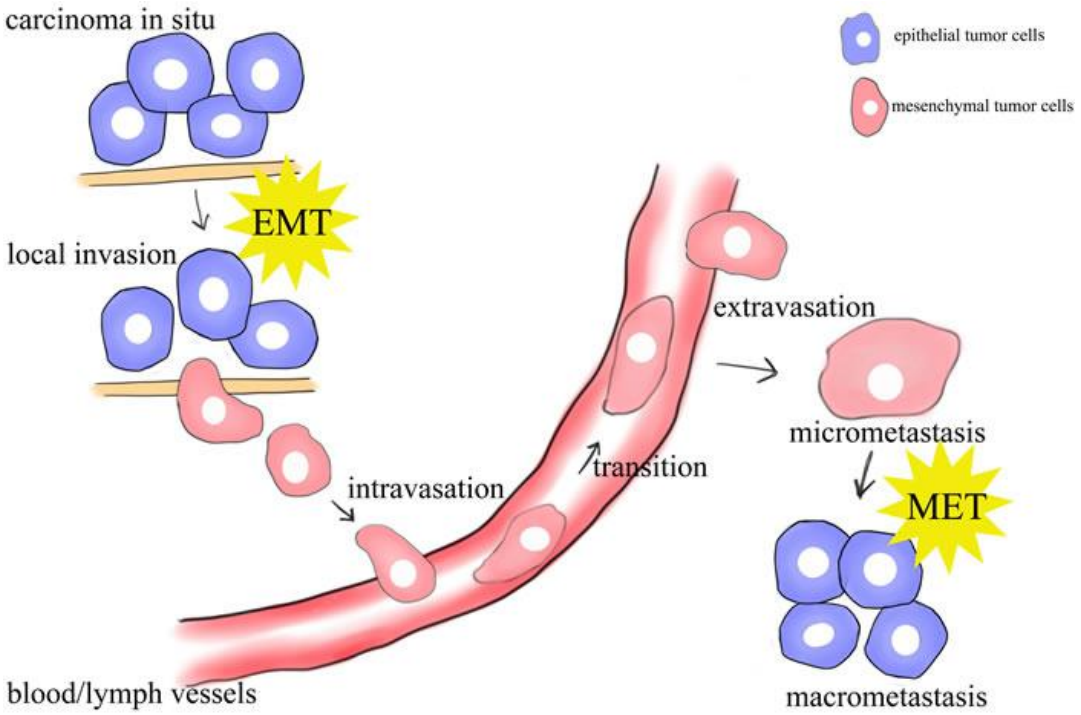


Figure 3: EMT in cancer metastasis (Cao MX, et al. Oncotarget. 2017).

Epithelial cells have apical-basal polarity, which allows cells to firmly arrange themselves on a basement membrane via intercellular junctions. During cancer spread, cancer cells lose cell-cell adhesion and develop motility and invasiveness as a result of EMT. These mesenchymal cancer cells can pass through blood

vessels, migrate down the blood vessel and eventually settle to become cancer. The extravasated cancer cells form metastasis via a reverse process known as a mesenchymal-epithelial transition (MET).

The 90-kDa heat shock proteins (HSP90s) are abundant molecular chaperones, accounting for 1%-2% of all proteins in the cell (Chatterjee and Burns, 2017; Welch and Feramisco, 1982). There are two major HSP90 isoforms, namely, HSP90α (the stress-inducible isoform encoded by *HSP90AA1*) and HSP90β (the constitutively expressed isoform encoded by *HSP90AB1*) (Chatterjee and Burns, 2017; Welch and Feramisco, 1982). In an ATP-bound state, HSP90α (referred to as HSP90 in this study) adopts a closed conformation and forms a mature complex with co-chaperones to promote tumor progression by protecting and stabilizing its client proteins, such as mutant p53, HER2, EGFR, BRAF, AKT, MET, VEGFR, FLT3, androgen/estrogen receptors, HIF-1α, and hTERT (Banerji, 2009; Eskander and Tewari, 2014) (Figure 4).

HSP90 exists in an activated form in tumor cells, while existing in a latent inactive form in normal cells (Miyata et al., 2013; Li et al., 2009). Thus, HSP90 was considered a unique target for cancer therapeutics.

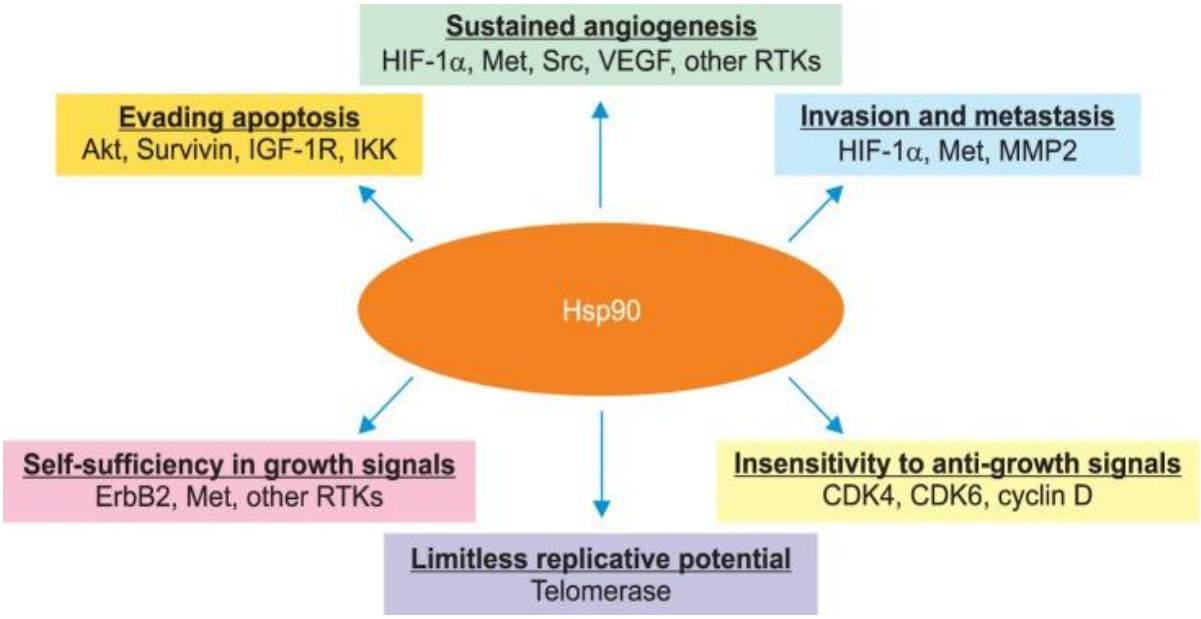


Figure 4: The cancer chaperone HSP90 (Eskander RN, et al. J Gynecol Oncol. 2014).

HSP90 is a molecular chaperone that is required for the folding, stability, and activity of numerous client proteins. By chaperoning a subset of client proteins, HSP90 plays a key role in supporting cancer hallmarks, including (a) the ability to avoid apoptosis, (b) the ability to promote angiogenesis, (c) the ability to invade surrounding tissues and metastasize to distant sites, (d) the ability to be self-sufficient for growth, (e) the ability to undergo limitless replication, and (f) the ability to ignore anti-growth signals.

HSP90 was shown to be expressed at higher levels in invasive breast cancer tissues compared with adjacent non-cancer tissues (Zagouri et al., 2010) and higher HSP90 expression correlated with a worse prognosis (Pick et al., 2007). Increased HSP90 protein expression was significantly associated with lymphatic invasion, lymph node metastasis, advanced stage, and poor survival in patients with gastric cancer (Lee and Kim, 2019). HSP90 interacts with its client protein STAT3 and facilitates the binding of STAT3 to the Twist1 promoter, leading to the transactivation of Twist1 and EMT induction in ovarian cancer cells and renal cancer cells (Chong et al., 2019). HSP90 triggers EMT in colorectal cancer cells through sustained activation of the NF-κB signaling pathway, and subsequent downregulation of the epithelial

marker E-cadherin and upregulation of the mesenchymal marker Vimentin (Nagaraju et al., 2015). HSP90 physically associates with OCT-4 (a key regulator of stem cell pluripotency and differentiation) and prevents it from degradation by the ubiquitin-proteasome pathway in mouse embryonic stem cells (Bradley et al., 2012).

The Cancer Genome Atlas (TCGA) is the largest and most comprehensive cancer genomics database in the world, which collected and molecularly characterized over 20000 primary cancer samples and matched normal samples from 33 cancer types (https://www.cancer.gov/tcga). Multiple gene expression data sets in TCGA indicated the upregulation of HSP90 in tissues of different cancer types (including cervical cancer) compared to the corresponding normal tissues (Elzakra et al., 2017; Zhang et al., 2019). High HSP90 expression is associated with poor prognosis in patients with head and neck cancer or colorectal cancer (Elzakra et al., 2017; Zhang et al., 2019). HSP90 protein was detected in cervical intraepithelial neoplasia (Castle et al., 2005). The levels of HSP90 increased gradually from the normal cervix to intraepithelial lesions, and consequently to CC tissues (Zhao et al., 2006). Although the pharmacological inhibition of HSP90 inhibited the growth of CC cells (Schwock et al., 2008), the exact function of HSP90 in CC development is still unclear.

The wide use of advanced genome analysis platforms in recent years has shown that, among all the RNAs transcribed from the genomic regions, only a small proportion of the transcribed RNA corresponds to protein-coding genes (<3% of the genome) (Parasramka et al., 2016). Non-coding RNAs (without evident protein-coding potential) account for a much larger portion of the human genome than those protein-coding RNAs (Parasramka et al., 2016) (Figure 5).

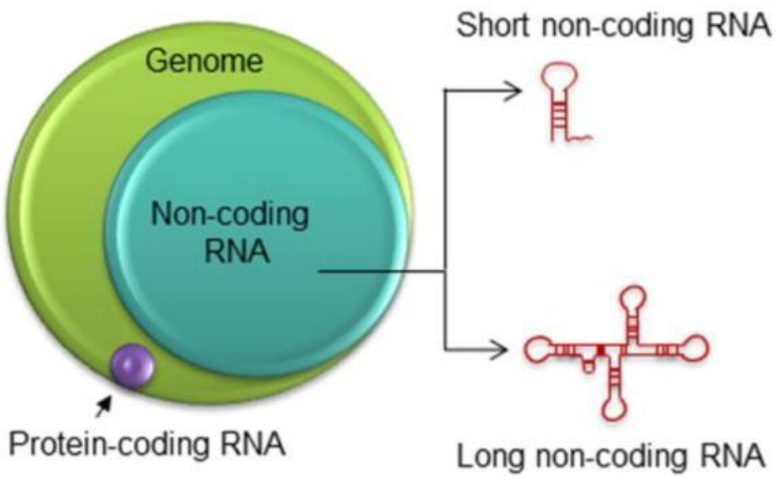


Figure 5: Protein-coding and non-coding RNAs in the human genome (Parasramka MA, et al. Pharmacol Ther. 2016).

Non-coding RNA makes up a far bigger proportion of the human genome than protein-coding RNA, which represents just around 3%. Non-coding RNAs are classified into two types based on their size. Small non-coding RNAs (such as miRNAs and Piwi-interacting RNAs) are below 200 nucleotides. Long non-coding RNAs are non-coding RNA transcripts that are longer than 200 nucleotides and have little or no coding potential.

There are several known types of non-coding RNAs, including lncRNAs, miRNAs, and circular RNAs. Previous work has shown that all these non-coding RNAs are involved in the tumorigenesis and metastasis of human cancers (Dong et al., 2011). MiRNAs function primarily as post-transcriptional regulators of mRNA, and they bind to the 3′-untranslated regions (3′-UTRs) of target mRNAs to inhibit the translation of their respective RNA targets or degrade their target genes (Van Roosbroeck and Calin, 2017; Dong et al., 2018a; Wienholds and Plasterk, 2005) (Figure 6). MiRNAs participate in the regulation of each cancer hallmark (Van Roosbroeck and Calin, 2017; Dong et al., 2018a; Wienholds and Plasterk, 2005). In human cancer, an increasing number of miRNAs have been linked to different steps of

metastasis (Kim et al., 2018). These miRNAs have critical roles in the modulation of cell migration, cell invasion, tumor proliferation, cancer stem cell-like properties, EMT, and tumor microenvironment (Kim et al., 2018).

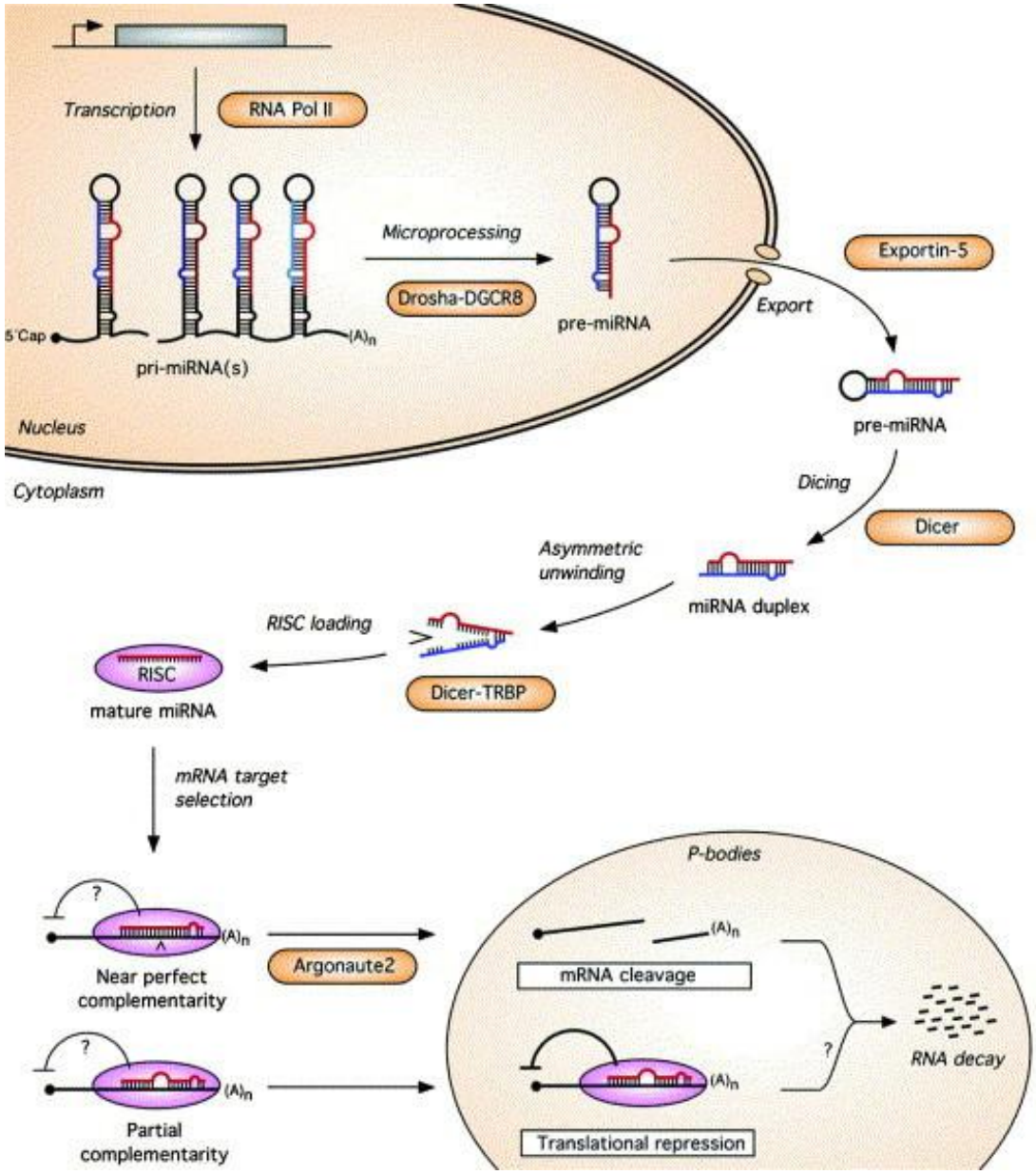


Figure 6: The biogenesis and mechanism of miRNA (Wienholds E, et al. FEBS Lett. 2005).

MiRNAs are produced from primary miRNAs (pri-miRNAs). The first nuclear ribonuclease III Drosha identifies pri-miRNAs and cuts the double-stranded RNAs, releasing precursor miRNAs (pre-miRNAs). Pre-miRNAs are then exported from the nucleus to the cytoplasm, and cleaved by the ribonuclease III

enzyme Dicer. Finally, mature miRNAs act either by degrading the mRNA targets or by inhibiting their translation.

LncRNA is a type of non-coding RNA longer than 200 nucleotides and lacks protein-coding potential (Dong et al., 2019a). It has become clear that approximately 80% of the transcripts in human cells are lncRNA species (Parasramka et al., 2016). A large number of lncRNAs have been discovered by high-throughput RNA sequencing technology (Parasramka et al., 2016). However, only a small part of lncRNAs have been studied in human cancer. LncRNAs can modulate the expression of genes by interacting with other molecules such as protein, DNA, and miRNA (Dong et al., 2018c) (Figure 7). LncRNAs have been implicated in the regulation of cancer pathways, thus affecting the malignant properties of tumor cells (Dong et al., 2018c). Multiple steps of the metastasis cascade (including EMT) were regulated by numerous lncRNAs (Liu et al., 2021). In addition, lncRNAs are also required for the remodeling of the tumor microenvironment, which contributes to the outgrowth of metastatic cancer (Liu et al., 2021). Nuclear paraspeckle assembly transcript 1 (NEAT1, a lncRNA) is abnormally overexpressed in many human cancer tissues and functions as a key tumor promoter in cancer cells (Dong et al., 2018c). However, how NEAT1 affects the expression of tumor-suppressive miRNAs in CC cells remains unclear.

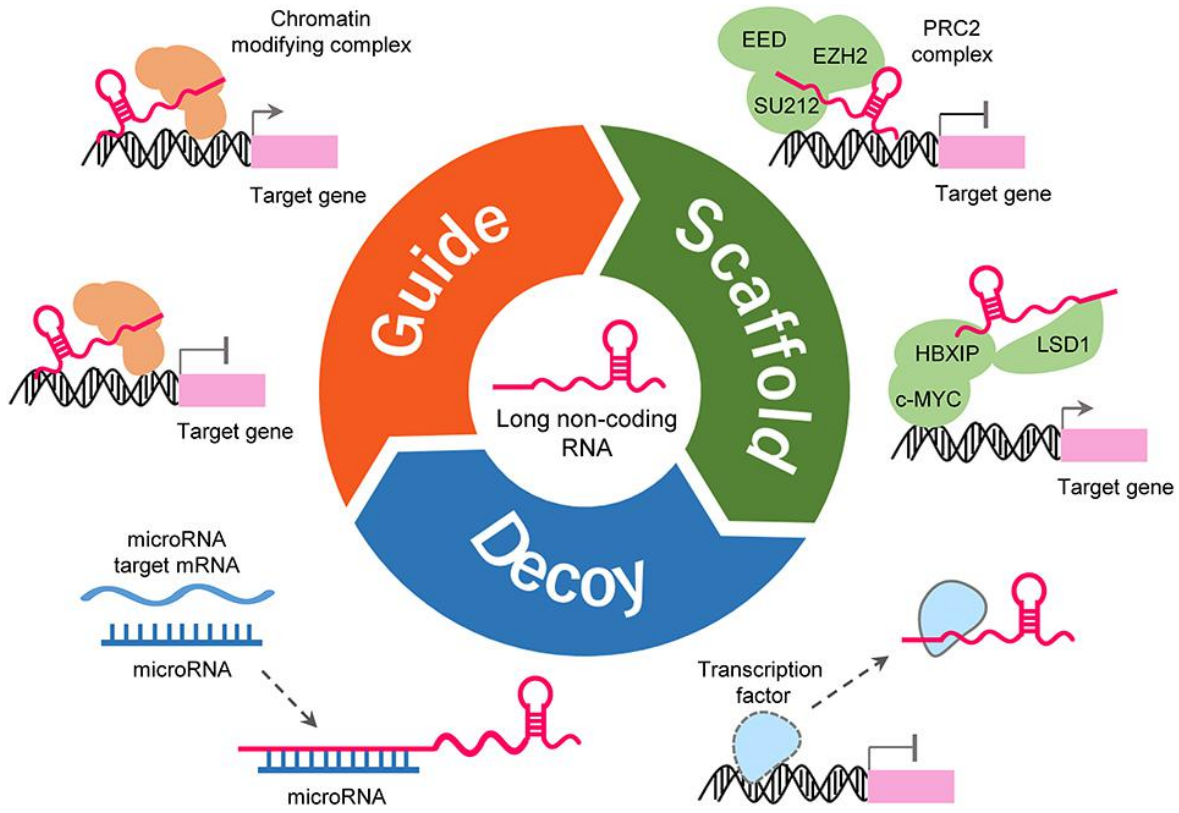


Figure 7: LncRNA functions and mechanisms (Dong P, et al. Front Genet. 2018).

LncRNA can guide transcription factors to particular genomic regions for gene expression control (upper-left). LncRNA acts as a scaffold to aid in the formation of chromatin remodeling complexes (upper-right). LncRNA can also act as a sponge, allowing miRNAs to be titrated away from their mRNA targets (lower-left). As a decoy, lncRNA can bind to transcription factors or other proteins and sequester them from chromatin (lower-right).

We conducted a preliminary literature review and found that miR-361 is frequently downregulated in diverse tumor types and exerts broad tumor-suppressing functions (Xu et al., 2019) (Figure 8). However, previous studies exploring the role of miR-361 in cervical cancer produced somewhat controversial results: an early study suggested that miR-361 was downregulated in CC tissues and reduced miR-361 expression was sufficient to promote CC cell proliferation (Yang and Xie, 2020), whereas another study indicated that increased

miR-361 expression was detected in patients with lymph node metastasis and stromal invasion and the introduction of miR-361 facilitates CC progression (Wu et al., 2013). Nevertheless, the precise role of miR-361 in cervical cancer and the mechanisms underlying its function in EMT have not been fully explored.

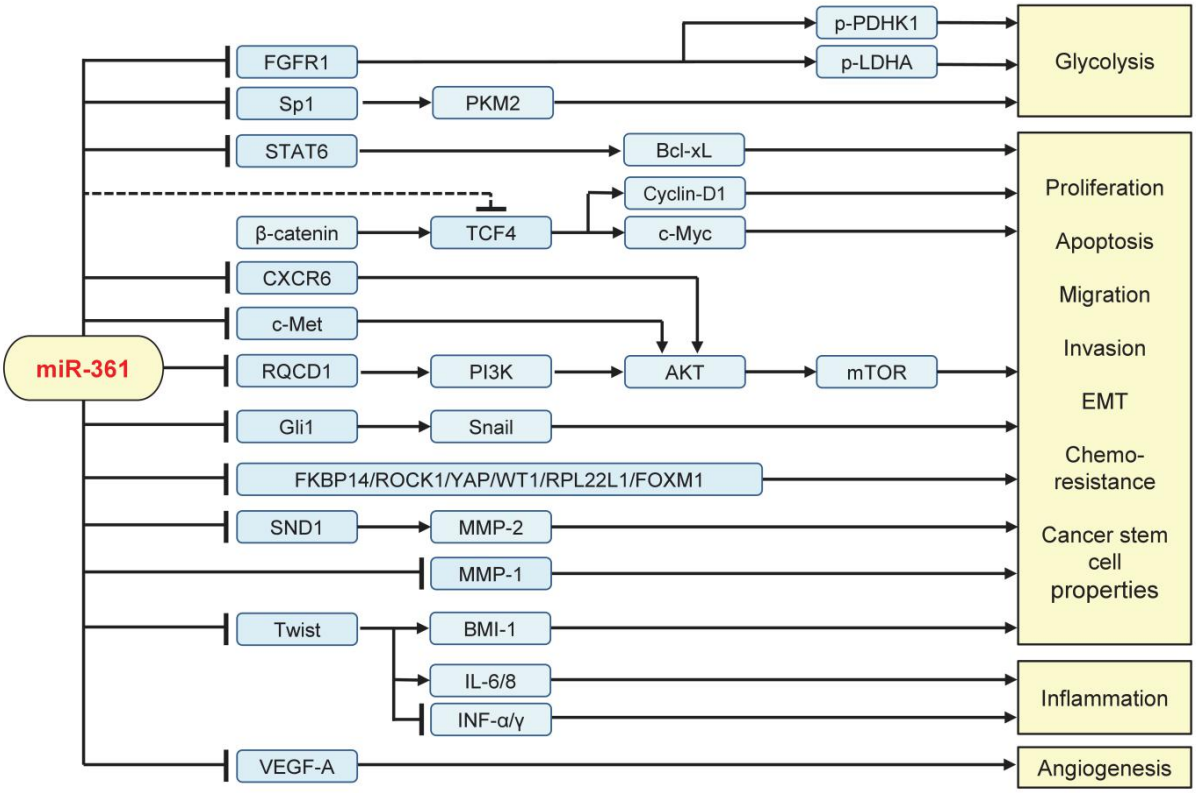


Figure 8: Validated targets and signaling pathways regulated by miR-361 in human tumor cells (Xu D, et al. Cancers (Basel). 2019).

MiR-361 functions as a novel tumor suppressor, repressing a wide range of downstream target transcripts involved in cellular proliferation, glycolysis, migration, invasion, EMT, chemoresistance, cancer stemness, angiogenesis, and inflammation. In accordance with its reported anti-tumor functions, ectopic expression of miR-361 was found to cause dramatic suppression of the EMT process in various cancer cells. In addition to targeting EMT-promoting transcription factors directly, miR-361 also modulated the expression of key mediators of the EMT program.

Our meta-analysis with TargetScan (Agarwal et al., 2015) (Figure 9, upper panel) and ENCORI database (Li et al., 2014) (Figure 9, bottom panel) indicated that the 3′-UTR of *HSP90* mRNA contains a sequence complementary to the seed region of miR-361, and that lncRNA NEAT1 may bind to miR-361.

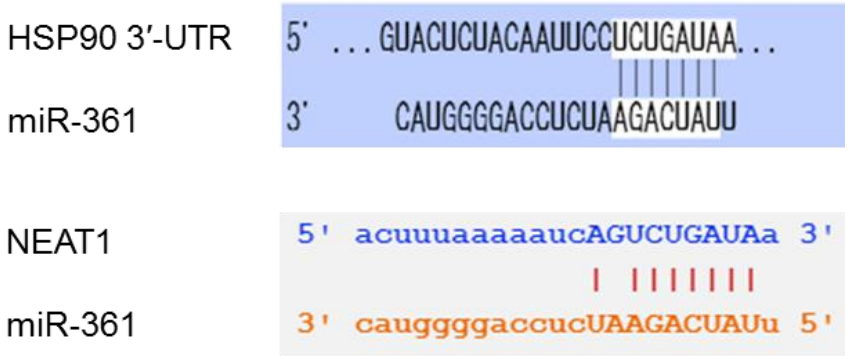


Figure 9: Our meta-analysis demonstrated that miR-361 may bind to *HSP90* and NEAT1.

Using the TargetScan and ENCORI databases, we found that the 3′-UTR of *HSP90* mRNA and NEAT1 sequence contain a sequence complementary to the seed region of miR-361, indicating that and *HSP90* mRNA might be a direct target of miR-361 and lncRNA NEAT1 potentially binds to miR-361.

Therefore, we speculated that miR-361 directly targets *HSP90* to inhibit the invasion and EMT features, and NEAT1 functions as an oncogenic lncRNA that suppresses miR-361 expression and induces EMT and sphere formation in CC cells (Figure 10). To investigate the accurate role of miR-361 in EMT and the mechanisms underlying its function in cervical cancer, we explored the functional roles of miR-361 in CC cells through a series of cell functional assays. We further used the luciferase reporter assays to demonstrate the potential interaction between miR-361, HSP90, and lncRNA NEAT1.

