An-Najah National University

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STATINS INHIBITS GROWTH AND METASTASIS OF MELANOMA THROUGH MIR-126/MMP-9

By

Rasha Essam Faris Nasser

Supervisor

Dr. Yousef Salama

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Rasha Essam Nasser

This Thesis was Defended Successfully on 11/3/2024 and approved by

Dr. Yousef Salama Supervisor	Signature
Dr. Adel Al Hidmi External Examiner	Signature
Dr. Awni Abu-Hijleh Internal Examiner	Signature

Dedication

This master's journey has been an experience that changed my life, and many people's contributions were necessary for the work contained in this thesis to be completed.

I dedicate this work to everyone who did not have the same opportunities to a good education and could not achieve his dreams.

I want to thank my husband, Forsan Zaroor, for his love and support, my daughter Bushra, and my sons Ayan and Abd Allazez, who were born during this time and have been my inspiration at every moment. Also, my family and my husband's family thank you for your support.

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I also want to thank the assistant, Asil Sawafta, for her valuable assistance with analytical work.

I want to express deep appreciation for all my friends who have brought me so much joy and happiness during this difficult time.

Declaration

I, the undersigned, declare that I submitted the thesis entitled:

STATINS INHIBITS GROWTH AND METASTASIS OF MELANOMA THROUGH MIR-126/MMP-9

I declare that the work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: Rasha Essam Faris Nasser

Signature: Rasha Nasser

Date: 11-3-2024

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STATINS INHIBITS GROWTH AND METASTASIS OF MELANOMA THROUGH MIR-126/MMP-9

By

Rasha Nasser

Supervisor

Dr. Yousef Salama

Abstract

Background: Melanoma is the deadliest type of skin cancer, with morbidity and mortality rates increasing globally. Melanoma has an elevated metastatic potential, leading to limited response to the available treatments in addition to poor prognosis. The transcription factor (NF-κB) for nuclear factor kappa B has been up-regulated in melanoma cases. Simvastatin has anticancer properties that can act as a repurposed drug for treating melanoma. Recognizing the signaling pathway and the anti-tumor properties of simvastatin is essential in discovering new kinds of tumors that might be intended by simvastatin. MicroRNA-126 is a significant microRNA family member; recent studies demonstrate its role in cancer as a new tumor suppressor gene because it can stop cancer cells from growing, migrating, and invading by suppressing important oncogenes like matrix metallopeptidase 9 (MMP9). Here, we inspected how simvastatin can affect melanoma growth, tumor cell migration, and angiogenesis. Mechanically, Simvastatin blocks the transcription factor NF-κB, which up-regulates MIR-126 and, therefore, down-regulates the expression of MMP9 as an oncogene of melanoma cancer responsible for cell proliferation, migration, and angiogenesis.

Aims: Eliminating melanoma from our lives is our objective. The only way to accomplish this is through study, and it is pretty bold and huge. We propose that, rather than finding a new drug, it would be better to use an existing one to prevent growth and metastasis so that we can investigate the impact of statin drug on melanoma growth and metastasis using an innovative approach. This goal is supported by our unique research technique, which describes how we use the newest ideas to advance the discovery of a new treatment for melanoma.

Methods: we use Simvastatin to block the transcription factor NF-κB, which then regulates the expression of MIR-126. Therefore, this micro-RNA inhibits the expression of MMP9 as an oncogene of melanoma cancer, responsible for cell proliferation, migration, angiogenesis, and metastasis.

Results: The results of the study demonstrate that simvastatin, via inhibiting the transcription factor NFκB; can influence the growth and spread of melanoma cancer at even low concentrations (5μM).

In conclusion: Through downregulation of the transcription factor NFκB, which restores the production of miR-126 that targets the MMP-9 mRNA, simvastatin possesses anti-proliferative characteristics that limit the growth and metastasis of the B16F10 melanoma cell line dependent on dosage and time approach.

Keywords: melanoma; microRNA (126); Simvastatin; metastasis; B16F10; MMP9.