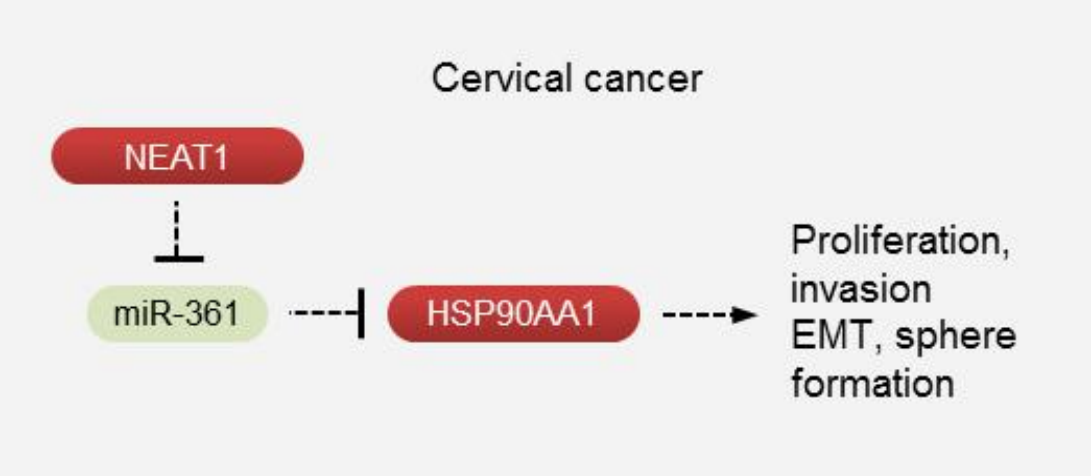


Figure 10: The hypothesis of our study.

We hypothesized that miR-361 inhibits invasion and EMT by directly targeting *HSP90* and that NEAT1 acts as an oncogenic lncRNA that reduces miR-361 production and stimulates EMT and sphere formation in CC cells.

CHAPTER 1: Methods

Cell lines and culture

Human CC cell lines (HeLa and SiHa) were obtained from the American Type Tissue Collection (Manassas, VA, USA). The normal endometrial epithelial cell line EM has been established as previously reported (Kyo et al., 2003). The cells were cultured in DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA) and 100 μg/ml Normocin (InvivoGen, San Diego, CA, USA). For subculture, these cells were detached from the plate with Tryple Express Enzyme (12604-013, Gibco, USA). Cells were subcultured 2-3 times per week at a 1:5 split ratio.

Cell transfections

The miR-361 mimic, control mimic, miR-361 inhibitor, control inhibitor, small interfering RNAs (siRNAs) targeting HSP90 and NEAT1, as well as control siRNA, were purchased from Ambion (Austin, TX, USA). The expression vector encoding *HSP90* and the corresponding empty control vector were obtained from OriGene (Rockville, MD, USA). CC cells were seeded into 24-well plates containing DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA) and 100 μg/ml Normocin (InvivoGen, San Diego, CA, USA) until approximately 70% confluence was reached. First, different densities of siRNA, miRNA mimic, miRNA inhibitor, plasmid, and different volumes of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were separately diluted with serum-free DMEM/F12 medium (without antibiotics). Next, siRNA, miRNA mimic, miRNA inhibitor, and plasmid were mixed with Lipofectamine 2000 dilutions,

and the mixture was incubated for 15 minutes at room temperature. Then, the prepared mixtures were added to CC cells by slowly dripping and gently rocking the plates back and forth. The cells were further incubated for 6 hours at 37°C in a 5% CO_2 incubator. After that, the Lipofectamine 2000-containing DMEM/F12 medium was replaced with DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA) and 100 μg/ml Normocin (InvivoGen, San Diego, CA, USA).

Quantitative Reverse Transcription PCR (RT-qPCR) analysis

Total RNA was extracted from CC cells and EM cells using TRIzol (InvivoGen, San Diego, CA, USA). Total RNA was reverse transcribed into cDNA using a Reverse Transcription Kit (Takara, Kusatsu, Japan). Real-time PCR was performed using SYBR Green Real-Time PCR MasterMix (Toyobo, Osaka, Japan) using the ABI 7500 Real-Time PCR Systems, and *GAPDH* was used as the reference. The primers used in this study (except NEAT1) were obtained from the PrimerBank Web-based database (http://pga.mgh.harvard.edu/primerbank/).

The primers used were as follows:

HSP90 Forward: 5′-AGGAGGTTGAGACGTTCGC-3′;

HSP90 Reverse: 5′-AGAGTTCGATCTTGTTTGTTCGG-3′;

E-cadherin Forward: 5′-CGAGAGCTACACGTTCACGG-3′;

E-cadherin Reverse: 5′-GGGTGTCGAGGGAAAAATAGG-3′;

ZO-1 Forward: 5′-CAACATACAGTGACGCTTCACA-3′;

ZO-1 Reverse: 5′-CACTATTGACGTTTCCCCACTC-3′;

Twist1 Forward: 5′-GTCCGCAGTCTTACGAGGAG-3′;

Twist1 Reverse: 5′-GCTTGAGGGTCTGAATCTTGCT-3′;

MCL-1 Forward: 5′-TGCTTCGGAAACTGGACATCA-3′;

MCL-1 Reverse: 5′-TAGCCACAAAGGCACCAAAAG-3′;

SOX-2 Forward: 5′-GCCGAGTGGAAACTTTTGTCG-3′;

SOX-2 Reverse: 5′-GGCAGCGTGTACTTATCCTTCT-3′;

MDR-1 Forward: 5′-TTGCTGCTTACATTCAGGTTTCA-3′;

MDR-1 Reverse: 5′-AGCCTATCTCCTGTCGCATTA-3′;

GAPDH Forward: 5′-AATCCCATCACCATCTTC-3′;

GAPDH Reverse: 5′-AGGCTGTTGTCATACTTC-3′.

The primers used to detect NEAT1 expression have been previously reported (Li et al., 2018b)

NEAT1 Forward: 5′-GACCTCTCACCTACCCACCT-3′;

NEAT1 Reverse: 5′-ATGCCCAAACTAGACCTGCC-3′.

The NCode miRNA qRT-PCR kit (InvivoGen, San Diego, CA, USA) was used to detect the miR-361 expression according to the manufacturer's instructions. U6 small nuclear RNA was used as the internal normalization control. Data was expressed as the fold change over control (set as 1).

U6 Forward: 5′-GCTTCGGCAGCACATATACTAAAAT-3′;

U6 Reverse: 5′-CGCTTCACGAATTTGCGTGTCAT-3′.

The color-coded scale was used to represent the level of expression of genes in CC cells after overexpression or knockdown of miR-361 (or HSP90).

Cell proliferation assay

Cell proliferation was measured using the Cell Counting Kit-8 assay (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells (2×10^3 each well) were seeded in a 96-well plate at 37°C. 10 µl CCK-8 reagents were added to each well and the plates were incubated at 37°C for 2 hours. Then, the absorbance was measured at 450 nm for each sample. The cell proliferation curves were plotted using the absorbance at each time point.

Cell invasion assay

Cell invasion assay was performed using 24-well transwell chambers (Corning Costar, Cambridge, MA, USA). 20000 CC cells were placed in the upper wells of 24-well Transwell chambers containing serum-free DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA). The lower wells were filled with DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco Laboratories, Grand Island, NY, USA). The chambers were incubated for 24 hours, allowing CC cells to invade. After incubation, non-invading cells on the upper surface of membranes were removed by gentle scrubbing, invaded cells on the lower surface of the membranes were fixed, stained and the number of invaded cells was counted in ten random fields using a microscope.

Cell sphere formation assay

CC cells (1000 cells/ml) were seeded into serum-free DMEM/F12 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with N-2 Plus Media Supplement Components (Invitrogen, Carlsbad, CA, USA), 20 ng/ml epidermal growth factor, 20 ng/ml basic fibroblast growth factor (Invitrogen, Carlsbad, CA, USA), and 4 mg/ml Heparin (Sigma-Aldrich, St. Louis, MO, USA) in Ultra-Low Attachment dishes (Corning Costar,

Cambridge, MA, USA). Mammospheres were grown for 14 days. The number of spheres larger than 50 μm was counted.

Western blotting analysis

Total cell lysates were extracted using the M-Per Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentration was measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Extracted proteins were separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk and incubated with the primary antibodies for total HSP90 (AA1 and AB1, #4877, 1:1000), E-Cadherin (#5296, 1:1000), Vimentin (#5741, 1:1000), and GAPDH (#5174, 1:5000). These antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). GAPDH was used as an endogenous control. Immunoblot images were digitized and quantified using the ImageJ software (Wayne Rasband, NIH, USA).

Luciferase reporter assay

The 3′-UTR of *HSP90* mRNA containing the putative miR-361 binding site was amplified using the following PCR primers (*HSP90* forward: 5′-TCTCTGGCTGAGGGATGACT-3′; *HSP90* reverse: 5′-ACGTGGACACTAAGAGAACACA-3′). The resulting products were cloned downstream of the firefly luciferase gene in the pGL3 luciferase reporter vector (Promega, Madison, WI, USA). Mutations of the miR-361 binding site in the *HSP90* 3′-UTR (TCTGATA to CACAGCG) were generated by PCR mutagenesis using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. The pGL3 luciferase reporter vectors containing wild-type (WT) NEAT1 fragment, or mutant (MUT) NEAT1 fragment with the mutated miR-361 binding site, were obtained from

IGEbio (Guangzhou, China). Luciferase reporter assay was performed as we previously described (Dong et al., 2011). In brief, CC cells were seeded into 24-well plates, and co-transfected after 24 hours with the above luciferase reporter vectors (100 ng) containing *HSP90* 3′-UTR (WT or MUT) or NEAT1 (WT or MUT) and miR-361 mimic, miR-361 inhibitor or their respective controls (30 nM) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), along with the Renilla luciferase plasmid (10 ng, pRL-CMV, Promega, Madison, WI, USA) used for normalization. Two days after transfection, luciferase activity was determined using a Dual-Luciferase Reporter assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical analysis

All results were expressed as mean ± SEMs of at least three independent experiments. Statistical analysis was conducted using a 2-tailed Student's *t*-test or one-way ANOVA using the SPSS version 16.0 software (SPSS, Chicago, IL, USA). *P*-values of < 0.05 were considered to be statistically significant.

CHAPTER 1: Results

1. Downregulation of miR-361 was found in cervical cancer

We first evaluated the expression pattern of miR-361 across different normal and cancer tissues using the dbDEMC v2.0 database (Yang et al., 2017). Our results suggested that miR-361 was downregulated in almost all cancer types, including cervical cancer (Figure 11).

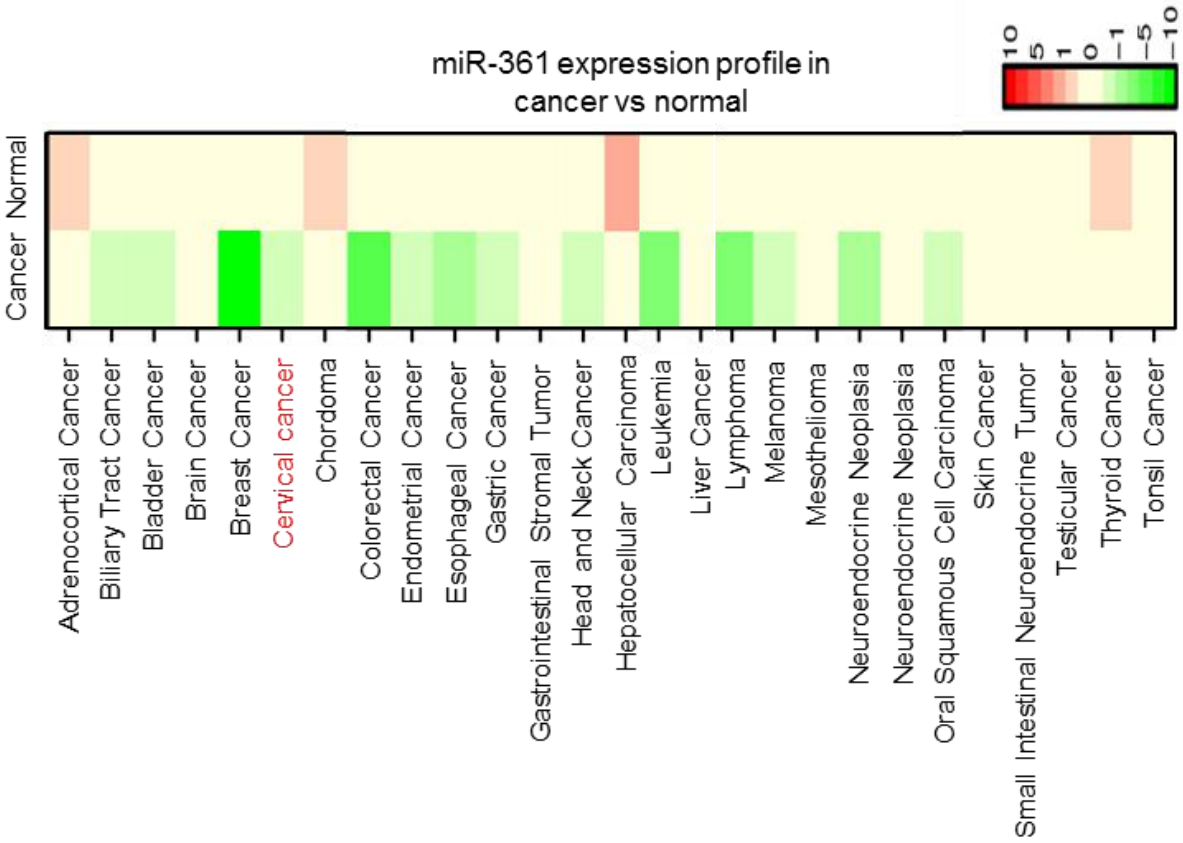


Figure 11: The expression patterns of miR-361 in different tumors and normal samples.

The dbDEMC v2.0 database contains 3268 differentially expressed miRNAs from 40 cancer types. We searched for miR-361 expression in various types of cancer in the dbDEMC v2.0 database. An expression heatmap shows that the expression of miR-361 was frequently downregulated in human cancer tissues (including cervical cancer) compared with the respective normal tissues. Green and red indicate

downregulated and upregulated miR-361 expression in the corresponding cancer tissues, respectively.

We then measured miR-361 expression in one immortalized but non-malignant human endometrial cell line (EM) and in two human CC cell lines (HeLa and SiHa) using RT-qPCR analysis. The expression of miR-361 in HeLa and SiHa cells was significantly lower than that in the EM cells (Figure 12).

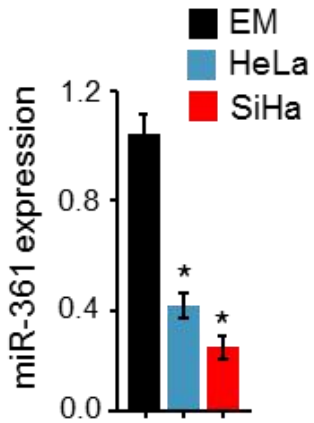


Figure 12: MiR-361 expression in human CC cell lines and normal cells.

MiR-361 expression was measured in a human endometrial cell line (EM) and two human CC cell lines (HeLa and SiHa) using RT-qPCR analysis. The expression of miR-361 was significantly reduced in two human CC cell lines compared with EM cells. *P<0.05.

Our Kaplan-Meier analysis using the KM Plotter database (Lánczky and Győrffy, 2021) showed that decreased miR-361 expression level indicated a worse overall survival outcome in CC patients from the TCGA cohort (Figure 13).

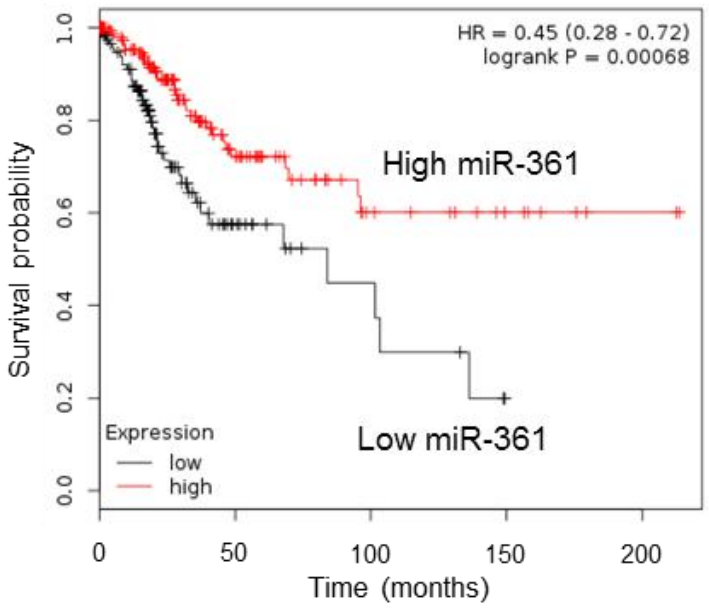


Figure 13: The clinical significance of miR-361 expression in CC patients.

The clinical significance of miR-361 expression in cervical cancer was evaluated by Kaplan–Meier survival analysis. The plots were generated using the KM Plotter database. Lower expression of miR-361 was significantly correlated with a poorer prognosis in CC patients.

The prognostic impact of miR-361 on CC patients was also analyzed using the PROGmiR database (Goswami and Nakshatri, 2012). Survival analysis revealed that lower expression of miR-361 was significantly associated with a poorer prognosis in cervical cancer (Figure 14). These results suggested that miR-361 was indeed downregulated in cervical cancer and its expression was positively associated with patient survival.

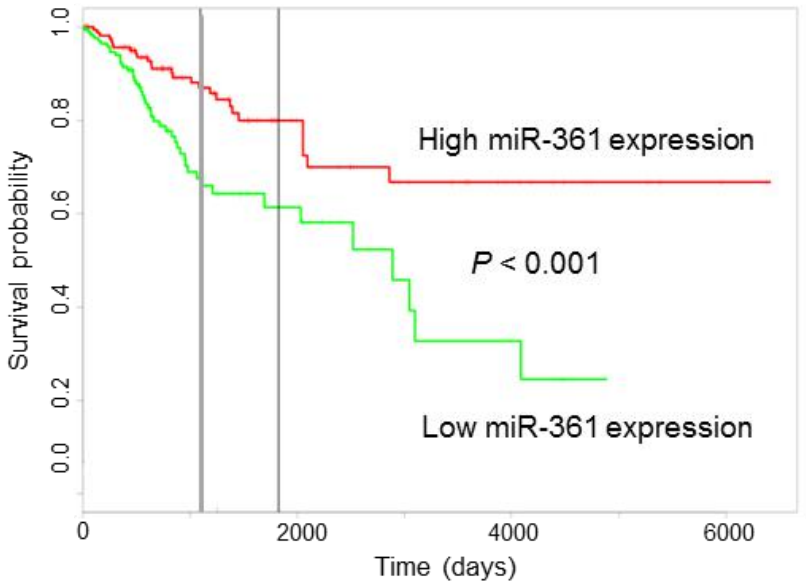


Figure 14: The prognostic impact of miR-361 in CC patients.

The prognostic impact of miR-361 on CC patients was also analyzed using the PROGmiR database. Decreased expression of miR-361 was significantly associated with worse overall survival in CC patients.

2. MiR-361 inhibits EMT and sphere formation in CC cells

We assessed the effects of miR-361 overexpression/knockdown on the proliferation, invasion, EMT, and sphere formation of SiHa/HeLa cells, which express relatively lower/higher levels of miR-361 (Figure 15A). The overexpression of miR-361 significantly attenuated the proliferation of SiHa cells, whereas the silencing of miR-361 markedly enhanced the growth of HeLa cells. Importantly, overexpression of miR-361 induced morphological changes in SiHa cells, from a spindle-shaped appearance to a cobblestone-like epithelial phenotype (Figure 15B). MiR-361-silenced HeLa cells became elongated and disassociated from their neighboring cells (Figure 15C).

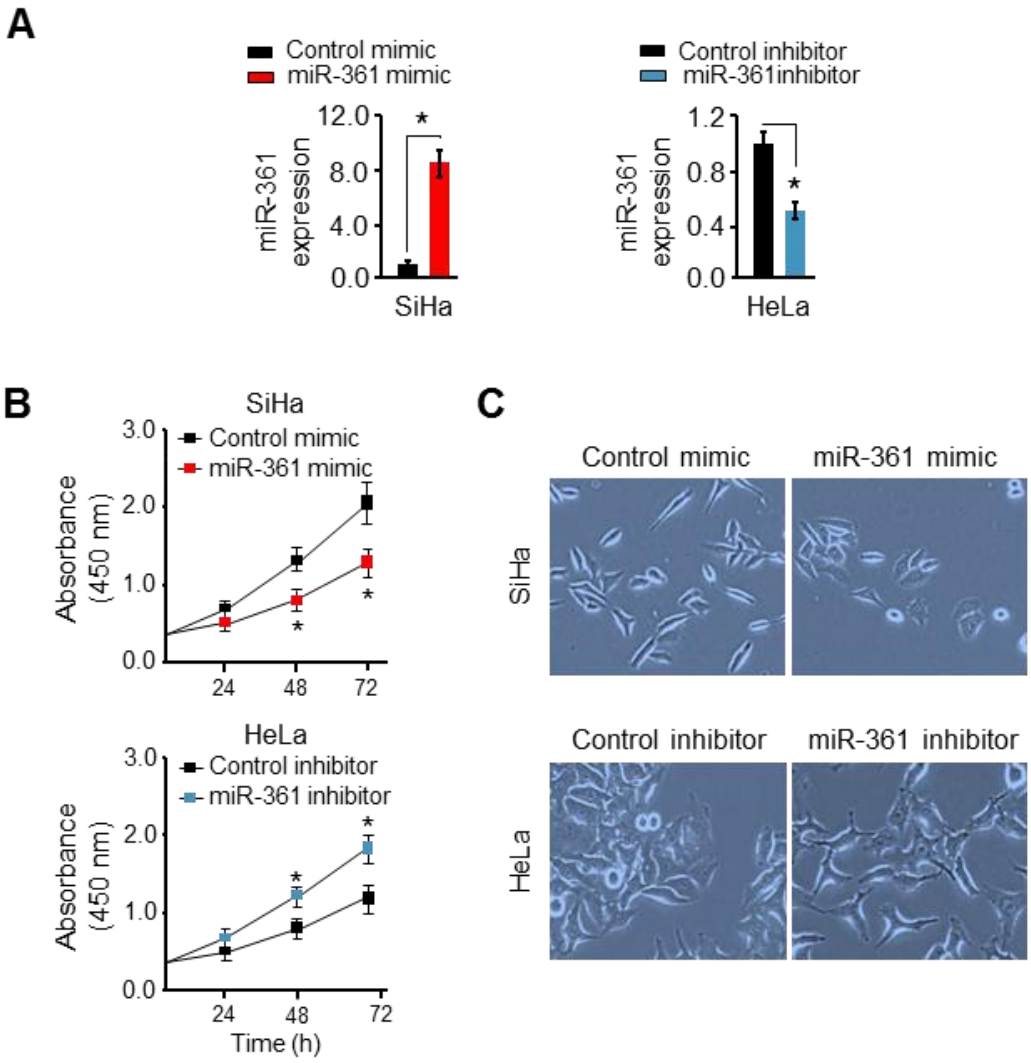


Figure 15: The effects of miR-361 expression on EMT and sphere formation in CC cells.

(A) MiR-361 expression was examined in SiHa cells transfected with miR-361 mimic or control mimic, and in HeLa cells transfected with miR-361 inhibitor or control inhibitor using RT-qPCR analysis. (B) Growth curves of CC cells transfected as indicated were determined using CCK-8 assays. (C) MiR-361 affected CC cell morphology. **P*<0.05.

The marked effects of miR-361 expression on cell morphology led us to examine its influence on cell invasion and cancer stem cell-like properties using Matrigel invasion assays and sphere formation assays. As shown in Figure 16A and 16B, the overexpression of miR-361

significantly decreased cell invasion and sphere formation, and miR-361 knockdown greatly induced these phenotypes.

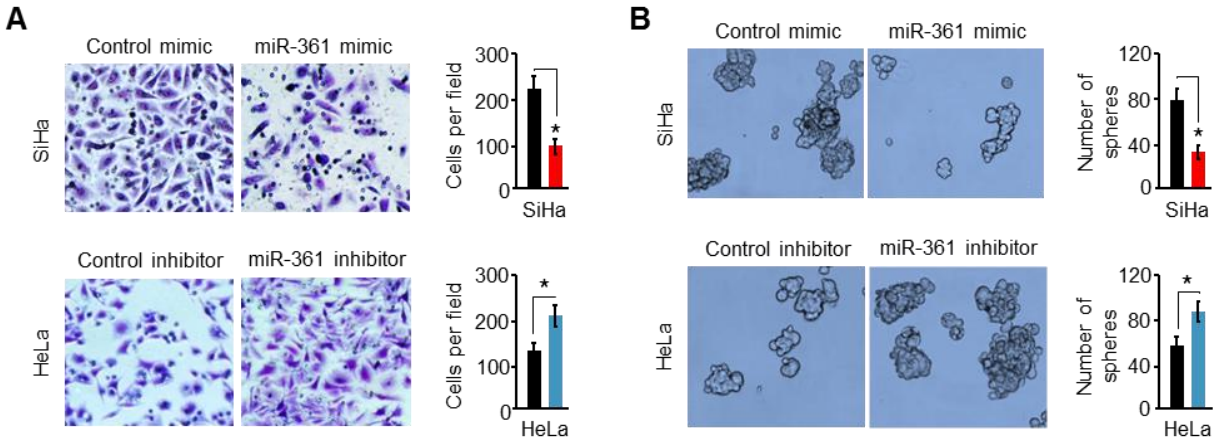


Figure 16: The impact of miR-361 expression on CC cell invasion and sphere formation.

The effects of either miR-361 overexpression or knockdown on CC cell invasion (A) and sphere formation (B) were assessed using the Matrigel invasion and sphere formation assays. **P*<0.05.

To investigate the mechanism by which miR-361 modulates EMT, we assessed the mRNA levels of known EMT markers, EMT-inducer genes, and the drug resistance-associated gene utilizing RT-qPCR analysis. The overexpression of miR-361 in SiHa cells increased the expression of two epithelial markers (*E-cadherin* and *ZO-1*) and reduced the expression of the mesenchymal marker *Vimentin* as well as *Twist1*, *MCL-1*, *OCT-4*, *SOX-2,* and *MDR-1* (Figure 17A). In contrast, silencing of miR-361 led to opposite effects on these phenotypes in HeLa cells (Figure 17A).

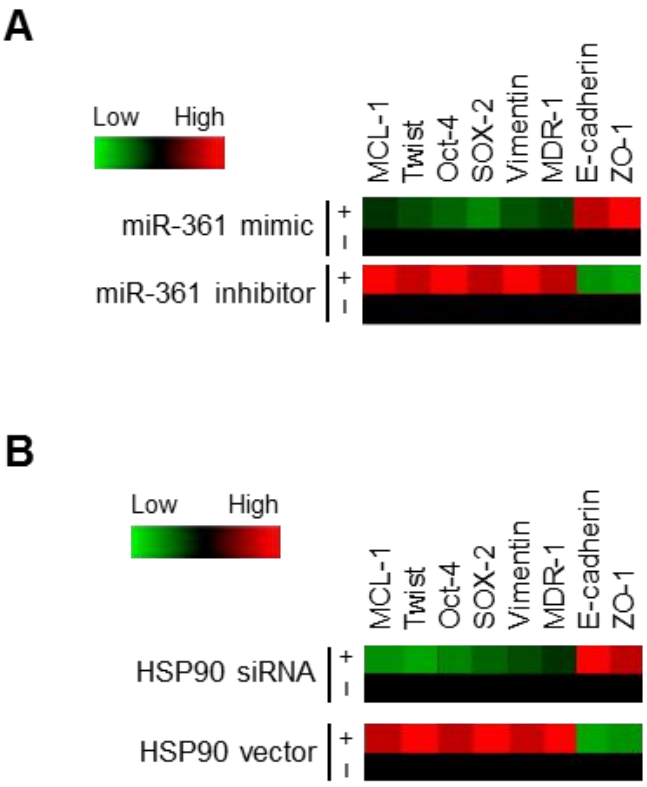


Figure 17: MiR-361 and HSP90 regulate the expression of EMT markers, EMT-inducer genes, and the drug resistance-associated gene in CC cells.

We modulated the expression of miR-361 (A) and HSP90 (B) in CC cells and assessed the mRNA levels of known EMT markers, EMT-inducer genes, and the drug resistance-associated gene utilizing RT-qPCR analysis. The color-coded scale depicts expression changes of indicated mRNAs in CC cells after overexpression or knockdown of miR-361/HSP90, as determined by RT-qPCR analysis. Red: upregulation; green: downregulation.

Our western blotting analysis showed that enforced overexpression of miR-361 led to increased E-cadherin expression and decreased Vimentin expression (Figure 18). However, inhibition of miR-361 reduced the expression of E-cadherin and enhanced the levels of Vimentin (Figure 18). Collectively, these results suggested that miR-361 negatively regulates EMT and sphere formation in CC cells.

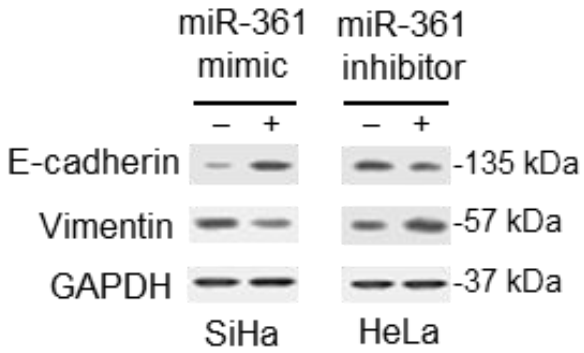


Figure 18: The protein expression of E-cadherin and Vimentin in CC cells after overexpression or knockdown of miR-361.

Using western blotting analysis, we examined the protein expression of E-cadherin and Vimentin in cervical cancer SiHa cells that were transfected with miR-361 mimic or control mimic, and in HeLa cells that were transfected with miR-361 inhibitor or control inhibitor.

3. Identification and functional annotation of potential miR-361 target genes

To elucidate the relevant miR-361 target genes in cervical cancer, we predicted the potential target genes of miR-361 using 3 existing miRNA-target prediction programs, namely Targetscan, microRNA.org (Betel et al., 2008), and miRTarBase (Hsu et al., 2011). Targetscan and microRNA.org are the most up-to-date sequence-based target prediction algorithms that consider the conservation of miRNAs (Agarwal et al., 2015; Betel et al., 2008). MiRTarBase is an up-to-date tool that contains information on experimentally validated miRNA-target interactions (Hsu et al., 2011).

Then, we combined the prediction data resulting from these databases and identified 160 overlapping genes as putative targets of miR-361. The web-based database DAVID (https://david.ncifcrf.gov/) was used to provide the biological functional interpretation of the

predicted targets of miR-361. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis revealed that these genes were significantly enriched in several pathways, including adhesion junction, microRNAs in cancer, regulation of actin cytoskeleton, cell cycle, pathways in cancer, and renal cell carcinoma (Figure 19A). The top significantly biological process Gene Ontology (GO) terms were involved in the regulation of gene expression, DNA repair, cell cycle, membrane organization, and cell migration (Figure 19B).

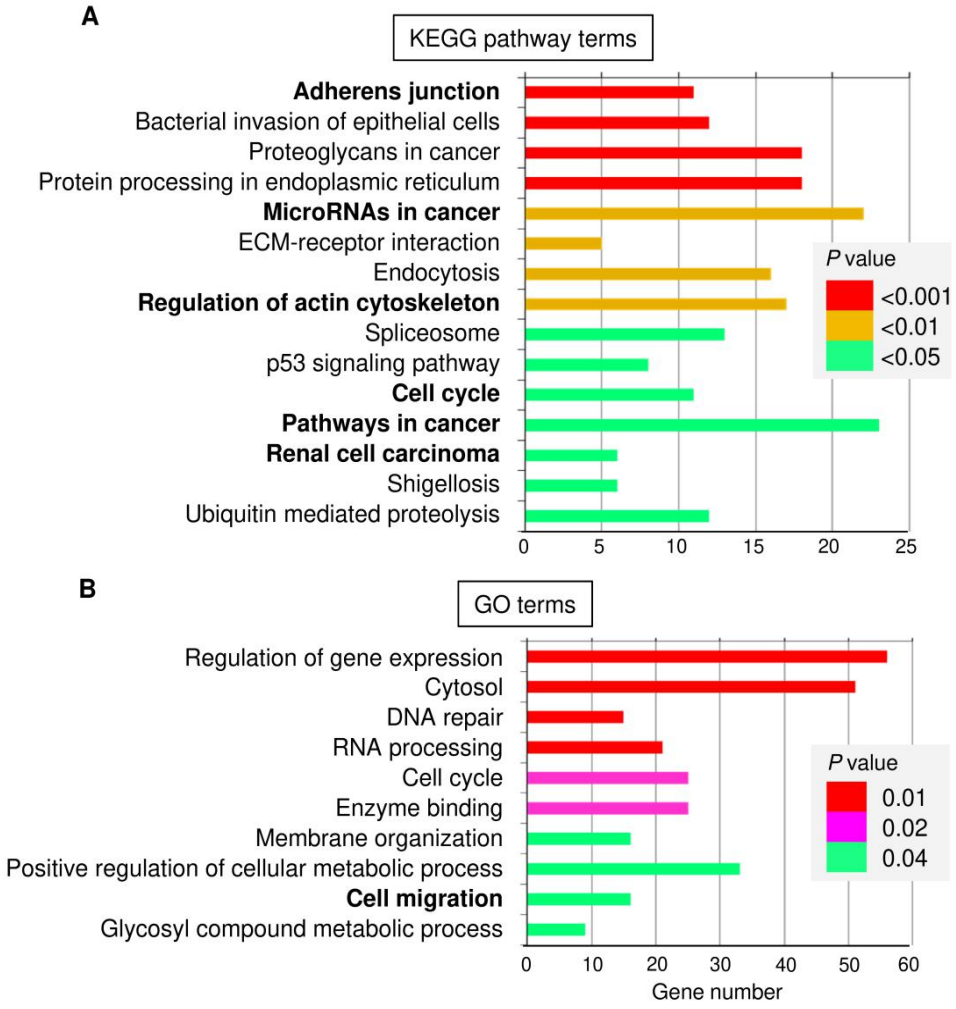


Figure 19: KEGG pathway and GO enrichment analysis for predicted miR-361 target genes using the DAVID database.

We predicted the potential target genes of miR-361 using 3 miRNA-target prediction programs and identified 160 overlapping genes as putative targets of miR-361. The DAVID database was used to provide the

biological functional interpretation of the predicted targets of miR-361. (A) Enrichment analysis for KEGG pathways. (B) GO analysis according to the biological process.

Considering the critical role of miR-361 in regulating CC cell invasion, we decided to focus on the 16 predicted genes belonging to the "Cell migration" GO term category, including *HSP90*, *ARF4*, *DEPDC1B*, *EPHA4*, *ABI2*, *BSG*, *GPC6*, *OGDH*, *PHACTR4*, *RAC1*, *SEMA6D*, *TMEM18*, as well as *Twist1* and *VEGF-A* (two known miR-361 targets) (Ihira et al., 2017; Kanitz et al., 2012). We explored their expression in human CC tissues and normal cervical tissues using the Oncomine database (https://www.oncomine.org/resource/login.html). Interestingly, among these 16 genes, 12 genes were significantly upregulated in CC tissues compared with normal tissues (Figure 20).

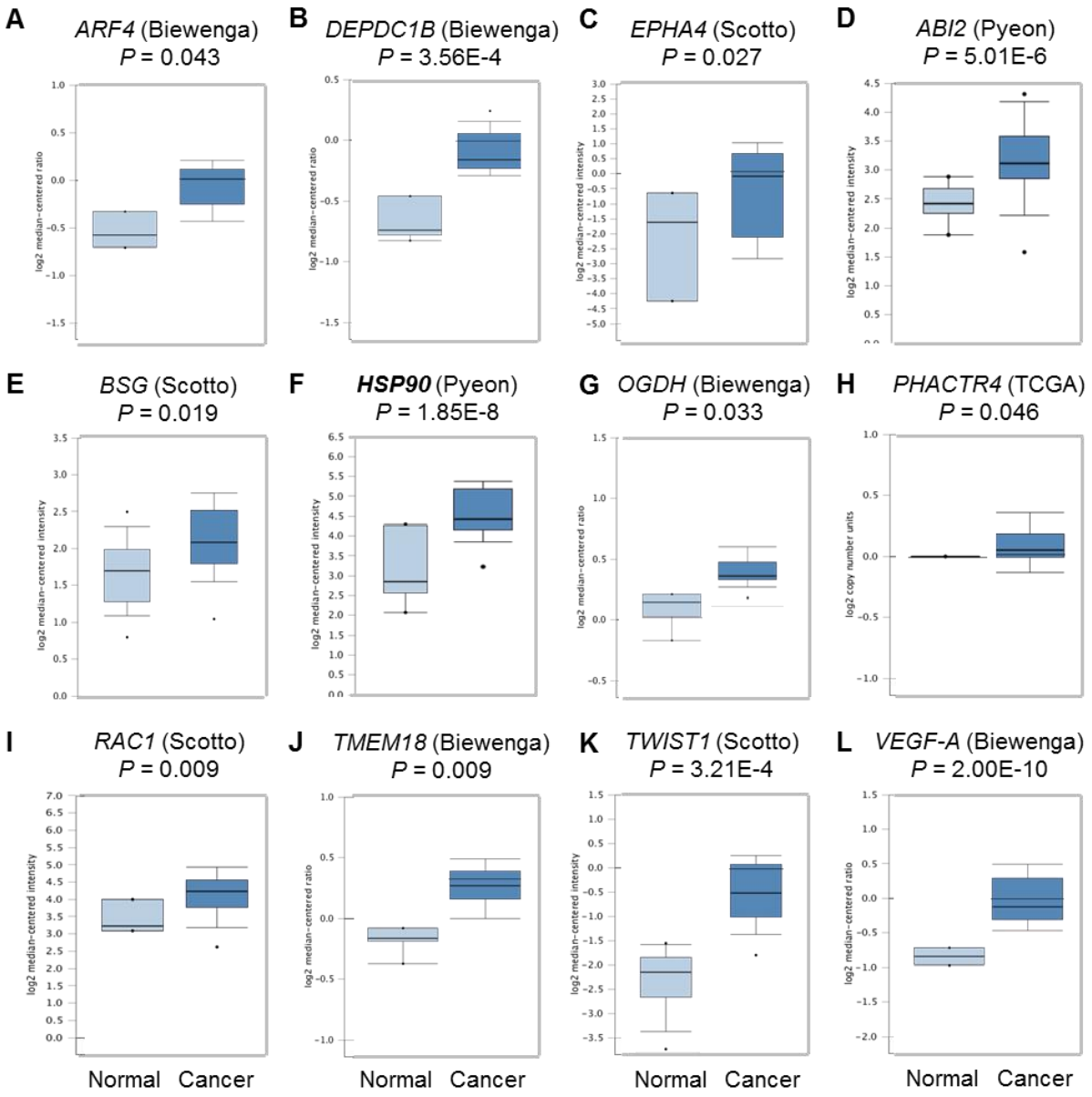


Figure 20: The expression of the predicted miR-361 target genes in CC tissues and normal cervical tissues.

Using the Oncomine database, we investigated the expression of the predicted miR-361 target genes in human cervical cancer and normal cervical tissues. 12 of these 16 genes (including (A) *ARF4*, (B) *DEPDC1B*, (C) *EPHA4*, (D) *ABI2*, (E) *BSG*, (F) *HSP90*, (G) *OGDH*, (H) *PHACTR4*, (I) *RAC1*, (J) *TMEM18*, (K) *Twist1*, and (L) *VEGF-A*) were elevated considerably in CC tissues as compared to normal tissues.

We further performed Kaplan-Meier analysis according to the mRNA expression of these genes using the KM Plotter database. Consistent with a previous study showing that *VEGF-A* was an indicator of poor survival in patients with cervical cancer (Zhang et al., 2017a), high *VEGF-A* expression negatively correlated with overall survival (Figure 21). Our results also demonstrated that increased expression of *ARF4*, *BSG*, and *HSP90* was significantly associated with shorter overall survival time in CC patients (Figure 21), suggesting that these genes might act as oncogenes in cervical cancer, and serve as potential biomarkers for predicting the prognosis of CC patients.

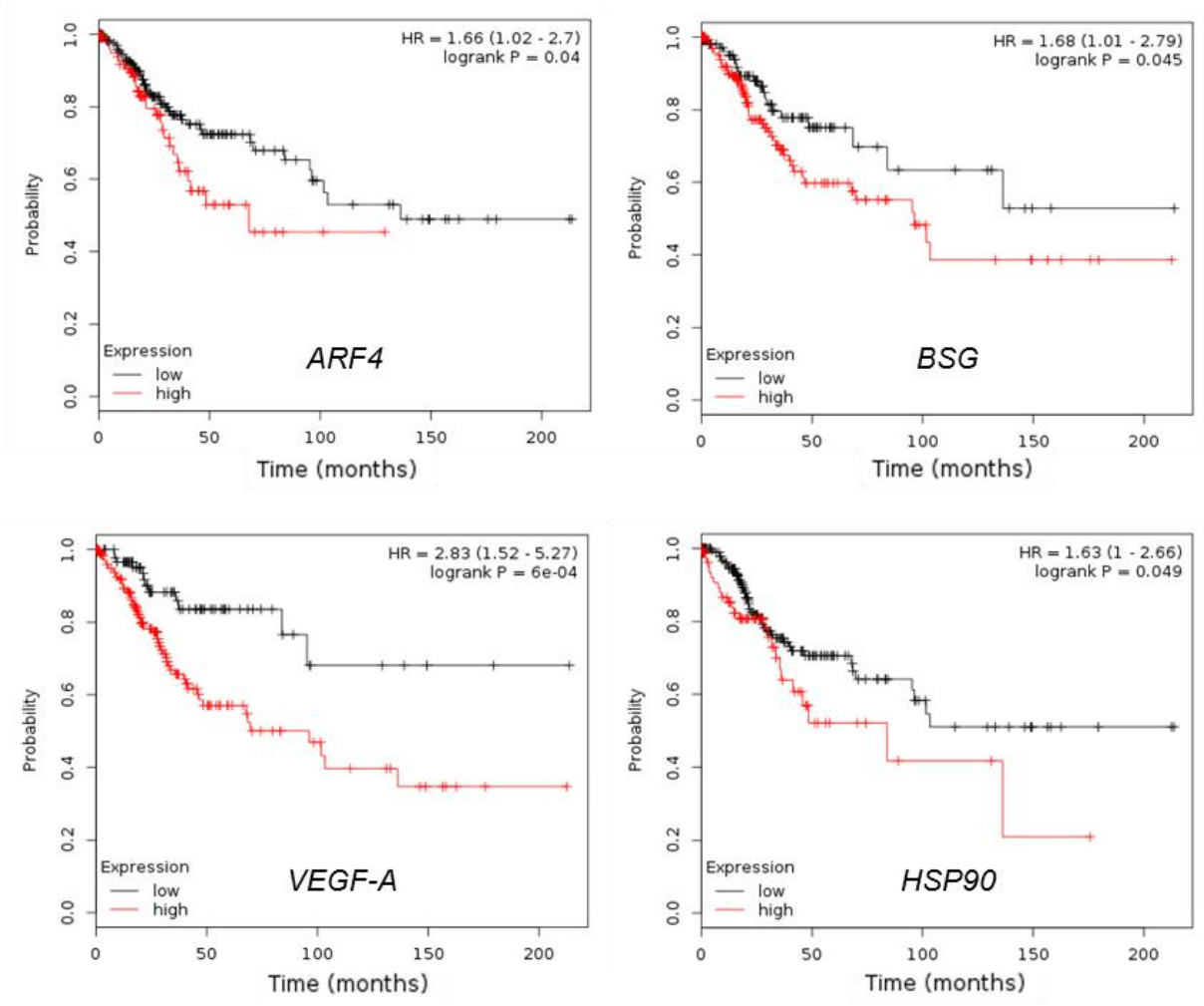


Figure 21: The prognostic value of *ARF4*, *BSG*, *VEGF-A*, and *HSP90* expression in cervical cancer.

Using the KM Plotter database, we explored the prognostic value of *ARF4*, *BSG*, *VEGF-A*, and *HSP90*

expression via a Kaplan-Meier analysis based on their mRNA expression.

4. HSP90 is overexpressed in CC tissues

Since *HSP90*, one potential miR-361 target, could modulate the EMT of cancer cells (Chong et al., 2019; Nagaraju et al., 2015), we selected this gene to determine whether miR-361 inhibits EMT phenotypes by targeting HSP90. We first estimated the expression pattern of *HSP90* in TCGA CC tissues and normal samples using the UALCAN web server (Chandrashekar et al., 2017). We found that *HSP90* expression was significantly higher in CC tissues than in normal cervical tissues (Figure 22).

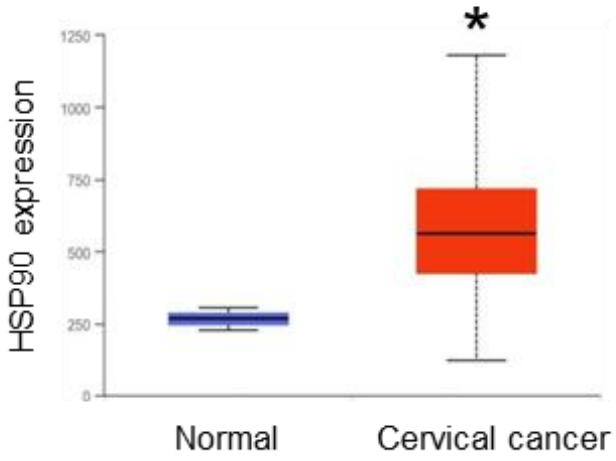


Figure 22: *HSP90* is highly expressed in CC tissues.

The mRNA expression of *HSP90* in CC tissues and normal tissues was investigated using the UALCAN database. *HSP90* expression was significantly higher in CC tissues than in normal cervical tissues. *P<0.05.

After that, the mRNA and protein expression of HSP90 were measured in HeLa and SiHa cells compared with the normal endometrial cell line EM using RT-qPCR and western blotting

analysis, and HSP90 was significantly upregulated in CC cells (Figure 23).

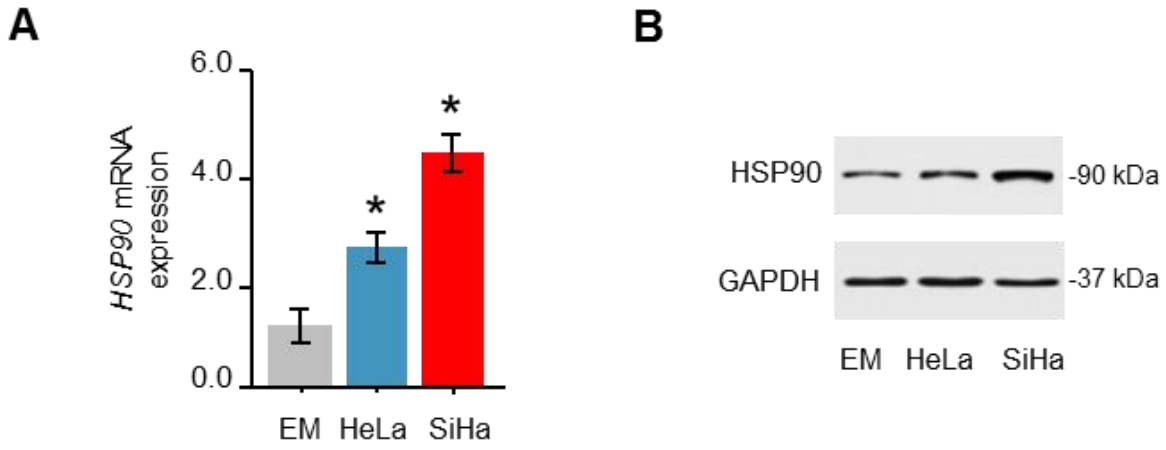


Figure 23: HSP90 expression in a human endometrial cell line (EM) and two human CC cell lines (HeLa and SiHa).

(A) The mRNA of *HSP90* was examined in EM cells and CC cells using RT-qPCR assays. (B) Representative western blotting images for HSP90 and GAPDH protein levels in CC cell lines and normal EM cells. *P<0.05.

To further verify the protein expression of HSP90 in CC tissues, we extracted the immunohistochemical staining images from the Human Protein Atlas (HPA) database (Uhlen et al., 2010). We verified that HSP90 expression in CC tissues was increased compared with adjacent normal cervical tissues (Figure 24).

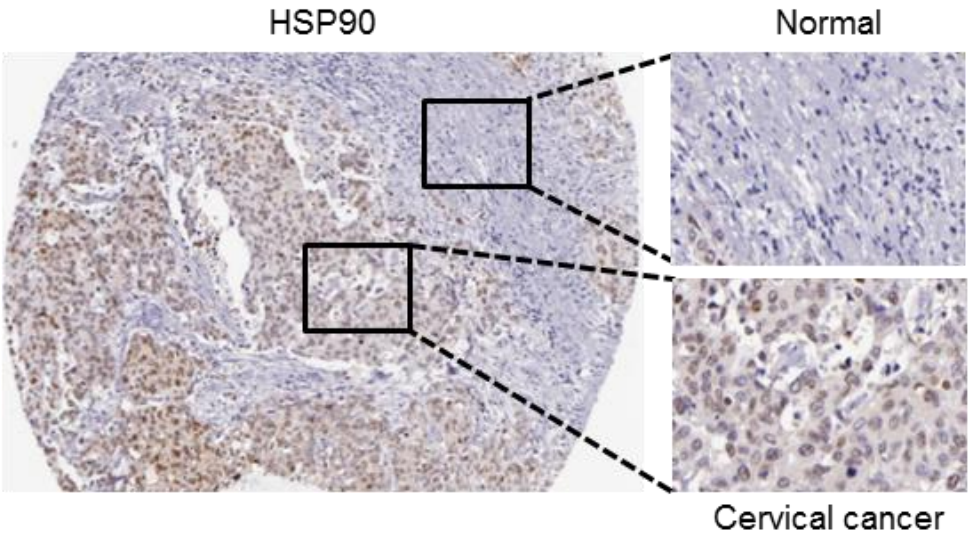


Figure 24: HSP90 protein expression in CC tissues and adjacent normal tissues.

We extracted the immunohistochemical staining images from the HPA database. The protein expression of HSP90 was upregulated in CC tissues compared with adjacent normal tissues.

Based on the data from the HPA database, CC patients with higher *HSP90* expression had worse overall survival than those with lower *HSP90* expression (Figure 25). Overall, these findings support the relevance of HSP90 for CC carcinogenesis and progression.

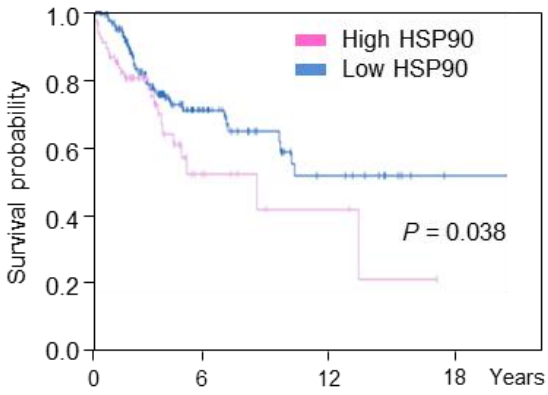


Figure 25: The prognostic value of HSP90 expression in CC patients.

The impact of *HSP90* mRNA expression on the survival of CC patients was investigated using the HPA

database. Those CC patients with higher *HSP90* expression had worse overall survival than those with lower *HSP90* expression.

5. MiR-361 binds to *HSP90* and suppresses its expression

Since the 3′-UTR of *HSP90* mRNA contains a sequence complementary to the seed regions of miR-361 (Figure 26A), we examined whether miR-361 regulates HSP90 expression in CC cells. Ectopic expression of miR-361 decreased, while inhibition of miR-361 increased the mRNA and protein expression of HSP90 in CC cells (Figure 26B, 26C, 26D). To determine whether *HSP90* is a direct target of miR-361, the pGL3 luciferase reporter vector containing the WT *HSP90* 3′-UTR or the MUT *HSP90* 3′-UTR was transfected into CC cells, with miR-361 mimic, miR-361 inhibitor, or the respective negative controls, respectively. Luciferase reporter assays showed that overexpression of miR-361 significantly decreased the luciferase activity of the WT *HSP90* 3′-UTR, but not the luciferase activity of the MUT *HSP90* 3′-UTR in SiHa cells (Figure 26E). Moreover, we found that transfection with miR-361 inhibitor markedly induced luciferase activity of the WT *HSP90* 3′-UTR in HeLa cells (Figure 26F). However, no obvious induction of luciferase activity was observed in HeLa cells transfected with miR-361 inhibitor and the luciferase reporter vector harboring the MUT *HSP90* 3′-UTR (Figure 26F). These results provided evidence that miR-361 directly reduces HSP90 expression in CC cells.

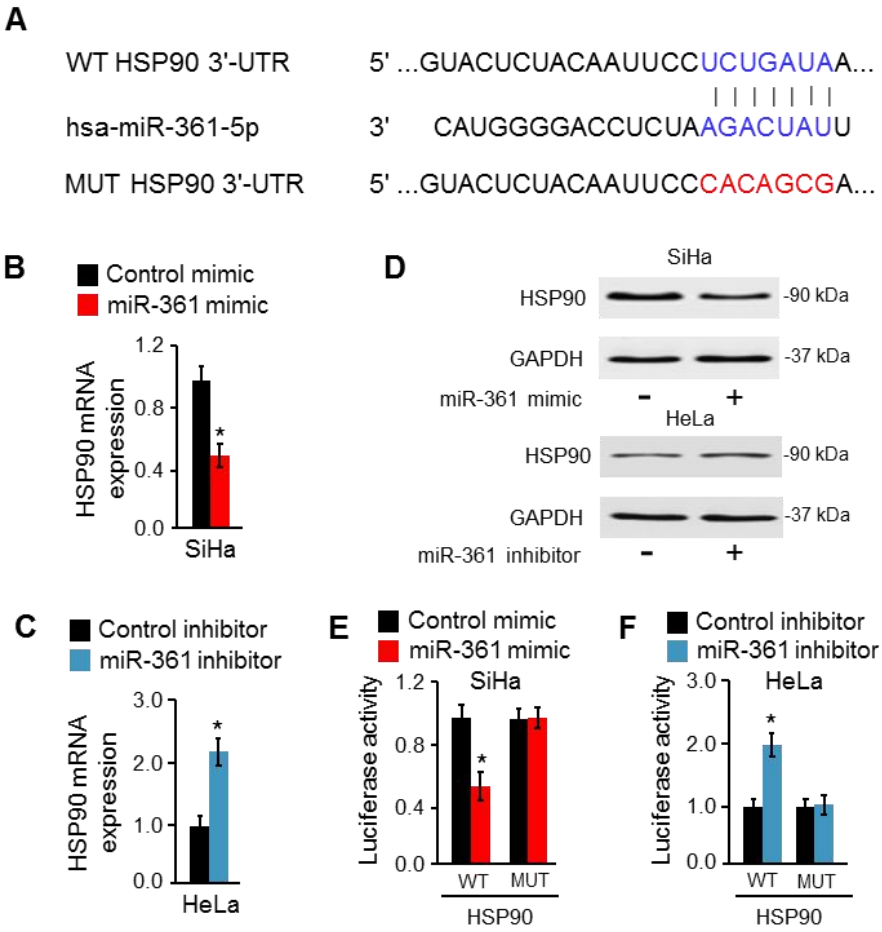


Figure 26: MiR-361 binds to *HSP90* mRNA and suppresses its expression.