Chapter One

Introduction

1.1 Background

Cancer remains the leading cause of death in every country. The global burden of cancer mortality and morbidity is continuously increasing [1]. The worldwide cancer burden is predicted to be 28.5 cases in 2040, which is a 47% increase over 2020 [1]. The creation of novel drugs can take a long time and is incredibly expensive. Repurposing existing drugs with prospective cytotoxic activity is a way to reduce both the time and cost of developing new cancer drugs [2]. For several years, repurposing of the drug has been utilized for the treatment of cancer, which is now commonly used for cancer treatment [2].

Skin cancer is the most common form of cancer in humans and is classified into three types: melanoma, basal cell carcinoma, and squamous carcinoma [3]. Both basal cell carcinoma and squamous carcinoma are called non–melanoma cancer, which accounts for the highest number of cases in different areas compared with melanoma cancer, which has the fewest cases. Melanoma is the most frequent cause of death caused by skin cancer due to its high tumor growth and resistance to chemotherapy and radiation therapy [3].

Melanoma is one of the most destructive tumors, affecting about 4% of all skin cancers but contributing to 75% of deaths caused by these fatalities. This aggressiveness is attributed to a strong invasive and metastatic potential [4].

Melanoma data from most of the nation show a significant rise in the occurrence of the disease. Melanoma is more common in women than males until they pass the age of 40. By age 65, the occurrence is about three times higher in men than women. The most prevalent places of cancer occurrence vary by gender. The back is the most common for men, whereas for women, the arms and legs are the most common [5].

Melanoma comes from the Greek words melas, which means dark, and the word oma, which means tumor. It was first mentioned in the fifth century by Hippocrates. It is a dangerous disease resulting from the malignant transformation and uncontrolled proliferation of melanocytes [6]. Melanocytes develop from the neural crest, which is an

existing formation in vertebrate embryos. Their primary function is the creation of melanin, which is stored while it is transmitted to keratinocytes [7]. Melanin is a pigment that determines the color of our skin, eyes, and hair. It also protects our skin from sun damage in addition to storing ions and neutralizing reactive oxygen species [8].

1.2 Epidemiology

More people are being diagnosed with cutaneous melanoma than with any other cancer. This is concerning because, in contrast to other solid cancers, where the average age of diagnosis is about 65, melanoma affects a significant number of younger patients, with a median age of diagnosis of 57. In terms of sex, often more women receive diagnoses when they are young, but the situation changes dramatically beyond the age of 55 because more males are receiving diagnoses in that age range [9]. Men had greater mortality rates than women [9], leading to a later admission of the disease to a physician and the resulting diagnosis [9].

1.3 Risk factors

The conversion of melanocytes into malignant cells is a complicated task caused by the interaction of various changeable and non-changeable risk factors [10].

Sunlight

The primary known epidemiological agent related to melanoma is ultraviolet radiation (UVR) [11]. Many people are unaware that distinct styles of sun exposure have different impacts on melanoma growth. For example, sun exposure received from daily outdoor work does not cause melanoma, while large doses of sun exposure received through the holiday or weekend are the most common type of UV light that increases the risk of melanoma [11]. Also, ultraviolet (UV) radiation affects the skin directly and indirectly. Ultraviolet B (UVB) light has a wavelength between 280 and 320 nanometer and is immediately absorbed by DNA, causing DNA damage and perhaps mutagenic alteration in the DNA sequences [12]. The involvement of ultraviolet A (UVA), which has a wavelength between 320 to 400 nanometer in the etiology of melanoma, is argumentative but is presented by its capacity to create intracellular reactive oxygen species that have the potential to be both genotoxic and cytotoxic, ultraviolet radiation

changes the intercellular controlling interaction among keratinocytes and melanocytes in the skin, influencing cell proliferation as well as apoptosis. Furthermore, UV light has immunosuppressive effects that may indirectly enhance tumor survival [12, 13].

Ethnicity

The population's ethnicity has a strong correlation with ultraviolet radiation. Evidence suggests that melanocytes in darker-complex individuals produce larger melanosomes and darker pigment than those in lighter-complex individuals. Eumelanin is a natural defense mechanism that deflects ultraviolet rays and decreases transmission across the epidermis [14]. It is essential to notice that populations with darker complexions have a high incidence rate of non-UV-linked melanoma and melanoma at particular sites [14].