(A) Illustration of the predicted binding site between miR-361 and *HSP90* mRNA, with mutant sites as shown in red. (B) The mRNA expression of *HSP90* in SiHa cells transfected with miR-361 mimic or control mimic. (C) The mRNA level of *HSP90* in HeLa cells transfected with miR-361 inhibitor and control inhibitor. (D) Western blotting analysis of HSP90 and GAPDH expression in CC cells transfected as indicated. (E) Luciferase assays in SiHa cells transfected with WT or MUT *HSP90* 3′-UTR, together with miR-361 mimic or control mimic. (F) Luciferase assays in HeLa cells transfected with WT or MUT *HSP90* 3′-UTR, together with miR-361 inhibitor or control inhibitor. *P<0.05.

6. HSP90 enhances EMT and sphere formation ability in CC cells

We then examined whether HSP90 induces EMT properties in CC cells by observing cell

morphology. As shown in Figure 27A and 27B, SiHa cells exhibited morphological changes from a spindle shape to a cobblestone-like shape upon knockdown of HSP90. On the other hand, the overexpression of HSP90 in HeLa cells altered the epithelial-looking morphology resulting in mesenchymal-like cells and triggering cell scattering (Figure 27A, 27B). We also found that knockdown of HSP90 significantly inhibited the ability of SiHa cells to invade, and forced expression of HSP90 promoted the invasion of HeLa cells (Figure 27C). Since EMT and stem cell properties are interconnected, we further assessed the impact of HSP90 expression on cancer stem cell properties by performing sphere formation assays. As expected, HSP90-depleted SiHa cells partially lost the ability to form spheres compared with control cells, and the overexpression of HSP90 in HeLa cells induced sphere formation by approximately 2-fold (Figure 27D).

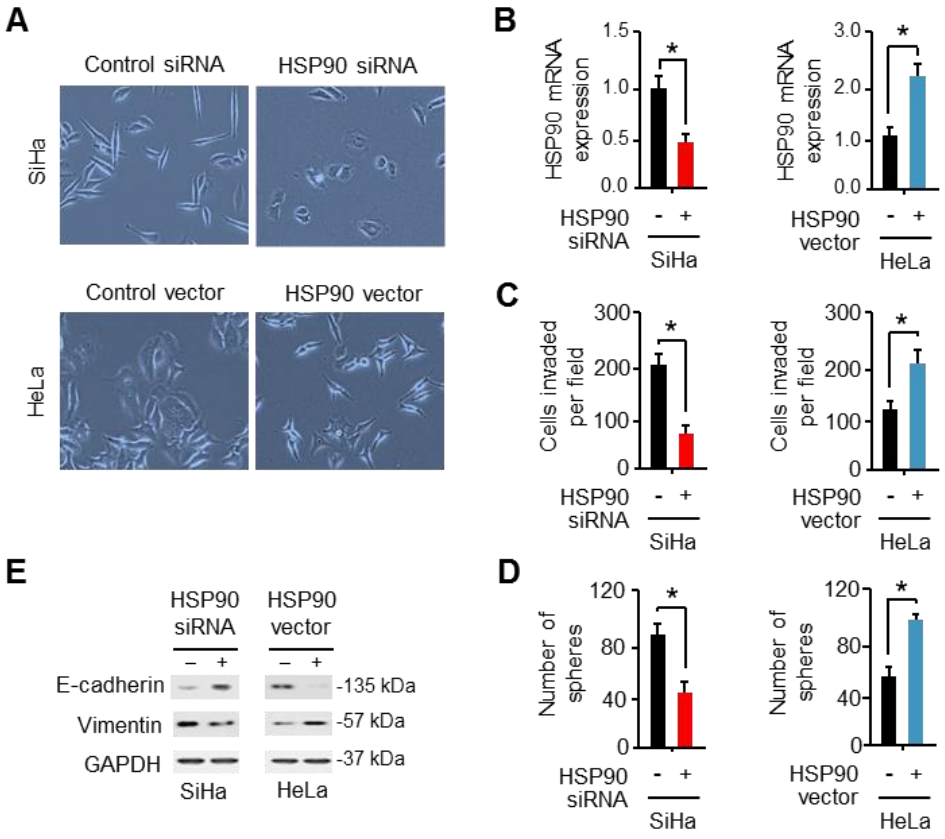


Figure 27: HSP90 enhances EMT and sphere formation in CC cells.

(A) The morphology of SiHa and HeLa cells transfected as indicated is shown. (B) RT-qPCR analysis of

HSP90 mRNA expression was examined in SiHa and HeLa cells transfected as indicated. (C, D) The impact of HSP90 expression on cell invasion (C) and sphere formation (D) was assessed using the Matrigel invasion assay and sphere formation assays. (E) Representative western blot images for E-cadherin, Vimentin, and GAPDH protein levels in CC cells after overexpression or knockdown of HSP90. *P<0.05.

Our RT-qPCR analysis confirmed that knockdown of HSP90 in SiHa cells significantly reduced the expression of mesenchymal marker *Vimentin* and the levels of *Twist1*, *MCL-1*, *OCT-4*, *SOX-2*, and *MDR-1*, but increased the expression of epithelial markers (*E-cadherin* and *ZO-1*) (Figure 17B). However, HSP90 overexpression in HeLa cells has the opposite effect on these genes (Figure 17B). As shown in Figure 27E, the silencing of HSP90 enhanced the protein expression of E-cadherin and reduced the protein expression of Vimentin, while overexpression of HSP90 decreased E-cadherin expression and increased Vimentin expression. Collectively, these findings indicated that HSP90 plays a critical role in promoting EMT and sphere formation in CC cells.

7. MiR-361 suppresses EMT and sphere formation in CC cells by inhibiting HSP90 expression

To examine the possibility that HSP90 is essential for miR-361-suppressed EMT and sphere formation in CC cells, we transfected SiHa cells with miR-361 mimic or control mimic, together with an HSP90 expression vector or control vector (Figure 28A). The overexpression of HSP90 largely abolished the inhibitory effects of miR-361 on SiHa cell invasion and sphere formation (Figure 28B, 28C). To better characterize whether HSP90 expression is required for miR-361 inhibitor-induced HeLa cell invasion and sphere formation, we co-transfected HeLa cells with or without miR-361 inhibitor, together with HSP90 siRNA or control siRNA (Figure

28A). Indeed, we found that the promotion of cell invasion and sphere formation by knockdown of miR-361 could be suppressed by the inhibition of HSP90 expression (Figure 28B, 28C). These results suggested that decreased HSP90 expression mediates the tumor-suppressive effects exerted by miR-361.

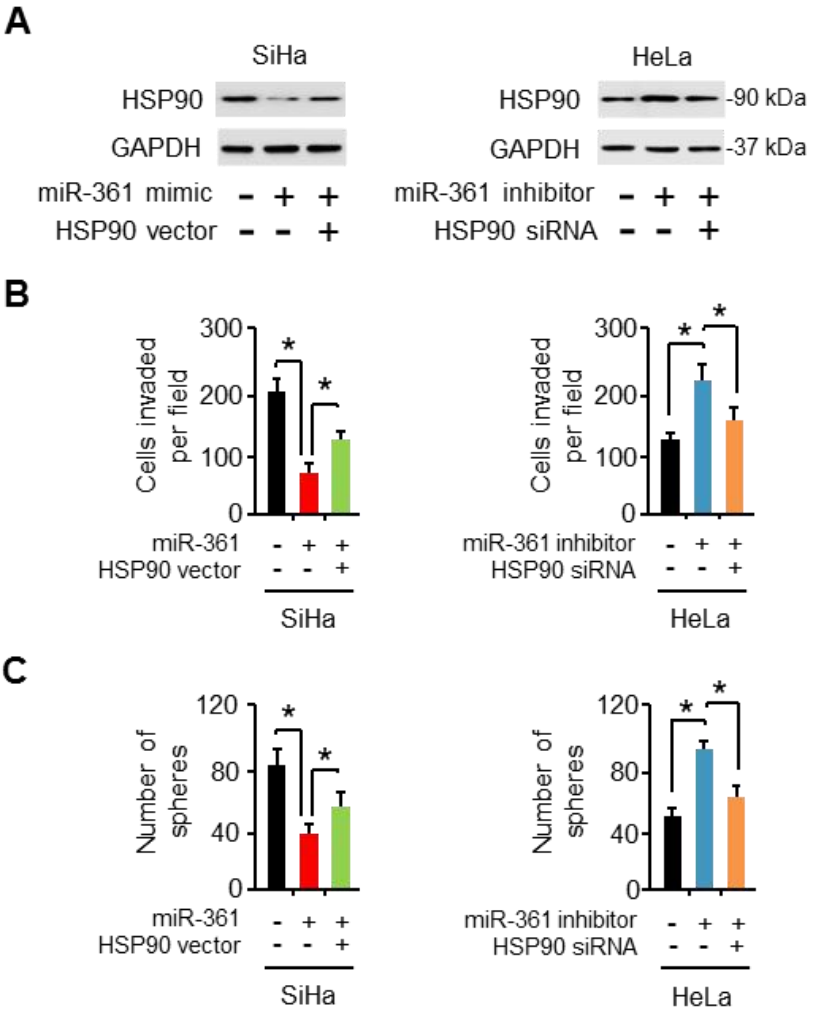


Figure 28: MiR-361 suppresses EMT and sphere formation in CC cells by inhibiting HSP90 expression.

(A) The protein levels of HSP90 and GAPDH in SiHa cells transfected with miR-361 mimic (or control mimic), together with (or without) HSP90 vector, and in HeLa cells transfected with miR-361 inhibitor (or control inhibitor), together with (or without) HSP90 siRNA. (B, C) Cell invasion (B) and sphere formation (C) capacity were detected in CC cells transfected as indicated. *P<0.05.

8. NEAT1-mediated miR-361 downregulation contributes to EMT and sphere formation of CC cells by increasing HSP90 expression

Recent studies have suggested that lncRNAs can regulate gene expression by competitively binding to miRNAs during cancer progression (Dong et al., 2019a). We have reported that the lncRNA NEAT1 functions as an oncogenic sponge for miR-361 in endometrial cancer (Dong et al., 2019b), raising the possibility that NEAT1 potentially interacts with miR-361 and suppresses its expression in CC cells. To test this possibility, we searched for NEAT1 expression in TCGA datasets from CC tissues and normal cervical tissues using the UALCAN database. The results revealed a significantly higher level of NEAT1 in CC samples compared with normal samples (Figure 29A). We then assessed the association between NEAT1 expression and overall survival of patients with cervical cancer using the online database SurvExpress (Aguirre-Gamboa et al., 2013), where CC patients from the TCGA dataset (n=191) were divided into low-risk and high-risk groups for poor prognosis. As expected, the overall survival of patients with high risk (red line) was significantly shorter than that with low risk (green line) (Figure 29B). As shown in Figure 29C, the expression of NEAT1 in the high-risk group was significantly higher than that in the low-risk group. Additionally, the expression of NEAT1 was measured in CC cell lines compared with the normal cell line EM, and NEAT1 was significantly upregulated in CC cell lines (Figure 29D).

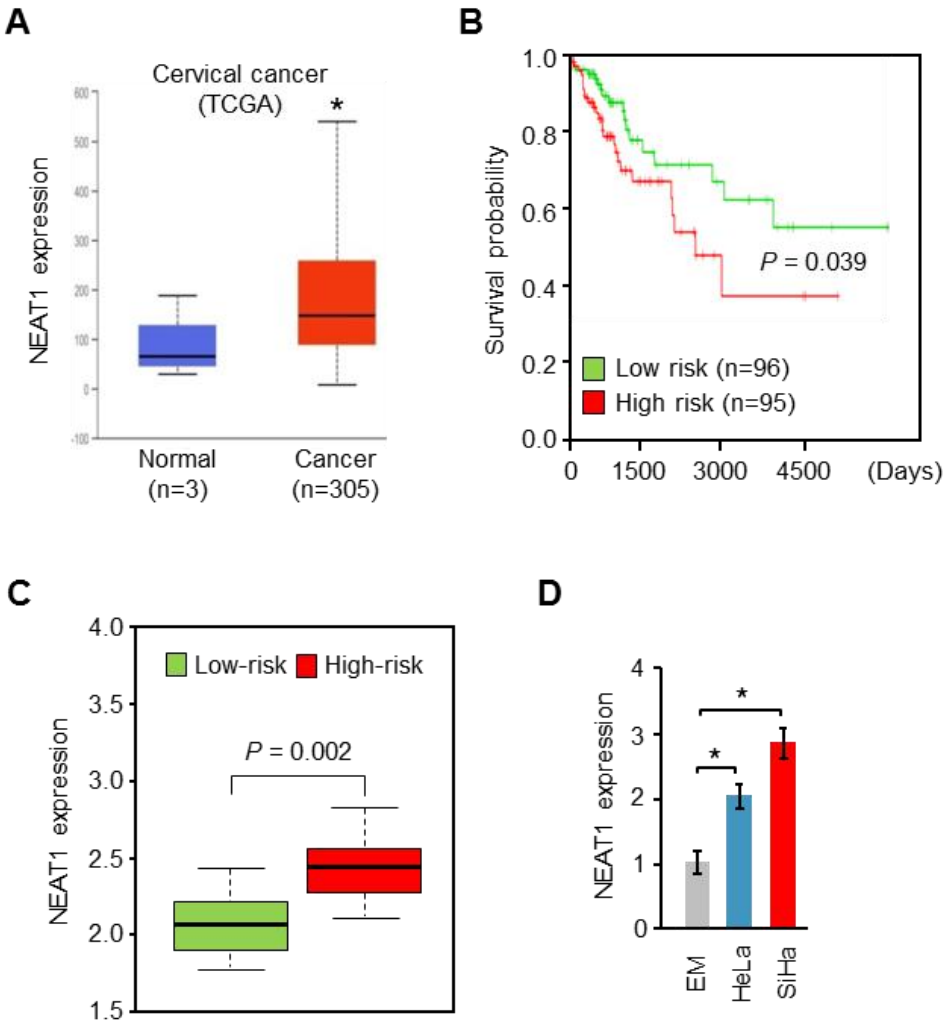


Figure 29: Upregulation of NEAT1 is associated with worse survival of CC patients.

(A) The expression of NEAT1 in CC tissues and normal tissues (UALCAN database). (B) The patients from TCGA cervical cancer dataset in the SurvExpress database were divided into low-risk and high-risk groups and survival differences between the two groups were demonstrated using Kaplan-Meier survival curves. (C) The expression level of NEAT1 in low-risk and high-risk groups. (D) NEAT1 expression was examined in a normal endometrial cell line (EM) and CC cell lines (HeLa and SiHa) using RT-qPCR analysis. *P<0.05.

We examined whether NEAT1 depletion influences miR-361 expression in CC cells using RT-qPCR analysis. As shown in Figure 30A and 30B, the depletion of NEAT1 by siRNA caused the upregulation of miR-361 in CC cells. To verify the direct binding relationship

between NEAT1 and miR-361, we performed a luciferase reporter assay by co-transfecting SiHa cells with luciferase reporter plasmids containing the WT NEAT1 or the MUT NEAT1, along with miR-361 mimic or control mimic. Our results showed that the overexpression of miR-361 significantly decreased the luciferase activity driven by the WT NEAT1, but caused no significant change in the luciferase activity of the MUT NEAT1 (Figure 30C). These results suggested that NEAT1 directly interacts with miR-361 and represses its expression in CC cells.

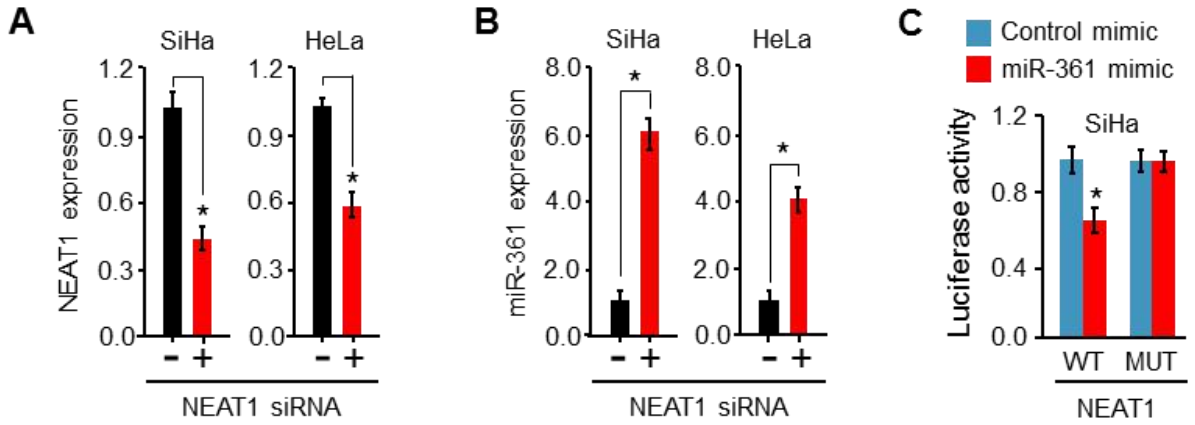


Figure 30: NEAT1 directly interacts with miR-361 and represses its expression in CC cells.

(A, B) NEAT1 (A) and miR-361 (B) expression were examined in SiHa and HeLa cells transfected with NEAT1 siRNA or control siRNA. (C) Luciferase activities were measured in SiHa cells co-transfected with the reporter vectors (WT or MUT NEAT1), together with (or without) miR-361 mimic. *P<0.05.

We further examined the effects of NEAT1 depletion on the protein expression of HSP90 and found that the silencing of NEAT1 greatly suppressed the expression of HSP90 in CC cells (Figure 31). Furthermore, western blotting analysis showed that knockdown of NEAT1 markedly increased the level of E-cadherin, but decreased the expression of Vimentin (Figure 31).

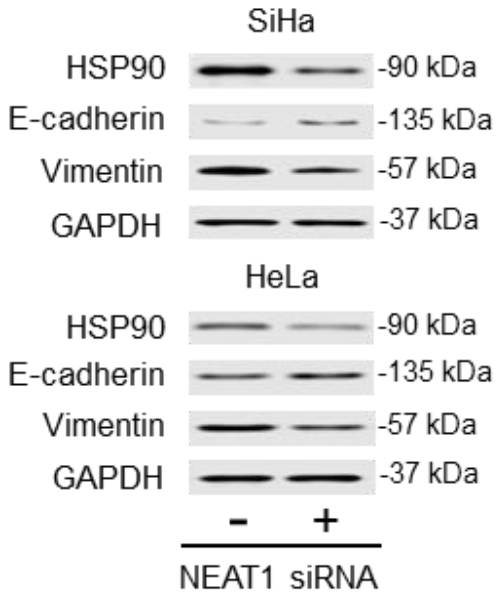


Figure 31: The protein expression of HSP90, E-cadherin and Vimentin in CC cells transfected with NEAT1 siRNA or control siRNA.

Western blotting analysis was used to measure the protein levels of HSP90, E-cadherin, Vimentin, and GAPDH in SiHa and HeLa cells transfected with NEAT1 siRNA or control siRNA.

Through cell invasion and sphere formation experiments, we found that NEAT1 knockdown led to decreased cell invasion and sphere formation (Figure 32). Taken together, these findings supported the notion that NEAT1 promotes CC invasion and sphere formation through up-regulating HSP90 expression via binding with miR-361, a tumor suppressor that directly suppresses HSP90 expression.

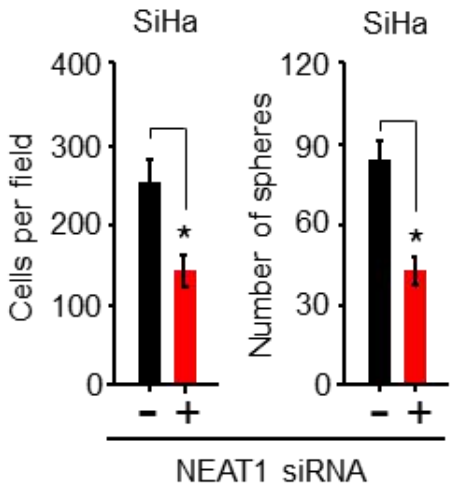


Figure 32: The effects of NEAT1 silencing on the invasion and sphere formation of SiHa cells.

Cell invasion and sphere formation capacity were detected in SiHa cells transfected with NEAT1 siRNA or control siRNA. *P<0.05.

CHAPTER 1: Discussion

Previous studies illustrating the important functions of miR-361 in the suppression of various malignant properties have established its widespread role in tumorigenesis and metastasis (Xu et al., 2019). However, its ability to mediate CC initiation and progression is still controversial (Wu et al., 2013; Gao et al., 2019). Consistent with an early report that has revealed that the overexpression of miR-361 in SiHa and HeLa cells has anti-proliferative effects by repressing the expression of its target gene *FOXM1* (Gao et al., 2019), in this study, our results suggested a role for miR-361 as a key suppressor in CC cell proliferation (Figure 33). Beyond that, our data showed that miR-361 serves as a novel regulator of EMT properties by inhibiting HSP90 in CC cells, thereby contributing to its tumor suppressor function (Figure 33).

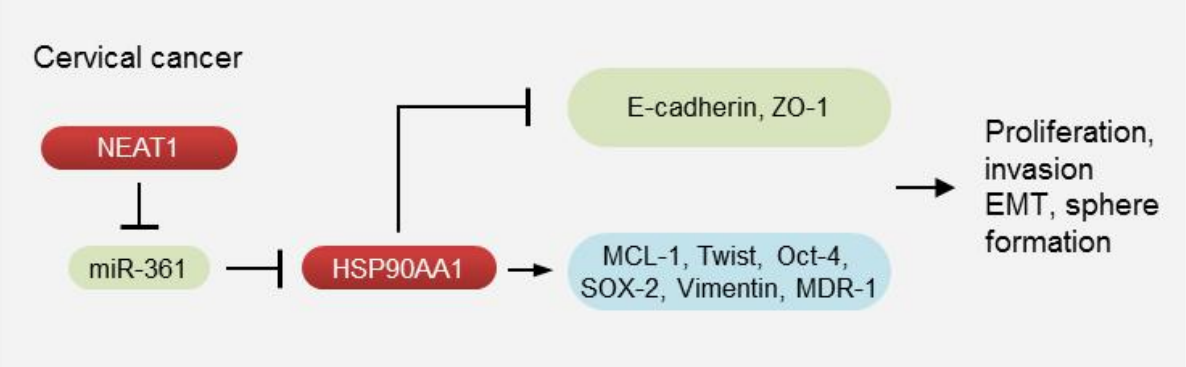


Figure 33: A schematic illustration of our findings.

This scheme illustrates our findings that the NEAT1/miR-361/HSP90 pathway regulates EMT and cancer stem cell properties in CC cells.

Recent and significant progress has been achieved in terms of the cellular functions of miRNA and their potential application in cancer diagnosis and targeted treatment (He et al., 2020). In tumor cells, the major functions of miRNAs are to regulate the expression of their

downstream target genes (Van Roosbroeck and Calin, 2017; Dong et al., 2018a; Wienholds and Plasterk, 2005). The expression of numerous miRNAs is known to be dysregulated in various cancers, including cervical cancer (Dong et al., 2011). A previous study has shown that the expression of miR-152 was extremely low in healthy control tissues, but its expression was higher in cervical intraepithelial neoplasia (CIN) tissues, and the highest in CC tissues (Yang and Zhang, 2019). A receiver operating characteristic (ROC) analysis revealed that miR-152 could preferentially distinguish CIN and CC patients from healthy controls (Yang and Zhang, 2019). These results suggested that miR-152 may be used as an early diagnostic biomarker in patients with cervical intraepithelial neoplasia and patients with cervical cancer.

More importantly, miRNAs can be released from cells to the extracellular environment, and a larger number of miRNAs have been identified in the various fluids of the human body, such as blood, urine, saliva, peritoneal fluid, amniotic fluid, cerebrospinal fluid, and tears (Condrat et al., 2020). A six-miRNA signature (including miR-21-5p, miR-155-5p, miR-199a-5p, miR-145-5p, miR-218-5p, and miR-34a-5p) in urine samples was reported to be valuable for early-stage CC detection and yielded high sensitivity and high specificity in distinguishing cervical cancer from pre-malignant lesions and healthy controls (Aftab et al., 2021). Therefore, we should investigate whether the expression of miR-361 in tissues and body fluids could serve as a sensitive diagnostic biomarker for cervical cancer.

The association between miRNA expression and CC prognosis has been reported, and many miRNAs can predict the clinical outcome in cervical cancer (Qi et al., 2020). In this study, our meta-analysis has confirmed that higher expression of miR-361 is correlated with a favorable prognosis in patients with cervical cancer. Further research would be needed to define the prognostic value of miR-361 in CC tissues.

It has been previously shown that miR-361 appears to be a molecular hub that participates in the control of multiple cancer cell signaling networks (Xu et al., 2019). Several reports have suggested that miR-361 plays a critical role in repressing tumor progression by targeting multiple components of many essential signaling pathways implicated in tumor growth, EMT, metastasis, drug resistance, glycolysis, angiogenesis, and inflammation, such as the PTEN/PI3K/AKT, Wnt/β-catenin, EMT/cancer stem cell-related pathways and VEGF pathway (Xu et al., 2019). These data, together with our findings, point to the complexity of miR-361-regulated signaling pathways that determine the phenotypes of human tumor cells. The detailed role of miR-361 in cervical cancer and molecular mechanisms underlying its action remains to be further clarified. In addition, the effectiveness of most conventional therapeutics is limited in cancer cells that exhibit stem cell-like features and EMT-like properties (Shibue and Weinberg, 2017). When overexpressed in CC cells, miR-361 significantly suppresses the EMT and sphere formation ability of CC cells, raising the possibility that miR-361 might be used as one of the future therapeutic agents that overcome chemoresistance, leading to an improved outcome of this disease.

Heat shock proteins, especially HSP90, are well known for their critical roles in modulating cell proliferation, apoptosis, migration, invasion, EMT, cancer stem cell features, metastasis, and angiogenesis (Chatterjee and Burns, 2017). HSP90 could function as an EMT-inducer in ovarian cancer, renal cancer, and colon cancer (Chong et al., 2019; Nagaraju et al., 2015). Our computational screening and experimental validation have identified HSP90 as a key downstream target of miR-361 in CC cells. Our study provided new evidence that HSP90 promotes EMT through increasing the expression of *Twist1* and *MCL-1*, which were demonstrated to accelerate the process of EMT and invasion of CC cells (Fan et al., 2015; Zhou et al., 2014). In addition, the expression of two cancer stem cell-related genes (*OCT-4*

and *SOX-2*) was elevated in CC tissues compared with normal tissues (Tulake et al., 2018; Kim et al., 2015), and their expression was significantly upregulated in HSP90-overexpressing CC cells, suggesting that loss of miR-361 expression elevates HSP90 levels, and causally increases the expression of *Twist1*, *MCL-1*, *OCT-4*, and *SOX-2*, leading to the acquisition of EMT phenotypes of CC cells. Since HSP90 represents a potential therapeutic target, various HSP90 inhibitors have been developed (Xiao and Liu, 2020). The HSP90 inhibitor Mycoepoxydiene can inhibit the function of HSP90 and trigger apoptosis in CC cells (Lin et al., 2015). Furthermore, the synergistic activities of HSP90 inhibitors and anti-cancer drugs in CC cells have been confirmed (Hu et al., 2019). Hence, the use of HSP90 inhibitors or the combination of HSP90 with other anti-cancer reagents might be a novel strategy for the treatment of cervical cancer.

In addition to its intracellular localization, HSP90 is also a secreted and cell surface protein. Extracellular HSP90 promotes EMT and cancer cell invasion and stimulates metastatic spread (Hance et al., 2012), and blocking the secreted HSP90 significantly inhibits melanoma metastasis (Wang et al., 2009), indicating the possibility that HSP90 secretion might be involved in CC progression and metastasis. Therefore, the effects of miR-361 and NEAT1 expression on the secretion of HSP90 by CC cells should be explored using the enzyme-linked immunosorbent assay. Whether the secreted HSP90 acts as a pivotal regulator of CC metastasis requires further investigation.

ADP Ribosylation Factor 4 (ARF4), a member of the RAS superfamily, is a small guanine nucleotide-binding protein and overexpression of ARF4 has been observed in human tumors, including ovarian cancer, lung cancer, and glioma (Casalou et al., 2016). ARF4 serves as a positive regulator of breast cancer cell migration (Jang et al., 2012). Another study

demonstrated that *ARF4* is a potential candidate target gene of miR-7 (Webster et al., 2009). In ovarian cancer cells, miR-221-3p was shown to inhibit cancer cell proliferation and migration, and *ARF4* was identified as a direct target of miR-221-3p (Wu et al., 2018). Further studies are required to reveal the role of ARF4 in CC progression and the mechanisms that regulate the expression of ARF4 in CC cells.

Basigin (BSG), also known as CD147 and EMMPRIN, is a glycosylated transmembrane protein that is overexpressed in cervical cancer (Xu et al., 2014). Furthermore, increased BSG expression was significantly correlated with poor survival in patients with cervical cancer (Huang et al., 2014). BSG was thought to enhance tumor metastasis, angiogenesis, and drug resistance through its association with various proteins (Xin et al., 2016). BSG was believed to interact with surrounding cells to increase the production of proteolytic enzymes (such as MMP-1, MMP-2, and MMP-9), thereby facilitating the destruction of the extracellular matrix (Gabison et al., 2005). In addition to extracellular matrix degradation, BSG also contributes to cancer angiogenesis by stimulating the secretion of VEGF in both tumor cells and endothelial cells (Tang et al., 2005). In previous research, the protein expression of BSG in CC tissues was found to be significantly higher than that in normal cervical epithelial tissues, and overexpression of BSG promoted the growth of SiHa cells (Wu et al., 2011). These results indicated that elevated expression of BSG may facilitate CC progression by enhancing cancer cell proliferation. Clarification of the pathological functions and molecular mechanisms of BSG will help achieve a better understanding of human CC development.

Our findings were consistent with these previous results (Casalou et al., 2016; Jang et al., 2012; Webster et al., 2009; Wu et al., 2018; Xu et al., 2014; Huang et al., 2014; Xin et al., 2016; Gabison et al., 2005; Tang et al., 2005; Wu et al., 2011) and showed that increased mRNA

levels of *ARF4* and *BSG* were abnormally overexpressed in CC samples and their expression correlated with poorer patient prognosis. More importantly, our study suggested the possibility that miR-361 inhibits CC growth and metastasis by simultaneously targeting ARF4 and BSG. Further investigations were needed to explore the functional link between miR-361 and ARF4/BSG in CC cells.

The dysregulation of lncRNA NEAT1 exerts its oncogenic functions in the majority of human cancers (Pisani and Baron, 2020). NEAT1 was reported to be upregulated in CC tissues compared to adjacent normal tissues, and enhanced NEAT1 expression was significantly correlated with larger tumor size, poor differentiation, progressed FIGO stage, lymph node metastasis, and decreased survival rate, thus it represents as a prognostic marker for cervical cancer (Wang et al., 2018b). Similarly, the expression of NEAT1 was increased in CC samples compared with normal samples, and the patients expressing high NEAT1 levels showed a shorter survival time (Wang and Zhu, 2018a). Recent mechanism experiments revealed that knockdown of NEAT1 inhibited CC development through repressing cell proliferation, migration, and invasion and also inducing cell apoptosis by regulating the miR-133a/SOX4 pathway (Yuan et al., 2019). Also, NEAT1 functioned as a molecular sponge for miR-9-5p to promote the proliferation and migration of CC cells (Xie et al., 2019). It has been also clarified that knocking down the expression of NEAT1 in CC cells resulted in the repression of cell proliferation and invasion via the PI3K/AKT signaling pathway (Guo et al., 2018). However, the study on the impact of NEAT1 on the EMT program and the self-renewal capacity of CC cells is limited. Here, we demonstrated that, by competitively binding to miR-361 and suppressing its expression, NEAT1 indirectly upregulates the expression of the EMT inducer HSP90, thereby promoting EMT, invasion, and sphere formation of CC cells. Future investigation will be devoted to determining the mechanism by which NEAT1 performs this

function in cervical cancer (especially the cross-talk between NEAT1 and other tumor suppressor miRNAs or the components of polycomb repressive complex 2).

Exosomes are extracellular vesicles that have a diameter of 30-150 nm (Doyle and Wang, 2019). Recent advances in exosome research have demonstrated that exosomes are secreted by cancer cells to the extracellular microenvironment, thereby facilitating the transmission of diverse molecules (such as mRNA, miRNA, and lncRNAs) to the surrounding cells (Pathania and Challagundla, 2020). Human bone mesenchymal stem cells (hBMSCs) secrete miR-361 in exosomes that alleviate chondrocyte damage and inhibit the NF-κB signaling pathway via targeting DDX20 (Tao et al., 2021). Moreover, exosomes isolated from prostate cancer cells could be transferred into hBMSCs, where NEAT1 induces the osteogenic differentiation of hBMSCs through the upregulation of RUNX2 by competitively binding to miR-205-5p (Mo et al., 2021). Therefore, the role of exosomal miR-361 or NEAT1 in the crosstalk between CC cells and the surrounding tumor microenvironment, and their clinical significance in CC diagnosis, prognosis prediction, and targeted therapy, should be investigated in our future research.

Since miRNAs and lncRNAs have diverse functional implications in human cancer, therapeutic targeting of miRNAs and lncRNAs would be a promising approach for the treatment of human cancers (Winkle et al., 2021). The re-introduction of tumor suppressor miRNAs with chemically synthetic miRNA mimics is expected to restore the functions of these miRNAs (Bader et al., 2011). For those miRNAs that promote the malignant phenotypes of cancer cells, the oncogenic activities of these miRNAs can be suppressed with antisense oligonucleotides (Li et al., 2017). LncRNAs can be targeted by multiple strategies, including modulation of the transcription of certain lncRNAs (Arun et al., 2018), targeting of the

post-transcriptional lncRNA degradation pathways (Li and Chen, 2013), the use of antisense oligonucleotides (Li et al., 2020), and the genomic editing of lncRNA genomic loci by the CRISPR-Cas9 and CRISPR-Cas13 system (Zhen and Li, 2019; Palaz et al., 2021). Therefore, there is an urgent need for developing efficient NEAT1-targeted therapy for CC therapeutics.

CHAPTER 2

Study on the role of MEG3/miR-216a/PD-L1 pathway implicated in epithelial-mesenchymal transition of aggressive endometrial cancer

CHAPTER 2: Introduction

Endometrial cancer is a kind of cancer that develops from the inner lining of the uterine cavity (Figure 34), and it is the most frequent gynecologic cancer in developed nations, accounting for 89,929 deaths in 2018 worldwide (Bray et al., 2018). Endometrial cancer is divided into two types: type I and type II (Gaber et al., 2016). Type I endometrial cancers are well-differentiated endometrioid endometrial cancers that tend to have a more favorable prognosis (Gaber et al., 2016). Endometrioid endometrial cancers account for approvingly 70% to 80% of newly diagnosed endometrial cancers (Gaber et al., 2016). These diseases are linked to persistent estrogen exposure and are frequently preceded by complex atypical hyperplasia (Gaber et al., 2016). Despite being a frequent malignancy, the mortality rate for type I endometrial cancers is relatively low, owing primarily to the early identification of this disease (Bray et al., 2018; Gaber et al., 2016).

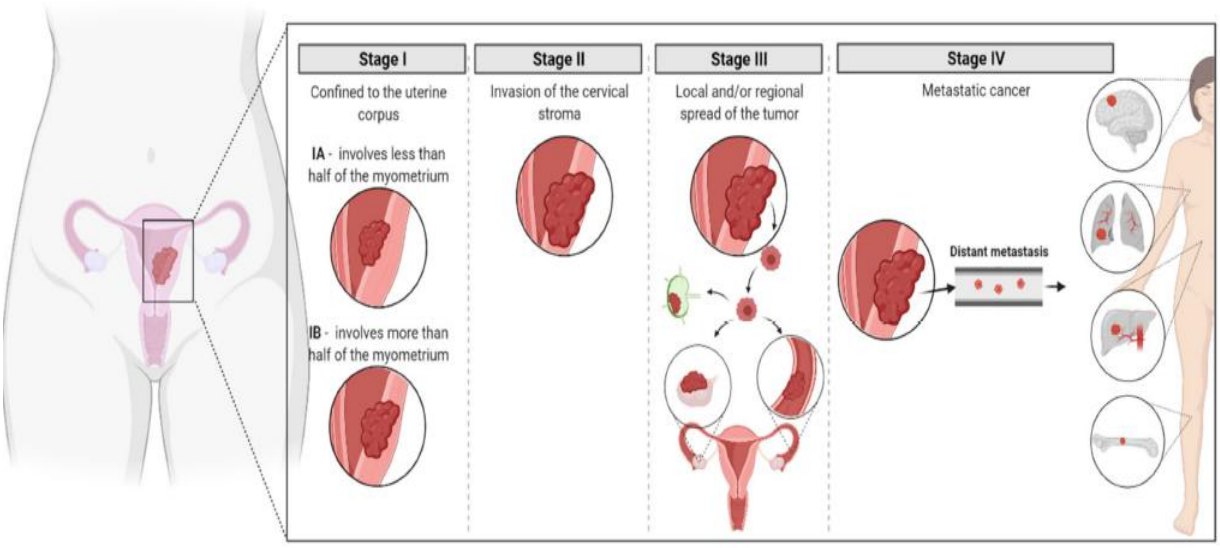


Figure 34: Endometrial cancer (Klicka K, et al. Cancers (Basel). 2021).

Endometrial cancer begins in the lining (endometrium) of the uterus. The FIGO staging and histological grading of endometrial cancer was shown.

In contrast, type II endometrial cancers, also known as non-endometrioid tumors, account for the remaining 10–20% of endometrial cancers (Samarnthai et al., 2010). The two major histologic subtypes of type II endometrial cancers are uterine papillary serous carcinoma and clear-cell carcinoma (Samarnthai et al., 2010). Both malignancies appear to arise from the atrophic endometrium (Samarnthai et al., 2010). Type II endometrial cancers are more aggressive, more common in older patients, and have a high rate of recurrence and metastasis (Gaber et al., 2016; Samarnthai et al., 2010).

It has been established that the molecular genetic changes involved in the development of type I endometrial cancers vary from those involved in the development of type II endometrial cancers (Figure 35). (Samarnthai et al., 2010). Recent molecular studies have shown that type I cancers have a greater prevalence of phosphatase and tensin homolog (*PTEN*) loss of function mutations, activation of the PI3K/AKT/mTOR pathway, and microsatellite instability (Samarnthai et al., 2010). First, *PTEN* is a tumor suppressor gene that is located on chromosome 10q23.3. It encodes a lipid phosphatase that inhibits the PI3K/AKT pathway (Samarnthai et al., 2010). Reduced *PTEN* activity promotes cell proliferation and survival via modulating signal transduction pathways (Samarnthai et al., 2010). *PTEN* can be inactivated by a variety of mechanisms, including gene mutation, loss of heterozygosity, and promoter hypermethylation (Samarnthai et al., 2010). Somatic *PTEN* mutations are prevalent in endometrial cancers and are almost exclusively seen in endometrioid endometrial carcinomas, accounting for up to 83% of endometrial cancers (Samarnthai et al., 2010). *PTEN* mutations were found in 15-55% of endometrial hyperplasia tissues with and without atypia, suggesting that *PTEN* mutations are early events in the development of type I endometrial cancers.

The most significant genetic changes in type II endometrial cancer appear to be *p53*

mutations and the overexpression of erb-b2 receptor tyrosine kinase 2 (*HER2*) (Samarnthai et al., 2010). The tumor suppressor gene *p53* is located on chromosome 17p13.1. p53 mutations are found in 90% of non-endometrioid endometrial cancers, while they are only found in 10-20% of endometrioid endometrial cancers, the majority of which are high-grade tumors (Samarnthai et al., 2010). In normal cells, p53 protein is rapidly degraded and thus cannot be detected by immunostaining. However, *p53* mutations in tumor cells often produce a non-functional protein that resists degradation and can be visualized by immunostaining (Samarnthai et al., 2010). Overexpression of the p53 protein is linked to a high histological grade, advanced stage, and a poor prognosis in endometrial cancer (Samarnthai et al., 2010). Endometrial intraepithelial carcinoma is considered as the putative precursor lesion to serous endometrial cancers, and p53 mutations are also detected in 75-80% of endometrial intraepithelial carcinoma (Samarnthai et al., 2010). It is hypothesized that *p53* mutation occurs early in the formation of serous endometrial cancer. *HER2* is located at the long arm of human chromosome 17q12, and codes for a transmembrane receptor tyrosine kinase involved in cell signaling (Samarnthai et al., 2010). *HER2* overexpression or amplification is more common in non-endometrioid endometrial cancers (18-80%) than in grade 2 and 3 endometrioid endometrial cancers (10-30%) (Samarnthai et al., 2010) and has been associated with advanced stage, high histologic grade, and poor overall survival in EC patients (Samarnthai et al., 2010).

Upregulation of epidermal growth factor receptor (*EGFR*) and vascular endothelial growth factor A (*VEGF-A*) is implicated in both type I endometrial cancer and type II endometrial cancer (Samarnthai et al., 2010). EGFR overexpression is found in 60-80% of endometrial cancers (Valastyan and Weinberg, 2011). EGFR overexpression has been reported in high-grade endometrial cancers with deep myometrial invasion, and poor survival (Samarnthai et al., 2010). In addition, VEGF-A expression has been found in 56-100% of

endometrial cancers and has been associated with high histologic grade, deep myometrial invasion, nodal metastasis, and short disease-free survival (Samarnthai et al., 2010).

Cadherins are a kind of adhesion proteins that are required for tight cell connections. E-cadherin is encoded by the *CDH1* gene, which is found on chromosome 16q22.1. It is assumed to be a tumor suppressor gene, and its absence has been connected to tumor invasion and metastasis. Endometrial cancer frequently has decreased E-cadherin expression, which may be caused by loss of heterozygosity or promoter hypermethylation (Samarnthai et al., 2010). Partially or completely lost E-cadherin expression corresponds with aggressive activity in endometrial cancer (Samarnthai et al., 2010).

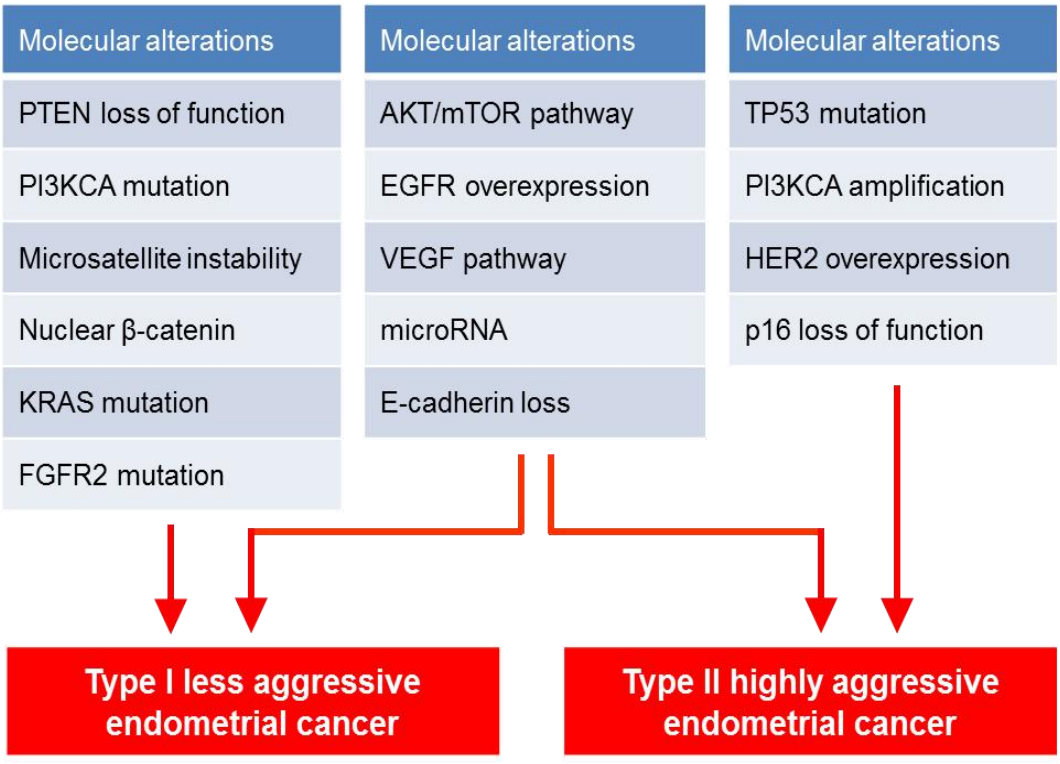


Figure 35: Molecular alterations in endometrial cancer (Samarnthai N, et al. Obstet Gynecol Int. 2010; Dong P, et al. J Transl Med. 2014).

Type I endometrial cancers are less aggressive and have a good prognosis. Type II endometrial cancers are more aggressive and have a worse prognosis. The molecular changes in type I endometrial cancers include

mutations in *PTEN*, *PIK3CA*, *KRAS*, and type II aggressive endometrial cancers are characterized by genetic alterations in *p53* and *HER2*.

An EMT process begins early in tumor metastasis and contributes to the acquisition of invasive capabilities, allowing epithelial cells to lose their polarity and adhesion potential while gaining the characteristics of mesenchymal cells (Valastyan and Weinberg, 2011). Twist1, ZEB1, Snail, and Slug are transcription factors that are known to impact EMT induction by modulating the expression of epithelial genes (such as *ZO-1* and *E-cadherin*) and mesenchymal genes (such as *Vimentin*) (Valastyan and Weinberg, 2011). MCL-1 , a member of the Bcl-2 family of proteins, has been shown to play an important role in enhancing cell survival and metastasis in various malignancies (De Blasio et al., 2018). Recent studies have also identified MCL-1 as the major EMT inducer in endometrial cancer (Konno et al., 2014).

In addition to genetic mechanisms, complex epigenetic regulatory mechanisms, including DNA methylation, histone modifications, as well as non-coding RNAs, including miRNAs, lncRNAs, also play crucial roles in the EMT process and cancer metastasis (Kiesslich et al., 2013; Xu et al., 2020). For instance, in oral cancer cells, hypermethylation at the *E-cadherin* promoter correlates negatively with E-cadherin expression, and treatment with a demethylation drug (5-azacytidine) promotes E-cadherin re-expression (Kiesslich et al., 2013). We have previously reported that EZH2 acts as a co-suppressor of YY1 to epigenetically suppress miR-361, upregulating *Twist1* (a direct target of miR-361). As a result, Twist1 upregulation promotes EMT, cell invasion and cancer stemness in EC cells. A large body of evidence demonstrated the miR-200 family and miR-34 perform critical regulatory roles in EMT (Kiesslich et al., 2013). Ectopic expression of miR-200b and miR-200c inhibits ZEB1 levels and results in increased E-cadherin expression (Kiesslich et al., 2013). Overexpression of

miR-34 caused mesenchymal-epithelial transition and Snail down-regulation via a conserved miR-34 seed-matching region in the *Snail* 3'-UTR (Kiesslich et al., 2013). However, the genetic and epigenetic mechanisms governing EMT in aggressive endometrial cancer remain unclear.

Evasion of the immune system is recognized as a characteristic of cancer, allowing cancer cells to avoid immune cell assault (Hanahan and Weinberg, 2011). Tumor cells can avoid immune cells by using the programmed death-1 (PD-1) immunological checkpoint mechanism (Okazaki and Honjo, 2007). The immune checkpoint ligand programmed death-ligand-1 (PD-L1) is expressed on the surface of tumor cells, and binding of PD-L1 to its receptor PD-1 on activated T cells reduces anti-tumor immunity by inhibiting T cell proliferation, cytokine release, and cytotoxic activity (Okazaki and Honjo, 2007) (Figure 36).

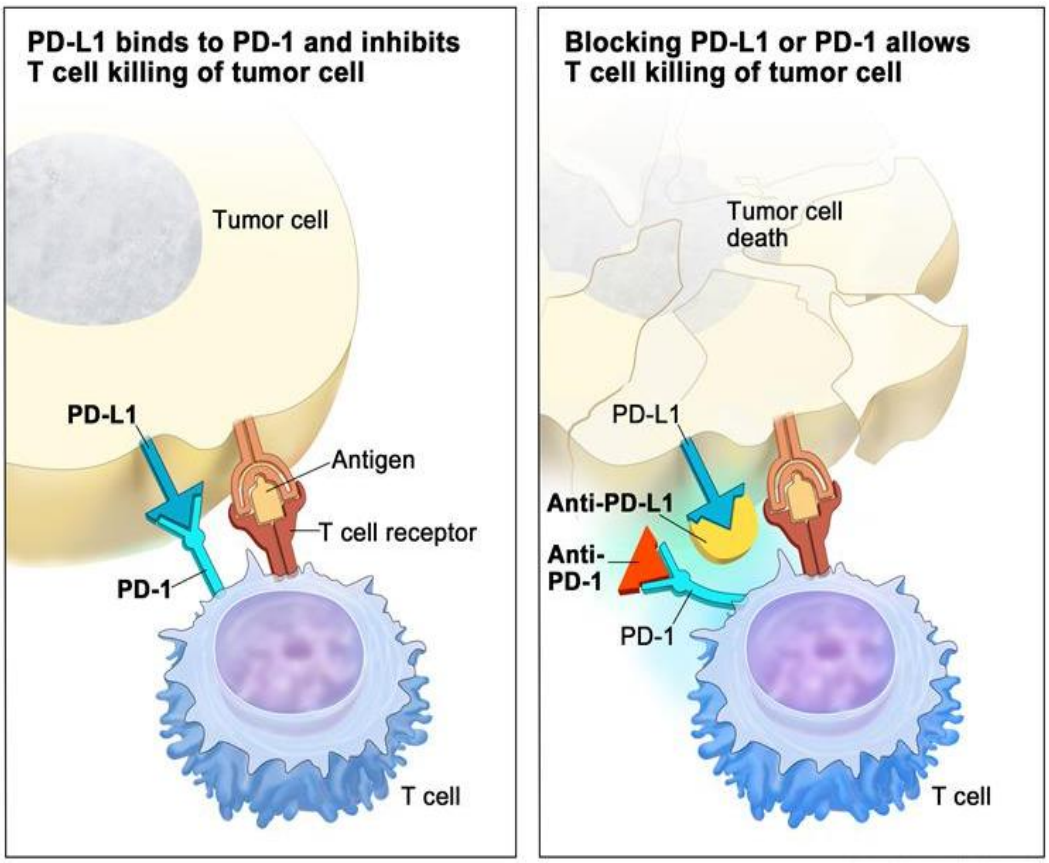


Figure 36: PD-1/PD-L1 signaling inhibits T cell proliferation and function (National

Cancer Institute, USA).

The binding of PD-L1 to its receptor PD-1 on activated T cells can suppress T-cell proliferation and reduce cytokine production. By preventing T-cell activation, PD-1 signaling contributes to the maintenance of tolerance to self-antigens and prevents anti-tumor immunity.

Despite this well-known function of PD-L1 in cancer immunity, new research shows that PD-L1 has a tumor cell-intrinsic role in triggering EMT and immune-independent tumor growth (Dong et al., 2018b) (Figure 37). PD-L1 expression levels were shown to be elevated in a variety of malignancies, and PD-L1 overexpression was found to be substantially associated with poor survival (Dong et al., 2018b). For example, immunohistochemistry analysis of 23 primary cervical cancer and paired adjacent normal tissue specimens suggested that protein expression of PD-L1 in CC tissues was significantly higher than that in adjacent normal samples (Dong et al., 2018a). Moreover, higher PD-L1 protein expression levels were associated with a higher incidence of lymphovascular space invasion in CC patients (Dong et al., 2018a). The subsequent cellular experiments suggested that short hairpin RNA (shRNA) -mediated knockdown of PD-L1 decreased CC cell proliferation and invasion, and stable overexpression of PD-L1 in CC cells promoted their proliferation and invasion (Dong et al., 2018a). The biological function of PD-L1 in CC tumorigenesis *in vivo* was then investigated by injecting PD-L1-knockdown CC cells or PD-L1-overexpressing CC cells into nude mice. The results showed that PD-L1-knockdown CC cells generated smaller tumors, and PD-L1-overexpressing CC cells formed larger tumors than control cells (Dong et al., 2018a). All these results demonstrated that PD-L1 is a novel oncoprotein that enhances the proliferation, invasion, and tumorigenesis of CC cells, and highlighted the therapeutic potential of agents that target PD-L1 in patients with cervical cancer.

On the other hand, suppression of PD-L1 was reported to increase tumor development in cholangiocarcinoma and lung cancer cells (Tamai et al., 2014; Wang et al., 2020a). Cholangiocarcinoma cells that express lower levels of PD-L1 are highly tumorigenic in nude mice compared with those cells with higher expression of PD-L1 (Tamai et al., 2014). Furthermore, depletion of PD-L1 expression by shRNA in cholangiocarcinoma cells enhanced their tumorigenicity and increased aldehyde dehydrogenase activity (Tamai et al., 2014). Consistently, those cholangiocarcinoma patients with lower expression of PD-L1 showed worse prognosis when compared with cholangiocarcinoma patients with high PD-L1 (Tamai et al., 2014). These data indicated that PD-L1 has a novel tumor suppressor function in repressing cancer stemness-related phenotypes in human cholangiocarcinoma. In line with these observations, the silencing of PD-L1 promoted lung cancer cell proliferation and colony formation i*n vitro* and tumor growth *in vivo* (Wang et al., 2020a). Conversely, overexpression of PD-L1 inhibited lung cancer cell proliferation and colony formation (Wang et al., 2020a). In this study, the tumor cell-intrinsic PD-L1 was shown to inhibit the AKT and ERK1/2 signaling pathways in lung cancer cells (Wang et al., 2020a). In accordance with these findings, higher PD-L1 expression was substantially connected with increased survival in patients with melanoma (Taube et al., 2012), colorectal cancer (Droeser et al., 2013), or Merkel-cell carcinoma (Lipson et al., 2013), demonstrating that tumor cell-intrinsic PD-L1 may perform either pro-tumor or anti-tumor activities, and that PD-L1 might have a tissue or tumor type-specific roles.

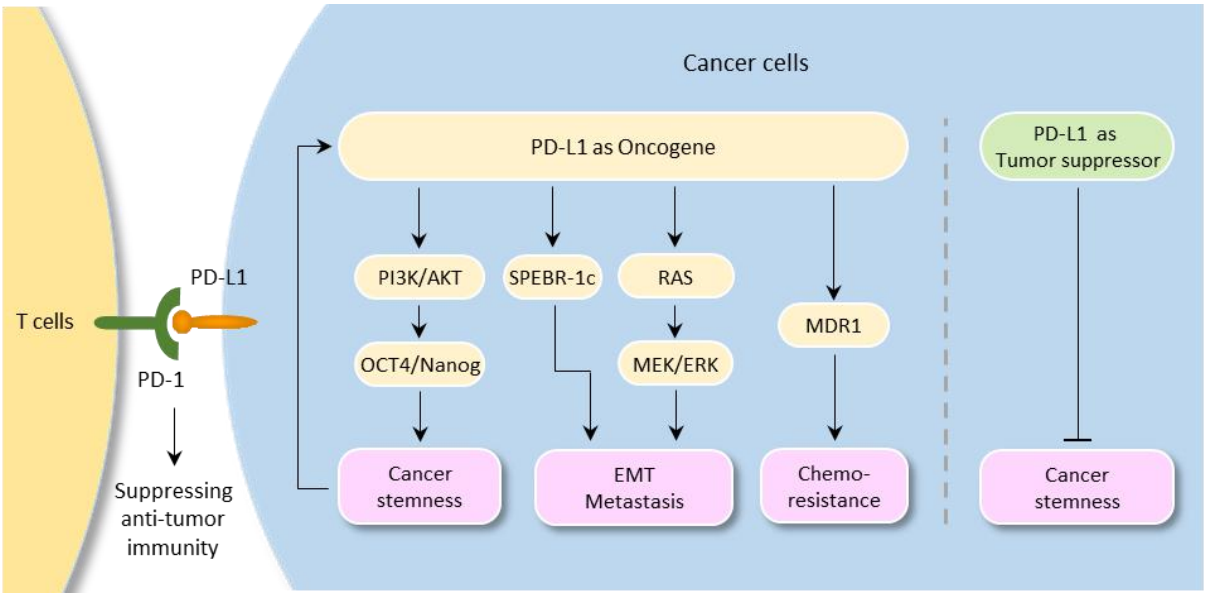


Figure 37: PD-L1 has a tumor-intrinsic role in human cancers (Dong P, et al. Front Oncol. 2018).