Phenotypic characterization

Light hair color, sunburn sensitivity, light skin color, freckles, and colored eyes (blue or green) are all risk factors for the growth of melanoma [11]. The most significant risk factor for melanoma, independent from other pigmentary features, is a phenotype with a large frequency of common naevi (CN) and dysplastic or atypical naevi (DN). Common naevi (CN) appears throughout the first ten years of life, rises significantly throughout the second, and reaches its highest by the third. Following that, CN was expected to grow up, retreat, and eventually disappear, becoming uncommon in the elderly. Dysplastic naevi (DN) is clinically classified as massive naevi (≥5 mm in diameter) with a wide margin and inconstant pigmentation. Histologically, dysplastic naevi (DN) is a colored naevi with cytological atypia and structural disorder [15]. DN is the transitional step between common naevi and cutaneous melanoma in a multistep tumor development process. DN is a risk factor for melanoma as well as the initial lesion in a subset of cutaneous melanoma [15].

Gene mutations

First of all, pigmentation genes like the melanocortin 1 receptor (MC1R), which is the major well-known pigmentary gene related to melanoma [16], have been linked to both phenotypes and melanoma, such as red hair, while others are linked to melanoma only, which suggests that this gene plays an essential role in melanoma [16]. Somatic genetic mutations that activate mutations in the BRAF gene are frequently detected in

melanoma tissue. BRAF is a component of an intracellular signaling pathway component generally stimulated by extracellular growth factors [17]. BRAF activation results in ERK activation, a crucial protein responsible for various critical cellular functions includes survival, proliferation, cell-cell interactions, and differentiation [18].

Age and gender

The chance of acquiring melanoma has been connected with a person's gender and age; melanoma incidence increases with age, according to many studies. Additionally, it has been shown that ultraviolet radiation (UVR) is not the sole key factor in the occurrence of melanoma in younger people when compared to older people. Furthermore, the individual's gender has been found to serve a crucial function. Compared to men, women were more common at the youngest ages. But men are more likely than women to get melanoma after the age of 40 [19].

1.4 Melanocyte development

The skin is separated into three layers from the outside to the inside: first, the epidermis, which contains primarily keratinocyte and dendritic cells like melanocytes, merkle, and Langerhans cells. The second layer is the dermis, consisting of connective tissue like collagen and elastic fibers in addition to blood vessels, glands like the sweating gland, and the hypodermis, which attach the dermis layer to the underlying organs [20].

Melanoma develops from specific cells called melanocytes [21], which can synthesize melanin derived from embryonic cells named neural crest cells. Melanocytes are a small cell population accounting for 1500 per square millimeter and are divided rarely fewer than two times each year [4]. Located at the epidermis's minimum level [4], they transfer as melanoblasts during embryogenic development and eventually end up in the body (skin, inner ear, brain uvea, heart), where they reach their mature stages [22]. All melanocytes share a common biology and important function: they produce melanin via a chemical process accomplished by tyrosinase enzymes within the melanosomes [22]. Based on the presence or absence of cysteine and glutathione in the process, either eumelanin or pheomelanin is also formed when exposure to the UV light keratinocyte release melanocyte-stimulating hormone (MSH), that links to the melanocortin 1 receptor (MC1R) on melanocyte to stimulate melanin formation [23], after that melanocyte distribute melanin to the surrounding keratinocyte via finger-like projection

which penetrates the cells [4], sun-exposed keratinocyte produce melanin that shields the nuclei against the carcinogenic effect as a result of UV radiation [4], keratinocyte matures by keratinization then they undergo anucleation. Finally, they die [4], so melanin pigment in keratinocytes and a layer of dead keratinocytes operate as a shield to protect the live cells under the skin surface [4].

There are two types of melanin: the (black or brown) pigment, which is called eumelanin, and the (red or yellow) pigment, which is called pheomelanin. Skin color determines the ratio of eumelanin to pheomelanin, which is more than the quantity of melanocytes, which is the same in allover the skin [4]. Because darker eumelanin provides a great ultraviolet screen, those with darker are less likely to get skin cancer [24]. Pheomelanin produces less ultraviolet protection and creates carcinogens through its formation [24]. Also, it demonstrates that the generation of more ultraviolet (UVA) produced reactive oxidative species (ROS), resulting in higher DNA damage when exposed to ultraviolet radiation [24].