The graphic depicts the functions of PD-L1 and the signaling pathways that occur downstream of PD-L1 activation in human cancer. PD-L1 plays an important role in increasing cancer stemness, EMT, tumor invasion, and chemoresistance in human cancers. However, the tumor-suppressive functions of PD-L1 were also reported in several tumor types.

Previous research has yielded inconsistent results in terms of PD-L1 expression patterns and prognostic significance in endometrial cancer (Marinelli et al., 2019). The proportion of endometrial cancers positive for PD-L1 expression ranged from 14% to 59% (Li et al., 2018a; Mo et al., 2016; Engerud et al., 2020). Although it was previously reported that PD-L1 expression does not affect patient survival (Engerud et al., 2020), a recent study has shown that increased PD-L1 expression in EC samples is related to enhanced survival (Zhang et al., 2020; Yamashita et al., 2017; Liu et al., 2019). Furthermore, greater PD-L1 expression in EC cells was associated with well-differentiated histology and a decreased risk of myometrial invasion and lymphatic dissemination (Zhang et al., 2020). To date, the biological role of PD-L1 and the

mechanisms behind PD-L1 expression in aggressive EC cells have remained unknown.

CHAPTER 2: Methods

Cell lines and culture

Human EC cell lines HEC-50 (JCRB Cell Bank, Osaka, Japan) and HOUA-I (RIKEN Cell Bank, Tsukuba, Japan) were generated from poorly differentiated endometrioid endometrial cancer. The invasive human EC cell line HEC-1 (JCRB Cell Bank, Osaka, Japan) was generated from a moderately differentiated endometrioid endometrial cancer. As previously described (Dong et al., 2011), a highly invasive subpopulation of HEC-50 cells (referred to as HEC-50-HI cells) was produced utilizing Matrigel invasion chambers. Dr. Satoru Kyo (Shimane University, Matsue, Japan) generously provided the immortalized human endometrial epithelial cell line (EM) (Kyo et al., 2003). The American Type Culture Collection provided the human CC cell line HeLa. These cells were grown in DMEM/F12 media supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) (Sigma-Aldrich, St. Louis, MO, USA). Dr. Fumihiko Suzuki (Tohoku University, Sendai, Japan) kindly provided the human serous EC cell line SPAC-1-L, which was grown in RPMI-1640 media (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA).

Transient transfection

The *PD-L1* cDNA expression vector pCMV6-PD-L1 (PD-L1-vector, RC213071), the *MCL-1* cDNA expression vector pCMV6-MCL-1 (MCL-1-vector, RC200521), the maternally expressed 3 (MEG3, a lncRNA) expression vector pCMV6-MEG3 (MEG3-vector, SC105816), and the pCMV6 control vector were obtained from OriGene (Rockville, MD, USA). The PD-L1-specific siRNA (s26547), MCL-1-specific siRNA (MCL-1-siRNA, AM51331),

MEG3-specific siRNA (MEG3-siRNA, n272552), negative control siRNA (Ctr-siRNA, AM4611), miR-216a mimic (PM10545), control mimic (AM17110), miR-216a inhibitor (AM10545), and control inhibitor were purchased from Invitrogen (Carlsbad, CA, USA). One day before transfection, EC cells were seeded in growth media at a density of 40-50%. The Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect the indicated vector, siRNA, miRNA mimics, or miRNA inhibitor into EC cells according to the manufacturer's instructions. The cells were extracted after 48 hours for the subsequent study.

Western blotting analysis

M-Per Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) was used to make protein extracts for western blotting analysis, which were subsequently separated by SDS-polyacrylamide gels and transferred to PVDF membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA). Membranes were incubated with the following primary antibodies: PD-L1 (1:1000, clone E1L3N, Cell Signaling Technology, Danvers, MA, USA), MCL-1 (1:1000, #4572, Cell Signaling Technology, Danvers, MA, USA), ZO-1 (1:1000, #5406, Cell Signaling Technology, Danvers, MA, USA), Vimentin (1:1000, A01189, GenScript, Edison, NJ, USA), and GAPDH (1:3000, sc-47724, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and then with HRP-conjugated secondary antibody. Finally, using the ECL detection kit, blots were created (Amersham Pharmacia Biotech, Buckinghamshire, UK). The loading control was GAPDH. The NIH Image program was used to quantify immunoblot pictures.

PD-L1 knockdown and overexpression in EC cells

Lentiviral particles containing two shRNAs (HSH064502 and HSH099746) targeting PD-L1 and a control shRNA (CSHCTR001) were obtained from GeneCopoeia (Guangzhou, China)

and used to suppress PD-L1 expression. Puromycin (1 μg/mL) was utilized to select stable-transfected HEC-50 cells (Sigma-Aldrich, St. Louis, MO, USA). To overexpress PD-L1 in SPAC-1-L cells, Lipofectamine 2000 was utilized to transfect SPAC-1-L cells with the PD-L1-vector and Ctr-vector (Invitrogen, Carlsbad, CA, USA). 0.5 mg/mL neomycin (Sigma-Aldrich, St. Louis, MO, USA) was used to select stable PD-L1-overexpressing SPAC-1-L cells and control cells, and PD-L1 expression was validated by western blotting assays.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) was used to assess cell proliferation according to the manufacturer's instructions. In a 96-well plate, 5,000 cells were seeded per well and cultivated for the timeframes stated. Each well received 10 μl of CCK-8 reagent and was incubated for 1 hour. A microplate reader (Bio-Rad, Hercules, CA, USA) was used to measure absorbance at 450 nm.

Wound-healing assay

The wound-healing experiment was carried out exactly as previously reported (Konno et al., 2014). In a nutshell, confluent cells were scraped using a 200 μl pipette tip to create a wound, and debris was washed away with PBS. The growth media was changed with new media containing Mitomycin C (5 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) and incubated for 12 hours. Cells were imaged both immediately after the wound was created and 12 hours later. The distance moved was measured by snapping photographs at 0 and 12 hours.

Transwell invasion assay and transwell migration assay

5×10^4 cells suspended in serum-free media were plated in the upper wells of Matrigel-coated Transwell plates (8 m pore size, Corning Costar Co., Lowell, MA, USA) for invasion assays. The insert was incubated in 750 µl medium containing 10% FBS. After 24 hours of culture, the membranes were treated with 10% formaldehyde for 3 minutes before being stained with 2% crystal violet for 15 minutes at room temperature. Swiping the top of the membrane with cotton swabs eliminated the non-invasive cells. In 10 randomly selected high-power fields, cells that invaded over the transwell membrane were counted using a light microscope. Transwell migration assays were carried out in the same way as transwell invasion assays, with the exception that the membrane was not covered with Matrigel, and the incubation time was 12 hours.

Caspase-Glo 3/7 assay

Caspase-3/7 enzymatic activity was measured using the Caspase-Glo 3/7 assay kit according to the manufacturer's instructions (Promega, Madison, WI, USA), as previously described (Konno et al., 2014). 3000 cells were plated in triplicate in 96-well plates and transfected as directed. The culture was then incubated for 3 hours with an equivalent quantity of Caspase-Glo 3/7 substrate. Following incubation, caspase-3/7 activity was measured using a GloMax-96 Microplate luminometer (Promega, Madison, WI, USA). The data was presented as a fold change in comparison to the control cells.

Gain-of-function screening for PD-L1 based on cell functional assays

The Lipofectamine 2000 reagent was used to transiently transfect the indicated EC cell lines or the human CC cell line HeLa with PD-L1-vector or Ctr-vector (Invitrogen, Carlsbad, CA,

USA). Cell proliferation, apoptosis, migration, and invasion were measured after a 48-hour incubation using the CCK-8 assay, Caspase-Glo 3/7 assay, transwell migration assay, and transwell invasion assay, respectively. The fold changes in cell proliferation, apoptosis, migration, and invasion of PD-L1-vector-transfected cells compared to control cells were reported.

Quantitative Reverse Transcription PCR

The TRIzol reagent was used to isolate total RNA (Invitrogen, Carlsbad, CA, USA). Using an M-MLV Reverse Transcriptase Kit, all mRNA was reverse transcribed into cDNA (Invitrogen, Carlsbad, CA, USA). SYBR Premix Ex Taq II (Takara, Kusatsu, Japan) was used for RT-qPCR in an ABI-7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA).

The primers were as follows:

Human *PD-L1*, sense: GTGGCATCCAAGATACAAACTCAA,

Human *PD-L1*, anti-sense: TCCTTCCTCTTGTCACGCTCA;

Human *ZO-1*, sense: GGAGAGGTGTTCCGTGTTGT,

Human *ZO-1*, anti-sense: GAGCGGACAAATCCTCTCTG;

Human *Vimentin*, sense: TGAGTACCGGAGACAGGTGCAG,

Human *Vimentin*, anti-sense: TAGCAGCTTCAACGGCAAAGTTC;

Human *Snail*, sense: GACCACTATGCCGCGCTCTT,

Human *Snail*, anti-sense: TCGCTGTAGTTAGGCTTCCGATT;

Human *MCL-1*, sense: CCAAGGCATGCTTCGGAAA,

Human *MCL-1*, anti-sense: TCACAATCCTGCCCCAGTTT;

Human *MEG3*, sense: TCCATGCTGAGCTGCTGCCAAG,

Human *MEG3*, anti-sense: AGTCGACAAAGACTGACACCC;

Human *GAPDH*, sense: GAAGGTG AAGGTCGGAGTC,

Human *GAPDH*, anti-sense: GAAGATGGTGATGGGATTTC.

GAPDH was used as an internal control.

The levels of miR-138/193a/216a/217 were measured using the NCode SYBR GreenER miRNA qRT-PCR analysis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The forward primers for miRNA analysis had the same sequences as the mature miRNAs. The reverse primer was provided by the NCode SYBR GreenER miRNA qRT-PCR analysis kit (Invitrogen, Carlsbad, CA, USA). The relative expression of these miRNAs was normalized against that of the U6 endogenous control.

Luciferase reporter assay

The human *PD-L1* 3′-UTR luciferase reporter vector and the Luc-Pair^{TM} Duo-Luciferase Assay kit were obtained from GeneCopoeia (Rockville, MD, USA). Using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA), the miR-216a-binding site in the *PD-L1* 3′-UTR was mutated. EC cells were co-transfected with the WT or MUT *PD-L1* 3′-UTR luciferase reporter vector, as well as a miR-216a mimic, miR-216a inhibitor, or the respective control, using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). Cell lysates were collected 48 hours later, and the Firefly and Renilla luciferase activities of each group were evaluated using the Luc-Pair^{TM} Duo-Luciferase Assay kit (GeneCopoeia, Rockville, MD, USA). Firefly luciferase activity was normalized with Renilla luciferase

activity.

Statistical analysis

All experiments were carried out with at least three replicates. Data are presented as the mean ± standard error of the mean. Statistical analysis was conducted using a 2-tailed Student's *t*-test, Mann-Whitney *U* test, or one-way ANOVA using SPSS 18.0 statistical software (SPSS, Chicago, IL, USA). The χ^2-tests and Fisher's exact tests were applied to analyze the relationship between PD-L1 expression and clinicopathological status. Differences were considered statistically significant when $P < 0.05$.

CHAPTER 2: Results

1. PD-L1 downregulation correlates with poorer survival in endometrial cancer

First, the mRNA expression of *PD-L1* was examined in different cancer tissue types vs. normal tissues utilizing the UALCAN portal, which provides TCGA gene expression data from 31 cancer types for further research. In line with our prior findings (Dong et al., 2018a), we found considerably greater levels of *PD-L1* in human CC tissues than in normal tissues (Figure 38A). However, *PD-L1* mRNA expression was lower in primary EC tissues than in normal samples (Figure 38A).

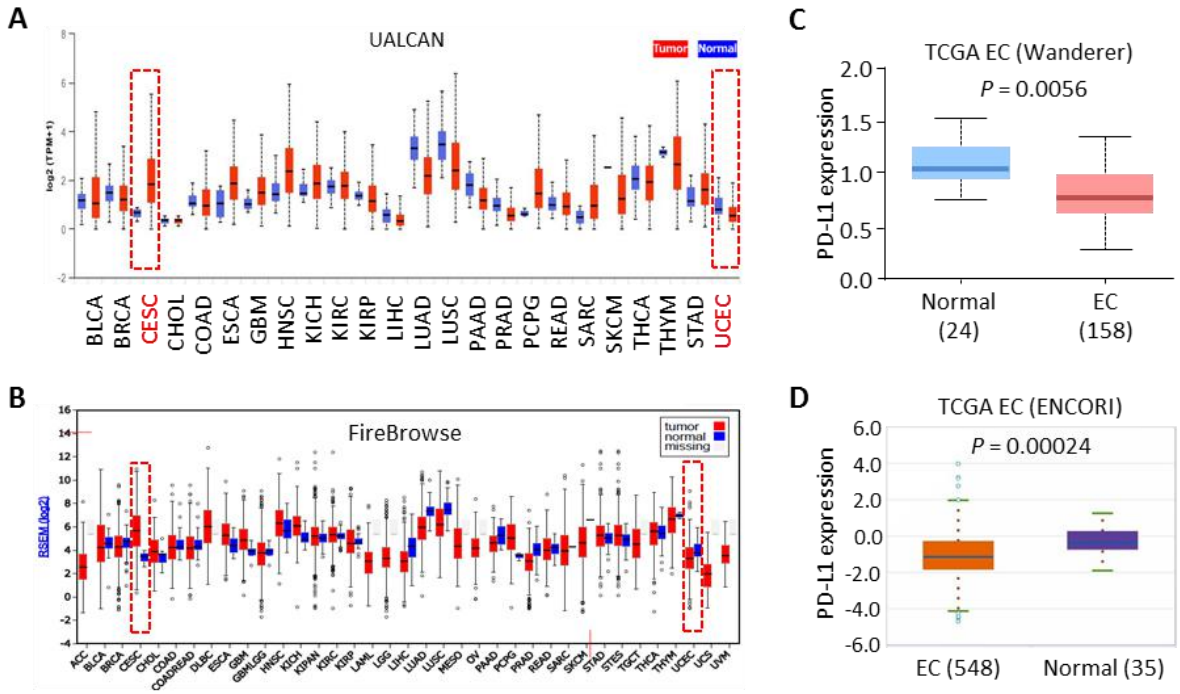


Figure 38: Downregulation of PD-L1 in endometrial cancer.

(A, B) Analysis of *PD-L1* expression in human cancer tissues and normal tissues (UALCAN database) (A) and FireBrowse database (B). CESC: human cervical cancer; UCEC: human endometrial cancer. (C, D) *PD-L1* mRNA expression in EC samples and normal samples was analyzed using the Wanderer database (C) and ENCORI database (D).

To validate these findings, we looked at the expression of *PD-L1* in the TCGA datasets on the Firebrowse website (Deng et al., 2017). The findings demonstrated that *PD-L1* expression was much higher in CC tissues than in normal tissues (Figure 38B). However, *PD-L1* levels in EC tissues were lower than in normal tissues (Figure 38B). Similarly, the comparison of *PD-L1* expression in EC tissues vs. normal tissues using the Wanderer (Díez-Villanueva et al., 2015) and ENCORI databases revealed that *PD-L1* expression was considerably lower in EC tissues (Figure 38C; 38D).

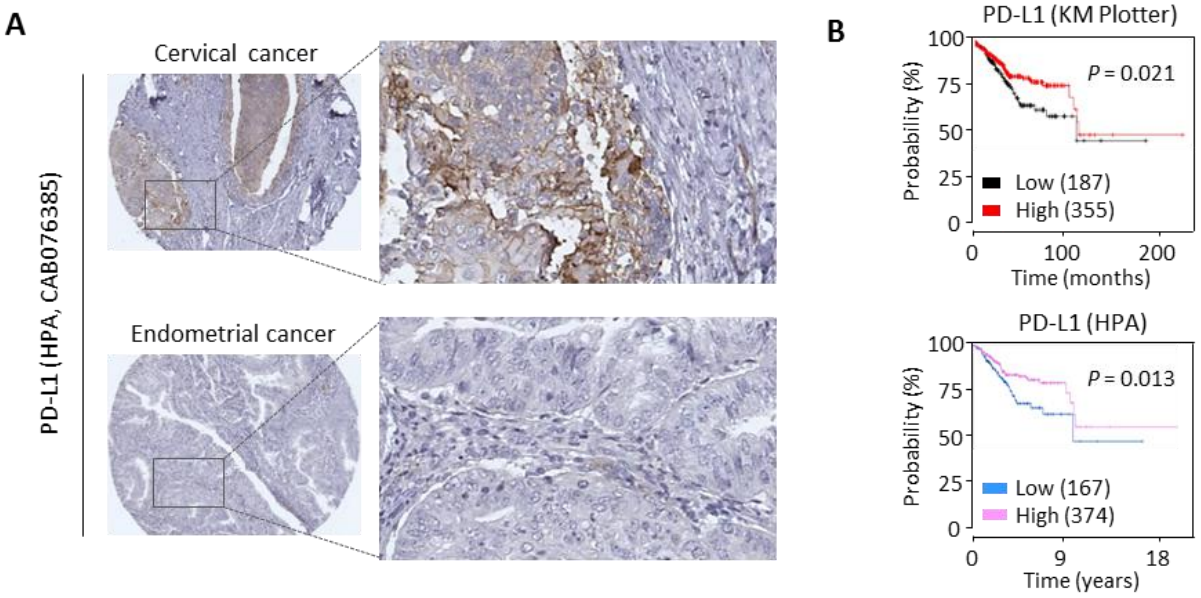


Figure 39: Downregulation of PD-L1 Correlates with Poor Survival in endometrial cancer.

(A) Immunohistochemical staining for PD-L1 in CC tissues and EC tissues with the same antibody (CAB076385, the HPA website) showed that high levels of PD-L1 were present in primary CC tissues. In contrast, PD-L1 was negatively or weakly stained in most EC tissues. (B) The probability of overall survival in EC patients expressing high or low *PD-L1* levels was assessed using the KM plotter database (upper) and the HPA database (bottom).

We retrieved immunohistochemistry pictures from the HPA database to see if PD-L1

protein expression was similarly differently regulated in EC tissues. PD-L1 was expressed at high levels in human CC tissues but not in surrounding normal tissues (Figure 39A). In contrast, immunohistochemical labeling of EC tissues and nearby normal tissues with the same antibody revealed that PD-L1 levels in most EC cells are very low or absent (Figure 39A). To determine the predictive significance of PD-L1 expression in endometrial cancer, we examined the KM plotter and HPA databases for the impacts of PD-L1 expression in EC patients. In EC patients, higher *PD-L1* expression was related to favorable overall survival (Figure 39B). These findings showed that higher PD-L1 expression predicts better survival in endometrial cancer.

2. PD-L1 acts as a tumor suppressor in aggressive EC cells

Our western blotting assays indicated that PD-L1 protein expression was decreased in all aggressive EC cell lines relative to a normal endometrial cell line (EM) (Figure 40). PD-L1 expression was much lower in the serous EC cell line SPAC-1-L than in other EC cells (Figure 40). HEC-50-HI cells, a subset of HEC-50 cells, are more invasive than parental HEC-50 cells and have mesenchymal characteristics (Dong et al., 2011). Interestingly, HEC-50-HI cells expressed less PD-L1 than parental cells (Figure 40), demonstrating that PD-L1 may control the invasive capabilities of aggressive EC cells.

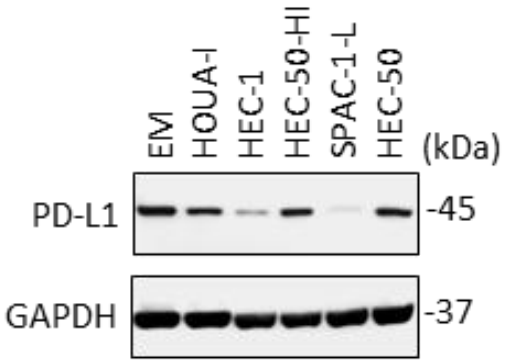


Figure 40: Loss of PD-L1 expression in aggressive EC cells.

PD-L1 protein levels in a normal endometrial cell line (EM) and human aggressive EC cell lines were determined by western blot analysis.

To investigate this possibility, we transfected the human *PD-L1* cDNA expression vector or the control vector into five aggressive EC cell lines and the CC cell line HeLa for 48 hours and then performed cell functional assays. Consistent with our earlier findings (Dong et al., 2018a), we discovered that overexpression of PD-L1 may greatly increase HeLa cell proliferation, migration, and invasion while decreasing apoptosis (Figure 41). Overexpressing PD-L1 expression in all aggressive EC cells dramatically reduced cell proliferation, migration, and invasion while causing cell death, according to our functional screening (Figure 41). These findings suggest that PD-L1 expression reduction is common in endometrial cancer and that PD-L1 performs tumor-suppressive activities in aggressive EC cells.

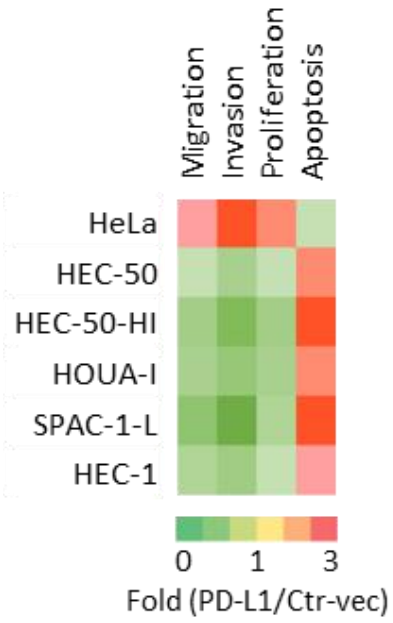


Figure 41: A gain-of-function screening for PD-L1 that affects the proliferation, apoptosis, migration and invasion of aggressive EC cells.

The indicated aggressive EC cell lines or human CC cell line HeLa were transiently transfected with a

PD-L1 expression vector (PD-L1-vec) or the control vector (Ctr-vec), and subjected to cell functional assays. A heatmap depicting the fold changes in cell proliferation, apoptosis, migration and invasion of the PD-L1-vec-transfected cells when compared with the Ctr-vec-transfected cells.

3. Loss of PD-L1 induces cell proliferation and triggers EMT in aggressive EC cells

After that, we looked at whether lower PD-L1 expression is essential for increased aggressive EC cell proliferation and invasiveness. We created SPAC-1-L cells that stably overexpressed PD-L1 and control cells (Figure 42), as well as HEC-50 cells that stably expressed control shRNA or PD-L1 shRNAs (PD-L1-sh-1 or PD-L1-sh-2) that repressed PD-L1 expression (Figure 42). Because PD-L1-sh-2 performed better than PD-L1-sh-1, we utilized it in all subsequent experiments.

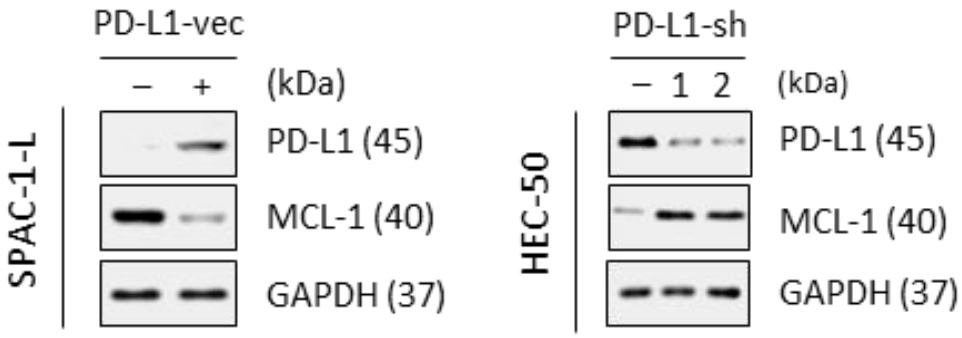


Figure 42: PD-L1 and MCL-1 expression in aggressive EC cells.

Western blotting analysis was used to examine PD-L1 and MCL-1 expression in SPAC-1-L cells overexpressing PD-L1 and in PD-L1-silenced HEC-50 cells.

SPAC-1-L cells are invasive, although they have an epithelial-like shape. PD-L1 overexpression did not affect the emergence of SPAC-1-L cells. On the other hand, PD-L1 knockdown caused cell scattering in HEC-50 cells (Figure 43).

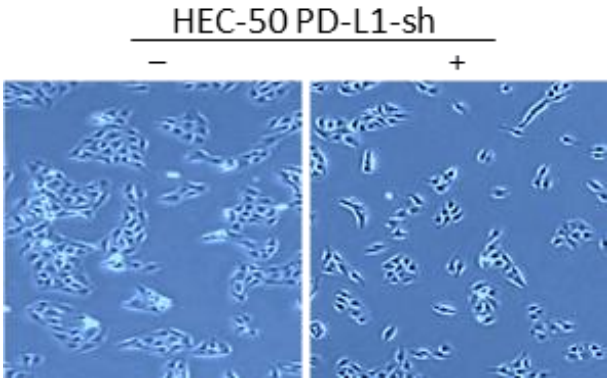


Figure 43: Knockdown of PD-L1 induces EMT in HEC-50 cells.

Cellular morphology of HEC-50 cells after knockdown of PD-L1.

Overexpression of PD-L1 in SPAC-1-L cells considerably reduced their proliferative and invasive capacities as compared to control cells, whereas silencing of PD-L1 in HEC-50 cells greatly boosted these abilities (Figure 44). These findings suggested that EMT may be involved in the PD-L1-suppressed migratory and invasive abilities of aggressive EC cells.

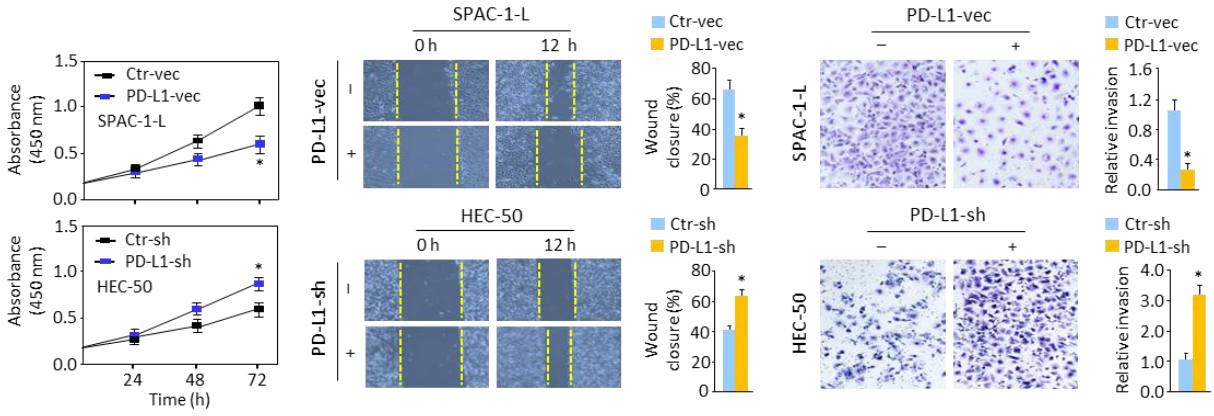


Figure 44: Loss of PD-L1 Induces the proliferation and triggers EMT in aggressive EC Cells.

Proliferation (left), wound-healing (middle) and invasion (right) assays in EC cells after overexpression or knockdown of PD-L1. *$P < 0.05$.

Western blotting and RT-qPCR research indicated that depleting PD-L1 from HEC-50 cells increased the expression of MCL-1, *Vimentin*, and *Snail* while decreasing the expression of *ZO-1* (Figure 42; Figure 45). We also discovered that overexpression of PD-L1 in SPAC-1-L cells inhibited EMT, as evidenced by activation of *ZO-1* and downregulation of MCL-1, *Vimentin*, and *Snail* (Figure 42; Figure 45). These findings show that PD-L1 inhibits EMT signaling-regulated migration and invasion in aggressive EC cells.

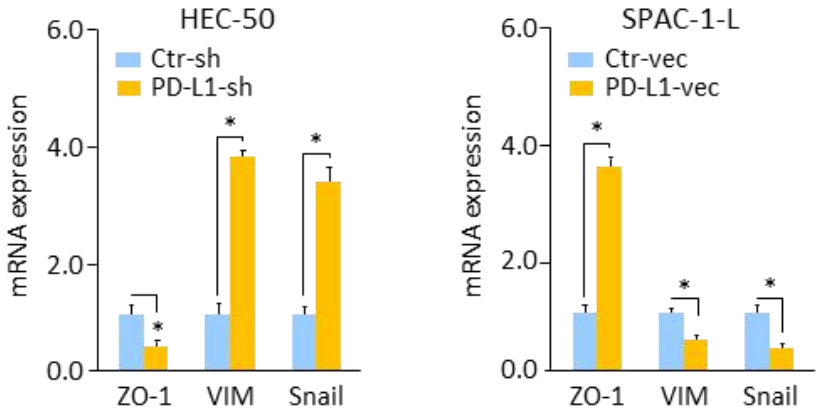


Figure 45: PD-L1 suppresses EMT in aggressive EC cells.

Gene expression in HEC-50 cells after knockdown of PD-L1 and in SPAC-1-L cells after overexpression of PD-L1 was performed using RT-qPCR assays. *$P < 0.05$.

4. PD-L1 represses EMT by decreasing MCL-1 expression

Because our earlier findings implicated MCL-1 in EMT and EC cell invasion (Konno et al., 2014) we investigated whether PD-L1 regulates EMT and HEC-50 cell invasion by modifying MCL-1. We discovered that inhibiting MCL-1 expression with MCL-1-specific siRNA dramatically decreased the migratory and invasive potential that was boosted by PD-L1 knockdown (Figure 46). Our western blotting results also showed that siRNA-induced downregulation of MCL-1 could abrogate the repression of ZO-1, and the induction of

Vimentin expression by PD-L1 inhibition (Figure 46).

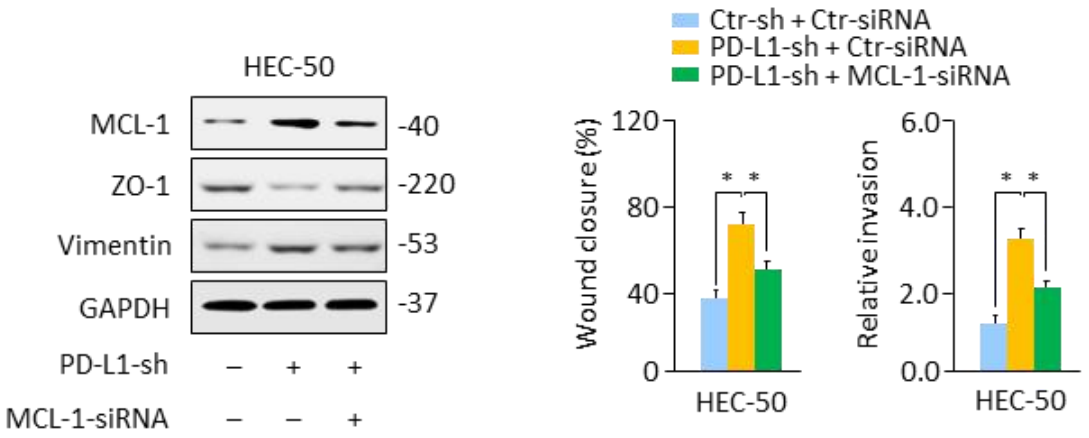


Figure 46: PD-L1 represses EMT by decreasing MCL-1 expression.

Left: Western blotting analysis of the indicated proteins in HEC-50 cells transfected as indicated. Right: wound-healing and invasion assays in HEC-50 cells transfected as indicated. **P* < 0.05.

We then investigated whether PD-L1 inhibits cell proliferation and invasiveness in SPAC-1-L cells by suppressing MCL-1 expression. Overexpression of PD-L1 in SPAC-1-L cells caused an upregulation of ZO-1 and a downregulation of Vimentin and significantly reduced cell growth and invasion. However, restoration of MCL-1 partially abolished these effects of PD-L1 (Figure 47).

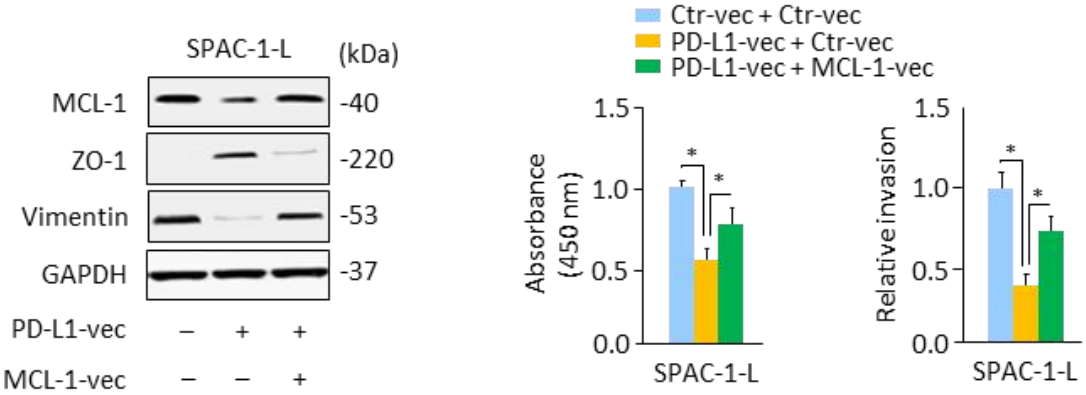


Figure 47: Overexpression of PD-L1 reduces the proliferation and EMT of SPAC-1-L

cells via suppressing MCL-1 levels.

Left: SPAC-1-L cells overexpressing PD-L1 or the control cells were transfected with MCL-1-vec or Ctr-vec, respectively. The protein levels of MCL-1, ZO-1, and Vimentin were assessed using western blotting analysis. Right: CCK-8 and invasion assays were performed to evaluate cell proliferation and invasiveness. **P* < 0.05.

The HPA database was used to get immunohistochemical staining pictures for MCL-1, and ZO-1. In EC tissues from the same patient, immunohistochemical staining analysis revealed high MCL-1 expression and low ZO-1 expression as compared to nearby normal tissues (Figure 48). The KM plotter database was used to examine the relationship between MCL-1 and ZO-1 expression and overall survival. According to the Kaplan-Meier survival study, EC patients with high levels of MCL-1 but low levels of ZO-1 had a considerably worse overall survival rate (Figure 48). These findings support that PD-L1 suppresses the EMT properties of aggressive EC cells via lowering MCL-1 protein expression.

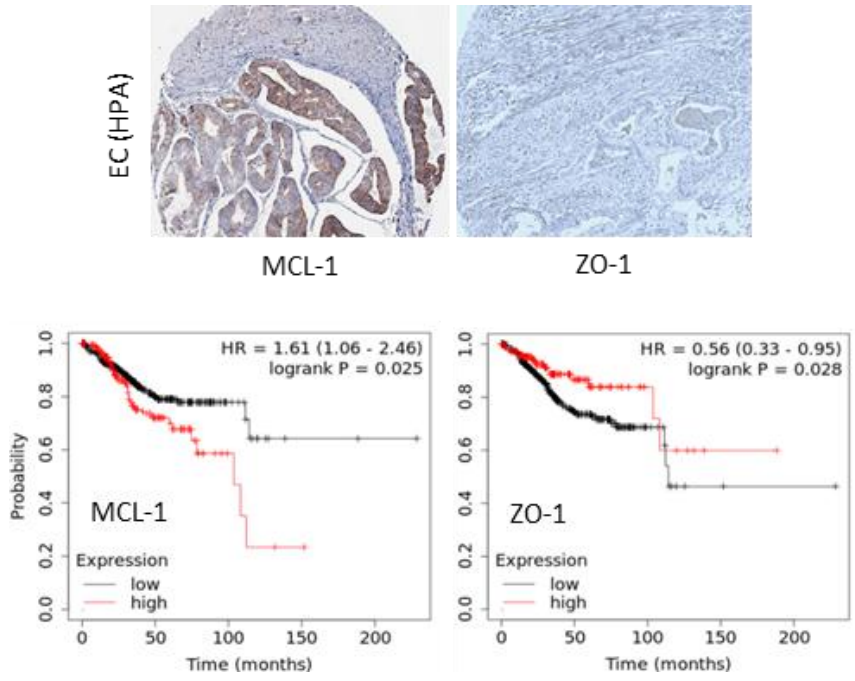


Figure 48: High MCL-1 and low ZO-1 expression predict poor survival in EC patients.

Upper: The immunohistochemical staining results were obtained from the HPA database. Immunohistochemical staining of EC tissues from the same patient showed a high MCL-1 expression and a low ZO-1 expression in EC tissues. Bottom: The correlation between the indicated genes and overall survival in patients with endometrial cancer (KM plotter database).

5. *PD-L1* is a direct target of oncogenic miR-216a

We wanted to find candidate miRNAs that target PD-L1 directly in aggressive EC cells. Figure 49 depicts a flowchart describing our miRNA screening procedure.

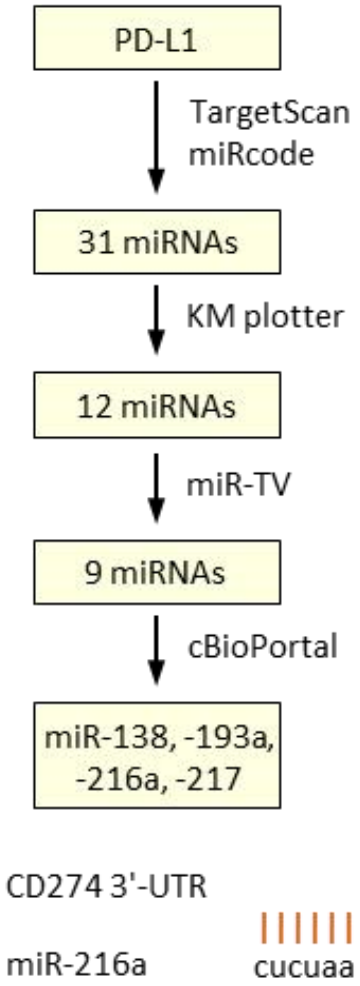


Figure 49: Identification of potential miRNAs that regulate the expression of PD-L1.

Upper: Workflow for identifying potential miRNAs that regulate the expression of PD-L1. Bottom:

Computational prediction of miR-216a duplex formation between with the *PD-L1* 3′-UTR sequence.

We started with a miRNA prediction analysis utilizing the TargetScan (http://www.targetscan.org/vert 72/) and miRcode (http://www.mircode.org/) databases. As a result, 31 miRNAs emerged in both databases at the same time. Following that, we searched the KM plotter database for 12 miRNAs whose expression is linked to a poor prognosis in EC patients (Figure 50).

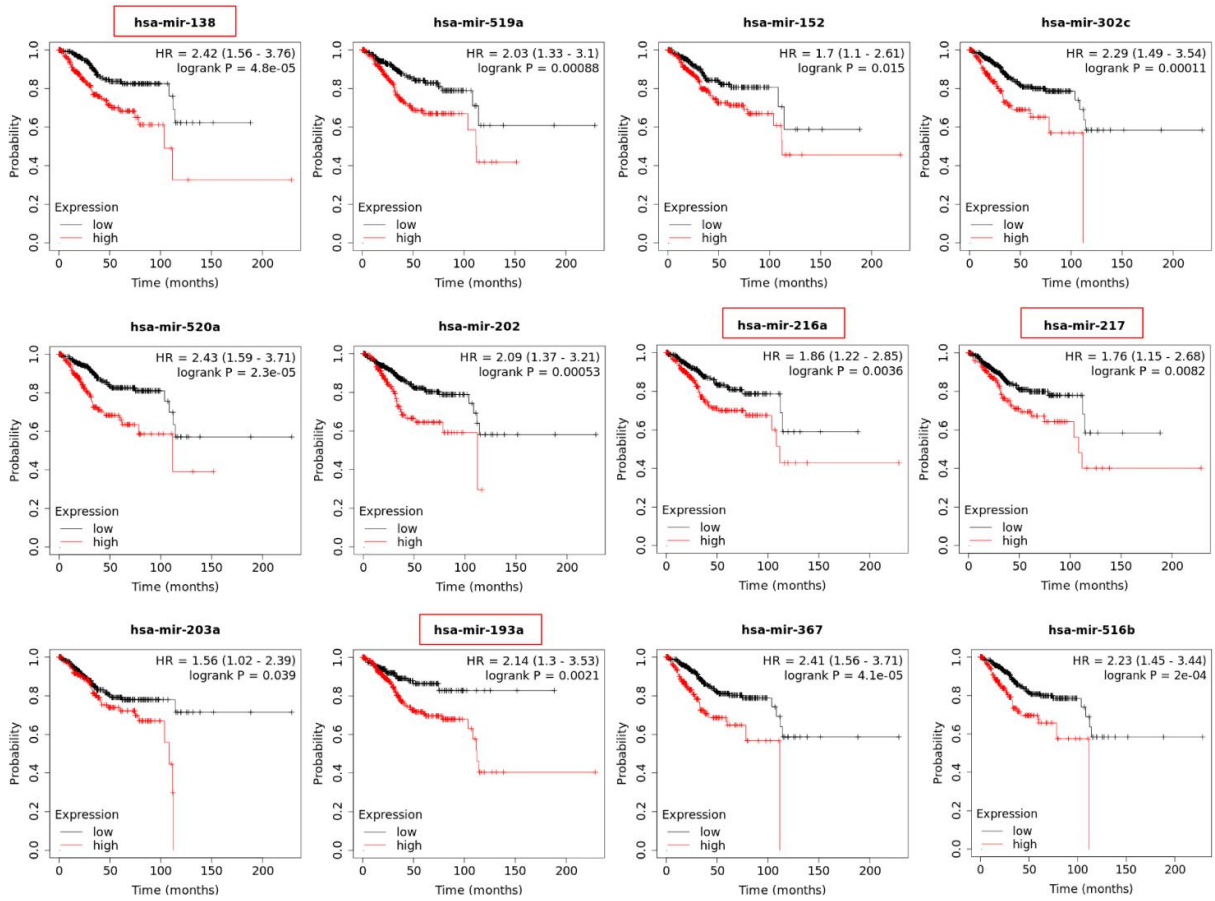


Figure 50: The prognostic significance of miRNAs in endometrial cancer.

The relationship between miRNA expression and EC patient survival was analyzed using the KM plotter database.

The miR-TV database (Pan and Lin, 2020) analysis of the TCGA endometrial cancer datasets revealed that 9 miRNAs had substantially increased expression in EC tissues relative to normal tissues (Figure 51). The cBioPortal database (Gao et al., 2013) was used to evaluate the expression of these 9 miRNAs in TCGA EC tissues. Four miRNAs, including miR-138, miR-193a, miR-216a, and miR-217, were amplified in patients with endometrial cancer (Figure 52).

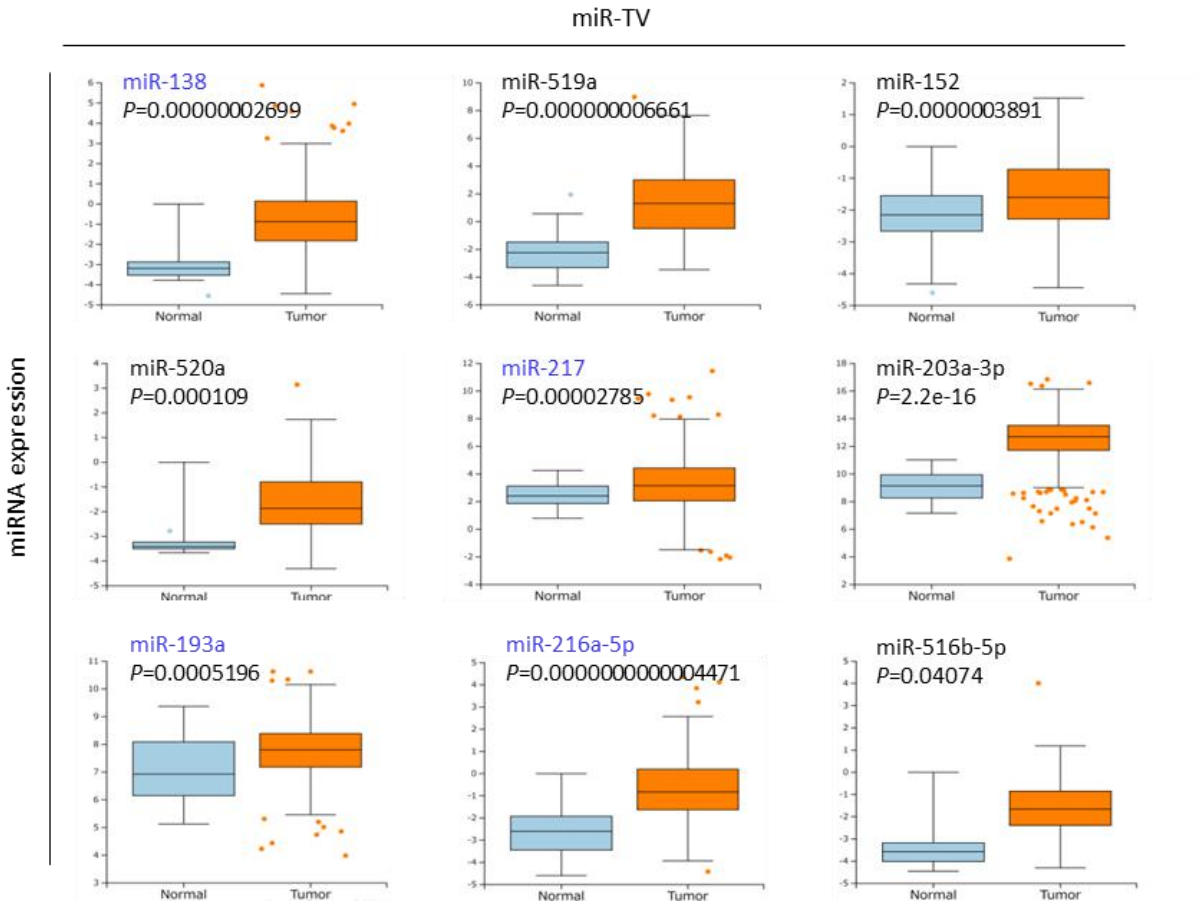


Figure 51: The expression of miRNAs in endometrial cancer and normal tissues.

The expression of miRNA in TCGA endometrial cancer samples and normal samples was analyzed using the miR-TV database.

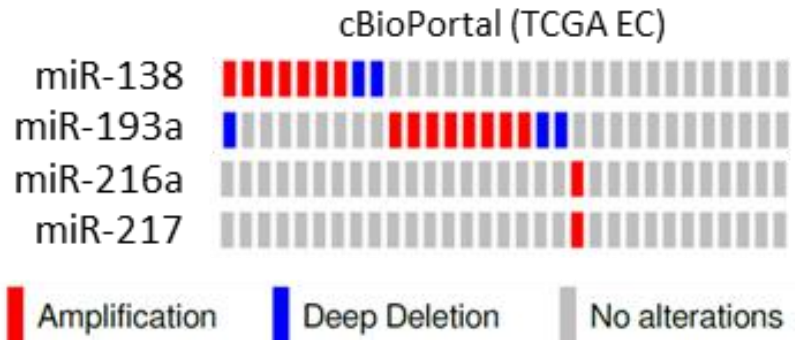


Figure 52: MiR-216a is amplified in patients with EC tissues.

Oncoprint plot showing an overview of the genetic alterations in four miRNAs from TCGA endometrial cancer samples (cBioPortal database).

Using RT-qPCR, we confirmed that only miR-216a and miR-217 were consistently increased in aggressive EC cells when compared to EM cells (Figure 53). As a result, we decided that miR-216a and miR-217 were likely candidates for additional research.

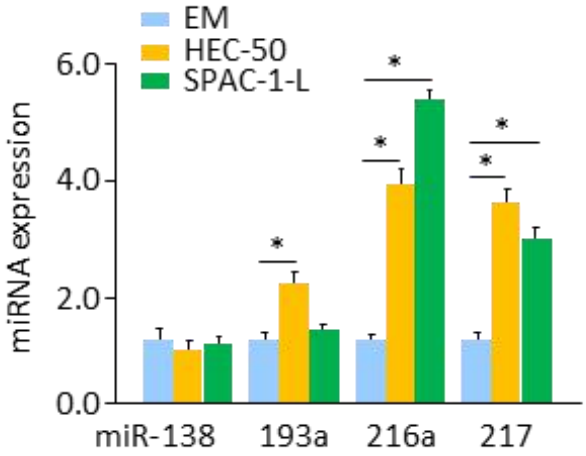


Figure 53: MiR-216a is consistently increased in aggressive EC cells.

The RT-qPCR analysis was used to measure the levels of miRNAs in HEC-50, SPAC-1-L and EM cells. *$P < 0.05$.

When miR-216a (but not miR-217) was overexpressed in HEC-50 cells, *PD-L1* mRNA expression fell significantly compared to the corresponding controls (Figure 54). In SPAC-1-L cells, *PD-L1* expression increased considerably when miR-216a (but not miR-217) was suppressed (Figure 54).

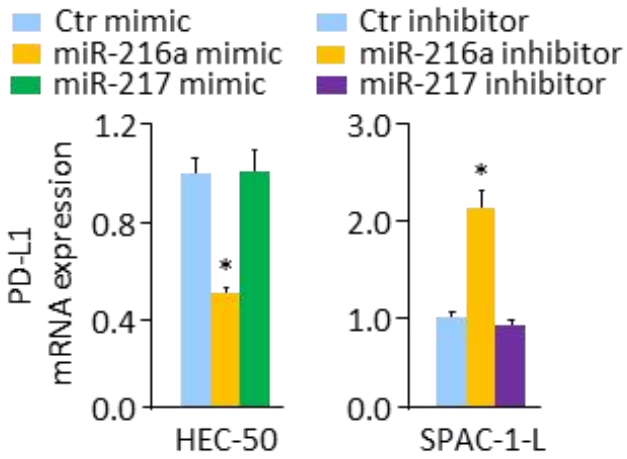


Figure 54: MiR-216a inhibits *PD-L1* levels in aggressive EC cells.

PD-L1 mRNA expression was examined using RT-qPCR assays in EC cells transfected as indicated. **P* < 0.05.

In addition, luciferase reporter tests were performed using a luciferase reporter vector containing the *PD-L1* 3′-UTR region surrounding the putative miR-216a binding site. As controls, mutations in the putative binding locations were produced. MiR-216a overexpression substantially decreased the luciferase activities of WT *PD-L1* 3′-UTR in HEC-50 cells, whereas miR-216a inhibition boosted the luciferase activities of WT *PD-L1* 3′-UTR in SPAC-1-L cells (Figure 55). Transfection of EC cells with miR-216a mimic or miR-216a inhibitor did not influence the luciferase activities of MUT *PD-L1* 3′-UTR (Figure 55).

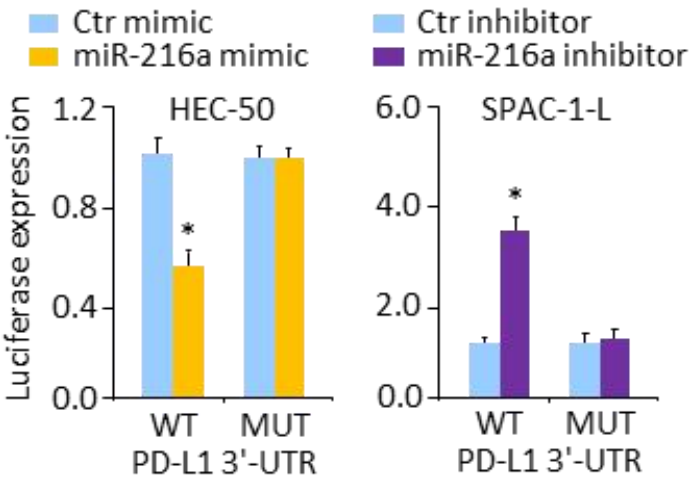


Figure 55: *PD-L1* is a Direct Target of Oncogenic MiR-216a.

Luciferase reporter assays in EC cells transfected with the WT or MUT *PD-L1* 3′-UTR, along with miR-216a mimic or miR-216a inhibitor, respectively. **P* < 0.05.

These findings were confirmed by western blotting experiments, which revealed that overexpression of miR-216a inhibited PD-L1 protein production while inhibition of miR-216a elevated PD-L1 levels in EC cells (Figure 56). These data suggested that *PD-L1* is a direct target of miR-216a in aggressive EC cells.

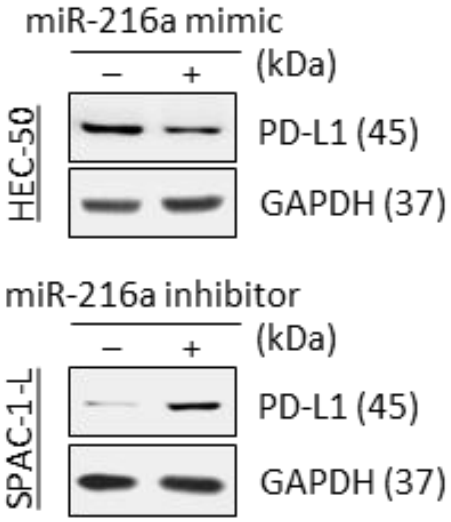


Figure 56: The protein expression of PD-L1 in EC cells with miR-216a overexpression or

knockdown.

Western blotting analysis of PD-L1 expression in HEC-50 cells transfected with miR-216a mimic (or control mimic), and in SPAC-1-L cells transfected with miR-216a inhibitor (or control inhibitor).

To investigate the role of miR-216a in aggressive EC cells, we overexpressed miR-216a in HEC-50 cells with low endogenous miR-216a expression and transfected miR-216a inhibitor into SPAC-1-L cells with high levels of miR-216a expression. Overexpression of miR-216a dramatically increased HEC-50 cell migration and invasion (Figure 57). Cell migration and invasion were drastically decreased in SPAC-1-L cells when miR-216a was knocked down (Figure 57).

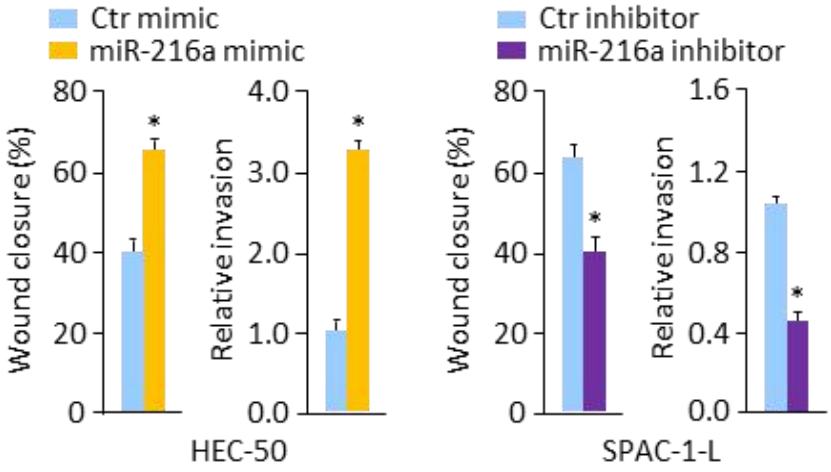


Figure 57: The impact of miR-216a on EC cell migration and invasion.

Wound-healing and invasion assays in HEC-50 cells after overexpression of miR-216a, or in SPAC-1-L cells after knockdown of miR-216a. *$P < 0.05$.