The changes that occur as normal melanocytes progress to malignant melanoma. The first level called a nevus, also known as the mole, is a melanocyte accumulation that is apparent at birth. Such groups of pigment-filled cells could not be obvious until puberty, implying that steroid hormones activate the pigmentation procedures during this period [21]. It is also defined as a benign lesion characterized by an excess of melanocytes compared to keratinocytes. A nevus growth is constructed, but the growth control of the cells that comprise the nevus is thought to be interrupted. The next level includes dysplastic nevus, distinguished by irregular nests of huge atypical melanocytes. In this level of development, molecular abnormalities affect DNA repair and cell growth.

The prognostic value of nevi is that nearly all melanomas develop from such seemingly benign lesions. Because of the special connection between nevi and melanoma, the physician must understand the clinical categorization of various neval shapes [21].

1.5 Screening

Specific screening could be the most successful, so people including one or two of the risk factors (red hair, the experience of more than three blisters from sunburns before the age of 20, freckling on the back) are 3.5 times greater risk of melanoma

development compared to overall population [25], and those who have three or more risk factors are twenty times more likely, variation in mole, skin, history of melanoma and being in the middle age or older age are also indicators of discovering confirmed melanoma through screening [25], in a dermatology setting the unsupervised self-admitting questionnaire was usefulness in determining patient with melanoma, so clinicians must use the physical examination to screen patients opportunistically because lesions discovered by doctors are thinner than those discovered by the patients. However, no validated risk assessment scale is available to screen melanoma until this time [25].

1.6 Types of melanoma

1.6.1 Lentigo Maligna Melanoma (LMM)

This is the least popular of the four growing conditions, accounting for around 10 percent of the overall cases [21]. LMM typically affects sun-exposed cutaneous areas like the head, neck, and arms [26]. It is 2 to 3 times more common in women than men [21]. This melanoma may be misdiagnosed as a benign age spot, also called sunspot, in the early stages, which can be treatable. LMM can go undetected for years because it is easily misidentified. This can be extremely dangerous [26]. As shown in the figure, the melanoma starts to spread as a flat patch with infrequent borders and varying brown color. It is frequently confused with lentigo simplex, a benign browned color patch that can grow in old people exposed to the sun for many years [26]. As the lesion becomes bigger and deeper in the skin, it may turn different shades of brown and form nodules, representing the invasive tumor [26].

1.6.2 Superficial spreading melanoma (SSM)

It is the most prevalent form of melanoma and appears more common among younger patients than other types [26]. It spreads along the epidermis layer for a while before going deeper into the skin. This type most commonly appears on the back, trunk, and extremities [26]. It manifests as a flat, slowly growing, infrequent lesion with varying pigmentation [26]. This is generally represented

Clinically mostly by the presence of a raised area [26]. This type will not become dangerous until it goes deeper into the skin [26].

1.6.3 Acral lentiginous melanoma (ALM)

This type includes acral areas, which mean extremities like the bottoms of the feet, subungual region, and the hands' palms [26]. They typically manifest as slow-growing and variegated colored macules. The dermoepidermal junction could be an important diagnostic indication of this type [26]. Lesions are typically black or brown, with color variations and irregular edges [26].

1.6.4 Nodular melanoma (NM)

This type is the most violent type of melanoma. It is most prevalent in older people and more popular in men than women. This type commonly manifests as a rapidly growing nodule with hemorrhage and ulceration [26], mostly found on the head, trunk, and neck [26].

1.7 Stages of melanoma

Cancer staging is important for providing physicians with prognostic data, assisting them in developing therapeutic options for patients, and assessing the surviving rates. This is critical, especially in melanoma, because the prognosis varies greatly in various stages of the disease. In 1998, the American Joint Committee on Cancer (AJCC) established a melanoma staging system (TNM system), which is a foundation for clinical classifications. The stages of melanoma are determined by factors like Breslow thickness, mitosis, ulceration, involvement of lymph nodes, and spread to another organ [27, 28].