When miR-216a was overexpressed, mRNA expression of *MCL-1* and *Vimentin* was consistently elevated, but *ZO-1* levels were decreased in EC cells (Figure 58). On the other

hand, SPAC-1-L cells transfected with miR-216a inhibitor displayed reduced *MCL-1* and *Vimentin* expression and elevated *ZO-1* expression (Figure 58). These findings show that *PD-L1* is a direct target of the oncogenic miR-216a in aggressive EC cells.

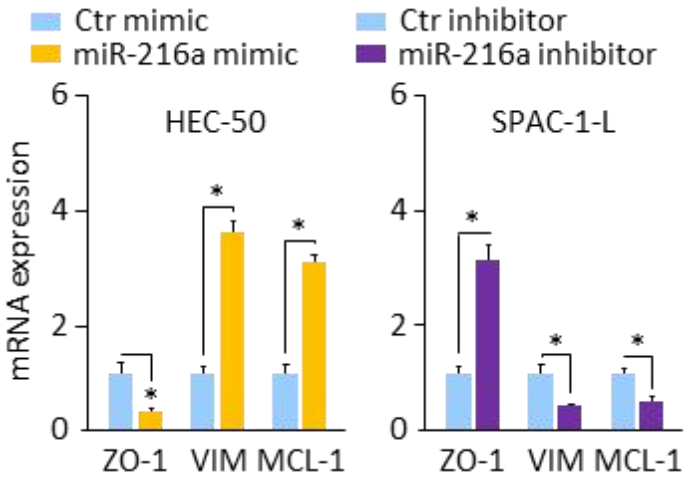


Figure 58: miR-216a downregulates *ZO-1* expression, while increasing *MCL-1* level.

The mRNA expression of the indicated genes was examined in HEC-50 cells after overexpression of miR-216a, and in SPAC-1-L cells after knockdown of miR-216a. VIM: Vimentin. *$P < 0.05$.

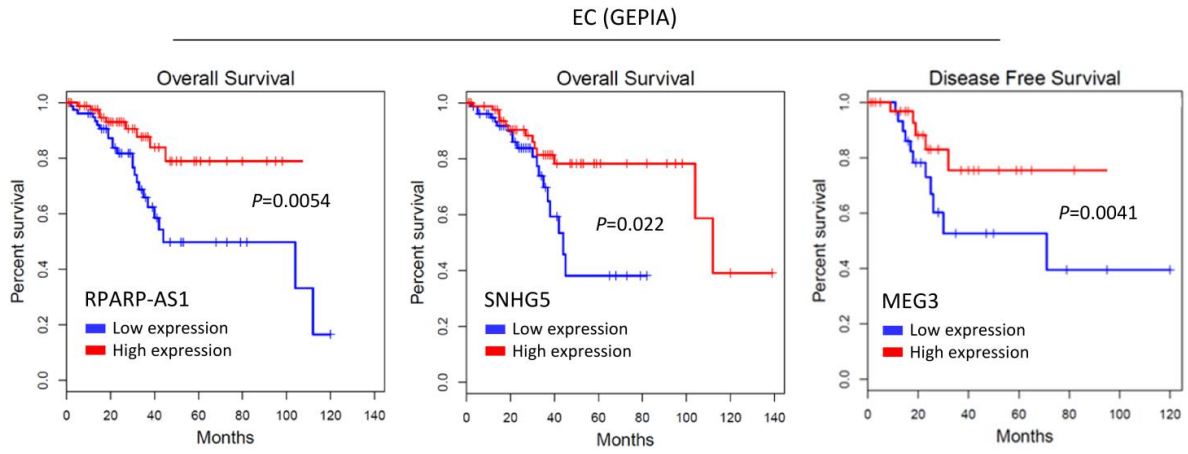
6. MEG3 acts as an upstream regulator of miR-216a and PD-L1

LncRNAs have important roles in a variety of human malignancies, including aggressive endometrial cancer (Dong et al., 2019a). Several lncRNAs, including NEAT1 and MEG3, have been shown to control cancer development and metastasis by interacting with DNA, RNA, and proteins (Dong et al., 2019a; Dong et al., 2018c; Paraskevopoulou et al., 2016).

To explore the processes driving PD-L1 expression, we performed a sequence alignment analysis using the ENCORI and LncBase Predicted v.2 databases (Paraskevopoulou et al., 2016). We identified 26 potential lncRNAs that might interact with miR-216a. An analysis of

the TCGA endometrial cancer database in the GEPIA database (Tang et al., 2017) revealed that high expression of three potential lncRNAs (including MEG3, RPARP-AS1, and SNHG5) was a good predictive factor for endometrial cancer (Figure 59).

A



B

miRNA	GeneID	GeneName	GeneType	TargetSite	Alignment
hsa-miR-216a-5p	ENSG00000214548	MEG3	lincRNA	chr14:101297378-101297398[+]	Target: 5' aucCAGGCCCCAG-UGAGAUUc 3' miRNA : 3' aguGUCAACGGUCGACUCUAAu 5'

Figure 59: The prognostic value of candidate lncRNAs in endometrial cancer.

(A) The correlation between lncRNA expression and overall survival in patients with endometrial cancer (GEPIA database). (B) Computational prediction of duplex formation between miR-216a with the MEG3 sequence (ENCORI database).

According to the results from the ENCORI database, only MEG3 (but not RPARP-AS1 and SNHG5) exhibited significantly lower expression in TCGA EC tissues as compared to the normal tissues (Figure 60).

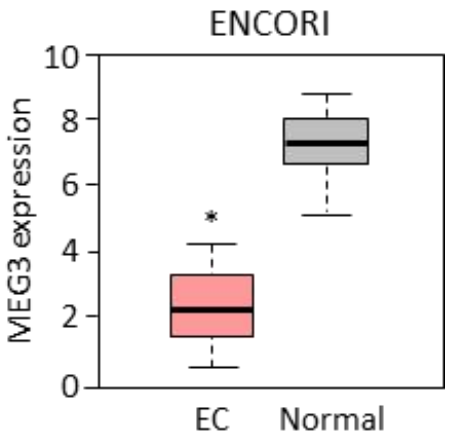


Figure 60: MEG3 expression in TCGA EC tissues and normal tissues

The expression of MEG3 in TCGA EC tissues and normal tissues was analyzed using the ENCORI database. *$P < 0.05$.

Using RT-qPCR analysis, we discovered that the levels of MEG3 were significantly downregulated in HEC-50 and SPAC-1-L cells compared with EM cells (Figure 61), suggesting a potential tumor-suppressor role for MEG3 in aggressive EC cells.

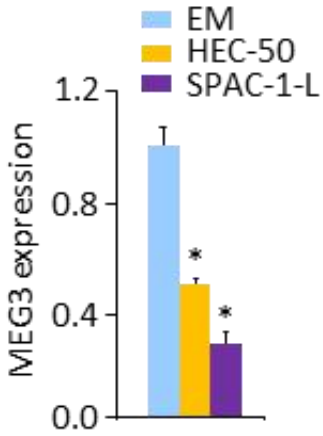


Figure 61: Downregulation of MEG3 in EC cells.

The RT-qPCR analysis was used to explore MEG3 expression in HEC-50, SPAC-1-L and EM cells. *$P < 0.05$.

LncRNAs have been proposed to behave as sponges for miRNAs, inhibiting their production (Dong et al., 2018c; Paraskevopoulou et al., 2016). Given that MEG3 functions as a tumor suppressor to regulate EC progression by acting as a competing endogenous RNA (Dong et al., 2019a), and that MEG3 contains the predicted miR-216a-binding site (Figure 59B), we hypothesized that MEG3 might positively regulate PD-L1 levels in aggressive EC cells by decreasing miR-216a expression. In keeping with this hypothesis, knockdown of MEG3 increased but ectopic MEG3 expression decreased miR-216a expression in aggressive EC cells (Figure 62).

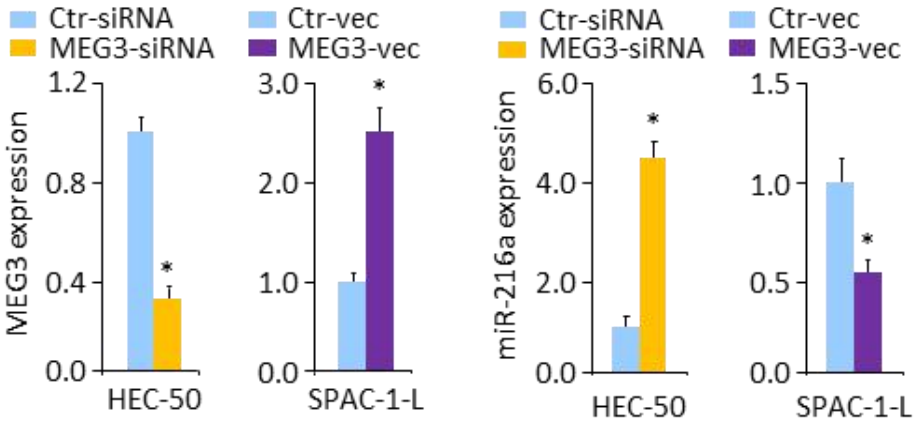


Figure 62: MEG3 suppresses miR-216a in aggressive EC cells.

MiR-216a expression was measured in HEC-50 cells transfected with MEG3 siRNA (or control siRNA), and in SPAC-1-L cells transfected with a MEG3 expression vector (or control vector). *$P < 0.05$.

The role of MEG3 in suppressing cell migration was confirmed by wound-healing assays in HEC-50 and SPAC-1-L cells (Figure 63).

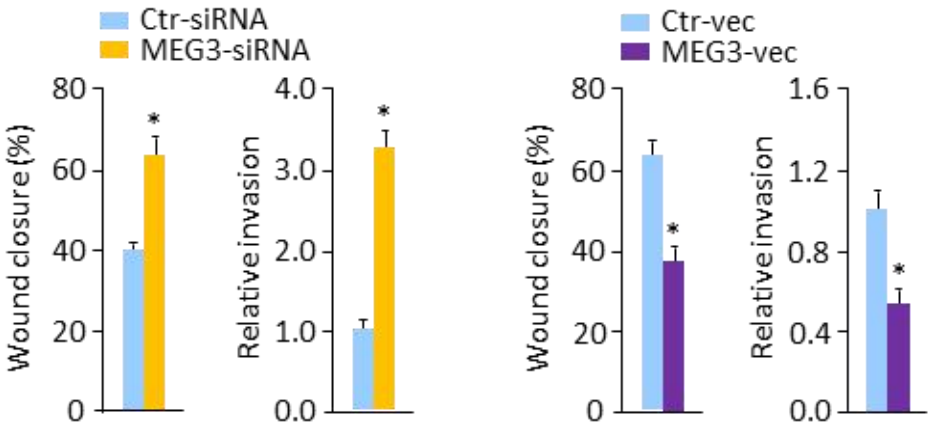


Figure 63: MEG3 represses the migration and invasion of aggressive EC cells.

Wound-healing and invasion assays in HEC-50 cells transfected as indicated. **P* < 0.05.

Consistent with these results, we found that knockdown of MEG3 inhibited the protein expression of PD-L1 compared to control cells, while overexpression of MEG3 increased the expression of PD-L1 in EC cells (Figure 64). Our RT-qPCR experiments showed that transfection with MEG3-specific siRNA downregulated *ZO-1*, and upregulated *Vimentin* and *MCL-1* in HEC-50 cells (Figure 64). However, overexpression of MEG3 had the opposite effects in SPAC-1-L cells (Figure 64).

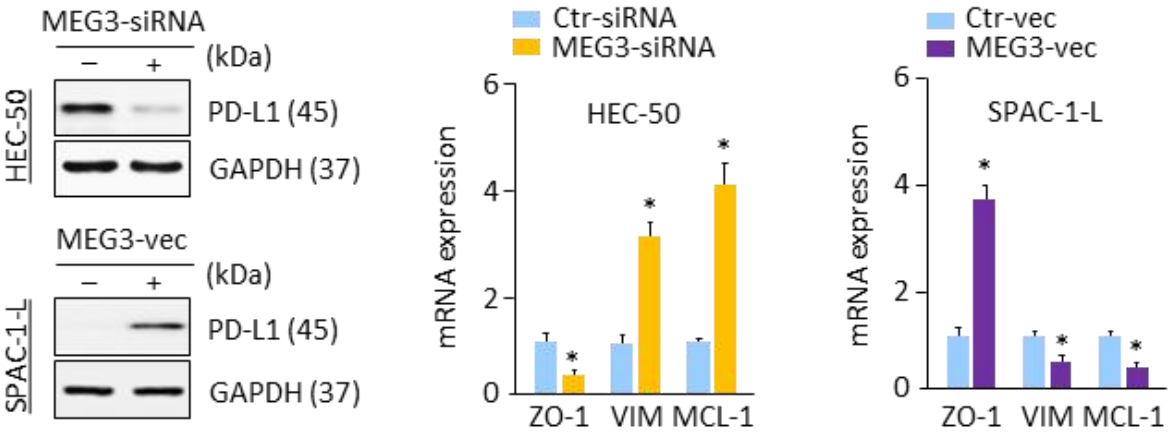


Figure 64: Overexpression of MEG3 increases the expression of PD-L1 in aggressive EC cells.

The expression of the indicated genes in HEC-50 cells following knockdown of MEG3, and in SPAC-1-L cells following overexpression of MEG3. VIM: Vimentin. *$P < 0.05$.

Taken together, our findings suggested that MEG3 negatively regulates miR-216a expression, thereby removing the miR-216a-induced restrictive effects on the *PD-L1* 3′-UTR.

CHAPTER 2: Discussion

The prevalence of aggressive endometrial cancer is quickly growing in the United States and Japan (Clarke et al., 2019; Yamagami et al., 2017). The highly metastatic and often treatment-refractory nature of aggressive endometrial cancer correlates with poor patient survival (Gaber et al., 2016). A better understanding of the mechanisms behind the tumorigenesis and metastasis of aggressive endometrial cancer is urgently needed to improve the early diagnosis and effective treatment of this cancer.

EMT induction and immune evasion have been demonstrated to promote cancer development, metastasis and chemoresistance (Valastyan and Weinberg, 2011; Gonzalez et al., 2018). Recent studies have linked EMT processes to immune escape (Dong et al., 2018b; Terry et al., 2017). Cancer cells undergoing EMT might contribute to immune escape through multiple mechanisms (Terry et al., 2017). In breast cancer cells, overexpression of Snail induces the EMT properties and also reduces the susceptibility to cytotoxic T cell-mediated lysis (Terry et al., 2017). In addition, colon cancer cells undergoing EMT are more resistant to natural killer cell-mediated lysis (Terry et al., 2017). Consistently, the inhibition of TGF-β signaling can increase the susceptibility of cancer cells to cytotoxic T cell-mediated lysis and natural killer cell-mediated lysis (Terry et al., 2017). EMT is a vital driver of cancer immunotherapy resistance. As a result, targeting EMT may open up new avenues for future cancer treatment.

In several human cancers, PD-L1 expression significantly promotes the EMT phenotypes (Dong et al., 2018b). However, little is known about the functional significance of PD-L1 in

aggressive EC cells. Our study demonstrates a new tumor-suppressor role of PD-L1 in repressing the proliferation and EMT-associated migration and invasion in aggressive EC cells, and that the downregulation of MEG3 and induction of its downstream effector miR-216a is likely a novel mechanism underlying the downregulation of PD-L1 observed in EC tissues (Figure 65).

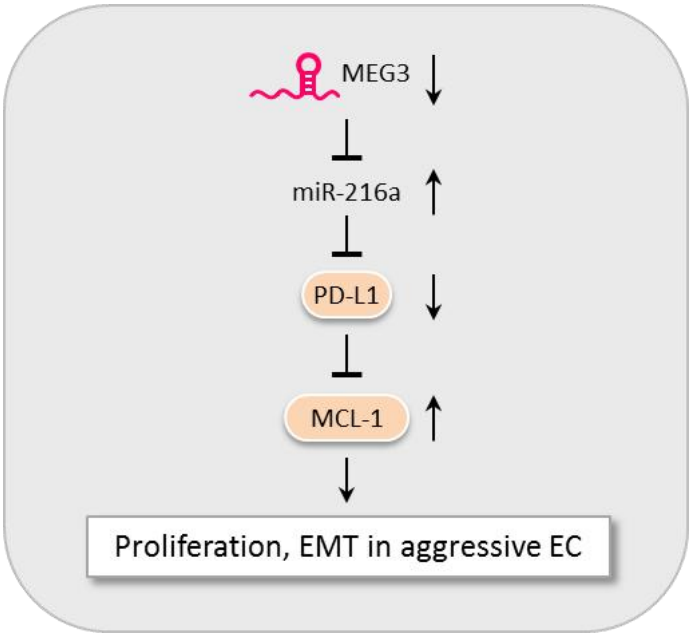


Figure 65: Model summarizing the role of MEG3, miR-216a and PD-L1 in controlling EMT and invasive potential of aggressive EC cells.

PD-L1 serves as a tumor-suppressor to inhibit EMT and invasive potential of aggressive EC cells via repressing MCL-1 expression. MiR-216a inhibits PD-L1 expression by targeting the 3′-UTR of *PD-L1* mRNA. The downregulation of MEG3 causes an elevation of miR-216a expression.

The pattern of PD-L1 expression and its prognostic value in endometrial cancer appear to be controversial in the literature (Marinelli et al., 2019; Li et al., 2018a; Mo et al., 2016; Engerud et al., 2020). These conflicting results may be attributed to either technical reasons or different clinical features of the analyzed samples (Marinelli et al., 2019). By employing a

validated antibody that exhibited high sensitivity and high specificity (Dong et al., 2018a; Lánczky and Győrffy, 2021), we found that, compared with normal endometrium samples, the protein expression of PD-L1 was frequently lost in EC tissues. In line with previous reports describing an inverse correlation between the levels of PD-L1 and the degree of tumor malignancy in human endometrial cancer (Engerud et al., 2020; Zhang et al., 2020), we found that higher protein expression of PD-L1 seems to correlate with younger patient age, low-grade disease, early-stage tumors, smaller tumor size, or superficial myometrial invasion. Future studies with a larger sample size are necessary to validate our current data.

Moreover, previous research has shown that, in patients with metastatic melanoma (Taube et al., 2012), colorectal cancer (Droeser et al., 2013), Merkel-cell carcinoma (Lipson et al., 2013), and endometrial cancer (Liu et al., 2015), higher PD-L1 expression was correlated with improved overall survival rates, suggesting that high PD-L1 expression may be a favorable prognostic marker in several types of cancer. Consistent with these reports, our data suggest that PD-L1 loss can identify EC patients with a worse probability of survival and that lower expression of PD-L1 was particularly associated with shorter overall survival in high-grade endometrial cancers, indicating that a PD-L1-negative expression signature might be an indicator of poor prognosis in endometrial cancers with aggressive behaviors.

PD-L1 exhibits pro-tumor effects via various mechanisms (Clark et al., 2016; Gato-Cañas et al., 2017; Gupta et al., 2016). Tumor cell-intrinsic PD-L1 promotes melanoma tumorigenesis in vivo through activating the mTOR signaling (Clark et al., 2016). In murine melanoma cells, PD-L1 confers resistance to interferon cytotoxicity and accelerates tumor progression via a STAT3/caspase-7-dependent pathway (Gato-Cañas et al., 2017). In glioma cells, PD-L1 is induced to prevent autophagic cytoskeleton collapse via Akt binding/activation, facilitating

glioma cell invasion upon starvation stress (Chen et al., 2019). PD-L1 was also shown to enhance tumor sphere formation of ovarian cancer cells possibly by increasing SOX2 expression (Gupta et al., 2016). The molecular link between high PD-L1 expression and EMT in cancer cells has been noticed (Qiu et al., 2018; Chen et al., 2017). In glioblastoma multiforme cells, PD-L1 activates the EMT process by upregulating Slug, β-catenin and Vimentin and by downregulating E-cadherin, via activation of the RAS/MEK/ERK signaling (Qiu et al., 2018). Overexpression of PD-L1 enhances the levels of mesenchymal genes (*ZEB1*, *N-cadhe*rin and Vimentin), and contributes to the EMT phenotypes of esophageal cancer cells (Chen et al., 2017). However, silencing PD-L1 in cholangiocarcinoma cells by shRNA can increase the tumorigenic potential and ALDH activity (Tamai et al., 2014). Furthermore, overexpression of PD-L1 significantly decreases the activities of PI3K/AKT and RAS/MEK/ERK pathways, leading to the suppression of lung cancer cell growth *in vitro* and *in vivo* (Wang et al., 2020a), providing evidence for the tumor-suppressive role of PD-L1 in specific tumor type. Our study revealed that, via repression of MCL-1, PD-L1 could induce the expression of ZO-1, while suppressing the expression of Vimentin. These results showed that tumor cell-intrinsic PD-L1 has tumor-suppressive functions in aggressive EC cells, at least through its negative modulation of EMT. Thus, the silencing of PD-L1 may underline the molecular mechanisms for inducing and maintaining the mesenchymal state of aggressive EC cells.

Activation of the PI3K/AKT axis is known to be a central feature of EMT in numerous cancers (Samarnthai et al., 2010). In addition, aberrant activation of the RAS/MEK/ERK pathway in human cancer cells allows them to undergo EMT via the upregulation of Snail (Tripathi and Garg, 2018). MCL-1 is an important downstream effector of the PI3K/AKT and RAS/MEK/ERK signaling (Xiang et al., 2018). In this study, we demonstrated that the

upregulation of MCL-1 caused by PD-L1 silencing contributes to EMT in aggressive EC cells. Further investigation will be required to determine whether PD-L1 represses the expression of MCL-1 to attenuate EMT in aggressive EC cells, through the PI3K/AKT and RAS/MEK/ERK signaling pathways.

Since PD-L1 has a critical role in suppressing anti-tumor immunity, cancer immunotherapy (in particular antibodies that block the PD-1/PD-L1 interaction) was considered to be a revolution in cancer treatment (Postow et al., 2015) and has generated clinical benefit in a subset of patients with endometrial cancer (Green et al., 2020). Recently, alternative strategies (such as combination therapies with chemotherapy and siRNA against PD-L1 (Yoo et al., 2019)), have been proposed. A nanocarrier-aided delivery of PD-L1 siRNA, together with gemcitabine, resulted in a significant reduction in pancreatic cancer growth (Yoo et al., 2019). However, our cell functional study revealed that tumor cell-intrinsic PD-L1 plays an anti-tumor role in multiple aggressive EC cell lines, and downregulation of PD-L1 is sufficient to stimulate the EMT features and cell invasion in an MCL-1-dependent manner. Thus, designing therapeutic strategies aimed at knocking down PD-L1 expression in aggressive endometrial cancer may lead to unexpected outcomes, possibly by accelerating EMT and metastasis. Future research is needed to explore this possibility.

Although a tumor-suppressive role for miR-216a has been reported (Zhang et al., 2017b), this miRNA was identified as an oncogenic miRNA in many cancers, including endometrial cancer (Wang et al., 2020b), ovarian cancer (Liu et al., 2017), hepatocellular carcinoma (Xia et al., 2013), and renal cell carcinoma (Chen et al., 2018). The direct target genes of miR-216a include *PTEN* in endometrial cancer (Wang et al., 2020b), and *PTEN* and *SMAD7* in hepatocellular carcinoma (Xia et al., 2013). We have validated that, by directly targeting

PD-L1 3′-UTR, miR-216a decreases PD-L1 levels to promote EMT and cell invasion in aggressive EC cells. It would be interesting to further determine the downstream targets of miR-216a and the clinical significance of miR-216a-regulated pathways in this clinically important subtype of endometrial cancer.

Prior studies demonstrated that MEG3 is located at human chromosome 14q32.3 and is a novel tumor-suppressor lncRNA in many tumors, including endometrial cancer (Dong et al., 2019b). MEG3 is expressed in normal tissues, but is absent or reduced in many human malignancies and tumor-derived cell lines (He et al., 2017). Reduced expression of MEG3 has been reported in lung cancer, liver cancer, prostate cancer, multiple myeloma, meningioma, gastric cancer, glioma, neuroblastoma, and renal cell carcinoma (He et al., 2017). In addition, loss of MEG3 expression has been observed in brain tumor, bladder cancer, breast cancer, cervical cancer, colon cancer, liver cancer, lung cancer, and prostate cancer-derived cell lines (He et al., 2017). Restoring the expression of MEG3 can suppress cancer initiation, progression, metastasis and chemoresistance (He et al., 2017). For instance, overexpression of MEG3 is known to inhibit cell proliferation and induce apoptosis in osteosarcoma cells, breast cancer cells, cervical cancer cells, lung cancer cells, liver cancer cells, colon cancer cells, and brain tumor cells (He et al., 2017). Moreover, enhanced MEG3 also suppresses the migration and invasion of cholangiocarcinoma cells by reversing EMT (Li et al., 2019). Consistently, knockdown of MEG3 significantly promotes cell proliferation, migration, invasion, and EMT by upregulating Twist1 and Vimentin expression and reducing E-cadherin expression in laryngeal squamous cell carcinoma cells (Zhao et al., 2019). In pancreatic cancer cells, MEG3 knockdown promotes cell proliferation, invasion, EMT, and sphere-forming properties, while decreasing the chemosensitivity to gemcitabine (Ma et al., 2018). MEG3 levels are downregulated in EC tissues compared with those in adjacent non-tumor tissues, and EC

patients with low MEG3 expression exhibit shorter overall survival compared with patients with high expression levels (Dong et al., 2019b). Here, we defined the anti-cancer function of MEG3 through the regulation of EMT in aggressive EC cells. Multiple molecular mechanisms, including gene deletion and promoter hypermethylation, contribute to the loss of MEG3 expression in tumor cells (He et al., 2017). Further studies are necessary to unravel the regulatory mechanisms of MEG3 expression in EC development.

Conclusion

In our research, we are aiming to exploring the network consisting of lncRNA–miRNA–mRNA in the EMT of cervical cancer and aggressive endometrial cancer. In CHAPTER 1, the NEAT1/miR-361/HSP90 pathway was discovered to modulate EMT and cancer stem cell characteristics in cervical cancer cells. After that, in CHAPTER 2, we began to reveal whether the lncRNA–miRNA–mRNA network could affect the EMT properties of aggressive endometrial cancer cells. Our main goal is to investigate the biological roles of PD-L1 in modulating EMT and the mechanisms associated with PD-L1 expression in aggressive endometrial cancer cells.

Our study has five new findings:

1. Firstly, our findings confirmed that miR-361 indeed functions as an important tumor suppressor by suppressing EMT, cell invasion, and stem cell phenotypes in cervical cancer cells.

2. Secondly, we have determined that miR-361 directly targets *HSP90* and down-regulates its expression in cervical cancer cells.

3. Thirdly, we further revealed that a lncRNA NEAT1 serves as an upstream inhibitor of miR-361 to promote EMT and sphere formation. These three findings defined a new functional determinant of mesenchymal/stem-like properties of cervical cancer cells and revealed previously unknown candidate therapeutic targets for cervical cancer treatment.

4. Our data also show that PD-L1 has a tumor cell-intrinsic role in reducing aggressive endometrial cancer cell growth and EMT.

5. Our work identifies MEG3 and miR-216a as important upstream regulators of PD-L1, revealing a previously unknown mechanism for PD-L1 dysregulation in aggressive endometrial cancer cells.

Taken all together, our studies highlight new insights into the molecular mechanisms of EMT in gynecological cancers. Those miRNAs, genes and lncRNAs identified in our studies, are key regulators of EMT, and they may represent potential biomarkers or therapeutic targets for cervical cancer and aggressive endometrial cancer.

We will continue to investigate the mechanisms that regulate the expression of miR-361 in cervical cancer, the effects of miR-361 on the cervical cancer microenvironment, the potential of HSP90 inhibitors as cervical cancer therapeutics, and the *in vivo* impact of miR-361 on cervical cancer progression. Also, we will continue to investigate the mechanisms that regulate the expression of PD-L1 in endometrial cancer cells, the effects of PD-L1 on endometrial cancer metastasis and chemoresistance, and the potential targets and underlying mechanisms of miR-216a and MEG3 in endometrial cancer cells.

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Disclosure of Conflict of Interest

The author declares no conflict of interest.

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