1.7.1 Localized Melanoma (Stages I and II)

Tumor characteristics (T) are used only to describe tumors at these stages in the TNM staging system. At this time, such features are divided according to the level of ulceration and tumor Breslow thickness [28]. Localized tumors are classified as T1, T2, or T3, depending on the tumor Breslow thickness, which is 1.0, 2.0, or 4.0 mm, respectively. The survival rate will decrease when the tumor thickness increases [28]. Additionally, ulceration is commonly used as a negative prognostic indicator because it is associated with more severe cases and causes a reduction in patient survival, so the presence or absence of ulceration is marked as “a” and “b,” respectively, in each T

subcategory; for example, T2a refers to non-ulcerated and T2b refers to ulcerated stage II melanomas [27, 28].

At each stage, the tumor is localized but invasive because the tumor cells have not spread to the tissue around it but have penetrated the dermis and gone beneath the epidermis [28]. Stage I tumors have Breslow thicknesses of less than 1 mm and might not have ulcers. Tumors in stage II were deeper than 1 mm, and they can or cannot be ulcerated; they remain localized, but compared to stage I, there is a great chance of spreading [28].

1.7.2 Regional Melanoma (Stage III)

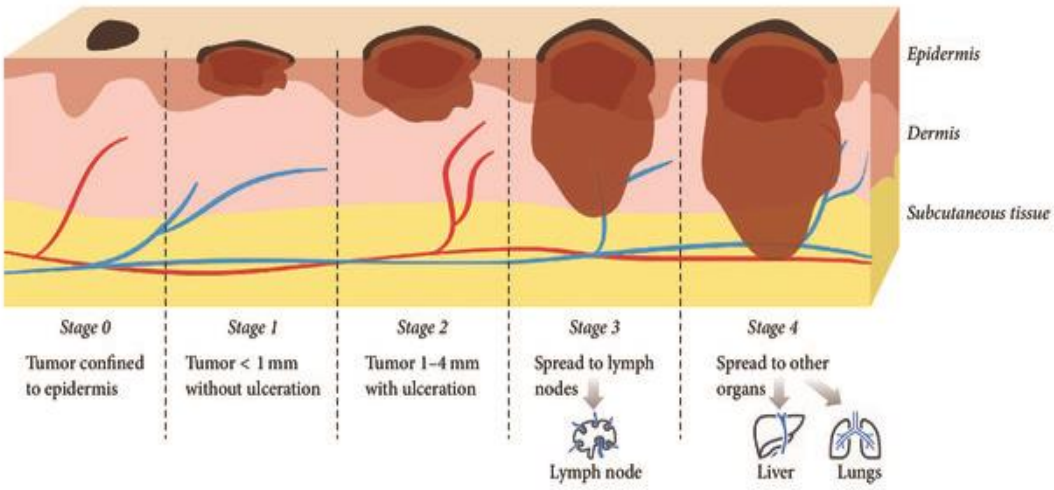
This stage indicates that the tumor has either not migrated to surrounding lymph nodes (non-nodal regional metastasis) or has spread to neighboring lymph nodes (regional metastasis). The degree of disease progression in stage III is determined by whether the tumor has reached the nodes, the number of nodes involved, the number of cancer cells identified, and whether the cells are microscopic or macroscopic. Local lymph nodes that are clinically visible are typically deleted. Breslow thickness is not utilized in staging [28]. The AJCC Staging System recently created four categories, including stage IIIA-D. Patients with stage IIIA have a 93% survival rate, while those with stage IIID have a 5-year survival rate of 32% [28].

1.7.3 Metastatic Melanoma (Stage IV)

The disease has metastasized from nearby tissues to distant organs or lymph nodes [28]. These tumors are categorized according to the development of metastases (M). There are four M subcategories (a-d) dependent on the site(s) of metastasis. The most recent version of the AJCC Staging System adds a new M subtype (d) to account for individuals who have central nervous system (CNS) metastases [28, 29].

Figure 1

The five stages of melanoma



1.8 Prevention

The most important aspects of melanoma treatment as a public health issue are early detection and prevention.

Prevention has three levels: primary, secondary, and tertiary. According to Malignant Melanoma, primary prevention focuses on increasing health education, seeking changes in behavior relating to changeable risk factors, and increasing public awareness about the importance of sun protection practices such as decreasing UV exposure and wearing protective clothes. Secondary prevention includes encouraging people to have an annual checkup in order to detect the cancer early [30].

1.9 Diagnosis

One of the most helpful approaches to melanoma diagnosis is the ABCDEs approach, which involves examining the skin from head to toe, searching for any moles, spots, or bumps that appears different such as being dark in color [31] if we find any changes in one of the moles or more, the physician should follow the ABCDEs roles of melanoma which include (A) ASYMMETRY: if one-half of the moles look differs in color or shape from the other half, (B) BORDER: if the moles represents any irregular or blurred border, (C) COLOR: if the moles vary in colors, (D) DARK: if the moles appear dark or its surrounding region seems dark, (E) EVOLVING: if the skin has moles, spots, or

bump evolved or changed in a different way than the rest of the skin in the body [31], most melanoma has at least one of these characteristics [31].

1.10 Treatment

The current treatment options involve chemotherapy, immunotherapy, photodynamic therapy, and surgical removal. The therapeutic strategies may include single agents or combination therapies based on the patients' health, location, and tumor stage [32].

Surgery

Surgical resection is the most prevalent therapy for melanoma in its initial stages. The recommended clinical zone for the primary diagnostic excision (diagnostic excisional biopsy) is 1 to 3 mm from the major tumor. After verifying a diagnosis, first-line treatment is determined to be a wide local excision (WLE) of 1 to 2 cm to the primary scar. Sentinel lymph node biopsy (SLNB) can also be performed for further staging in patients with melanomas that have moderate to elevated Breslow thickness (>1 mm). This procedure evaluated the possibility of microscopic metastatic dissemination in the next regional lymph node (RLN). The original Breslow thickness of the tumor determines the excision margin (WLE). Although wider margins are required for larger Breslow thickness, most in-suit melanomas can be adequately treated with a 5 mm wide local excision margin.

Patients with RLN metastases confirmed cytologically are additionally checked for further metastases in faraway organs. Lymph node elimination is performed per clinical standards if the condition is localized. When a patient is diagnosed with a distant metastatic illness, systemic treatment is recommended rather than lymph node removal.

Chemotherapy and radiotherapy

The primary option for therapy among patients at late-stage melanoma was chemotherapy. In 1974, the FDA approved decarbonization as a melanoma chemotherapeutic agent [33]. Despite being mainly ineffective in terms of therapeutic response, it was nevertheless an essential type of treatment prior to the introduction of specific treatment as well as immunotherapies [34]. Even so, in terms of therapeutic response, it is usually ineffective. Temozolomide (TMZ) was also utilized to treat

metastatic melanoma and had a better progression-free survival rate than decarbonize. However, such a finding was not designed for the general survival [34, 35].

As opposed to chemotherapy, radiotherapy is infrequently utilized as first-line treatment; however, radiotherapy can be applied as a complement to therapy as an alternative option for advanced inoperable cases [36].

Immunotherapy

Recombinant antibodies directed against checkpoint proteins on the cytotoxic T cells, which are the immune system's natural protectors, are the foundation of modern immunotherapy. Two monoclonal antibodies that block PD-1 (programmed cell death 1), a co-inhibitory molecule on T cells that inactivates the T cells when it comes into contact with ligands (PD-L1, PDL2) found on tumor cells and stroma cell, are Nivolumab and Pembrolizumab [37].

Adaptive T-cell therapy

Steven Rosenberg and colleagues created adaptive T-cell treatment in 1988 for patients with metastatic melanoma by utilizing autologous TILs. ACT has not been demonstrated to be an effective primary therapy for melanoma, but multiple clinical studies have shown its efficiency in managing progressed stages of melanoma is not responding to the target treatment [38].

Target therapies

The importance of oncogenic driver mutations in melanoma led to the development of corresponding inhibitory drugs, specifically in the MAPK cascade, which stands for mitogen-activated protein kinase. BRAF mutations are common in a substantial proportion of melanoma driver mutations. Two selective inhibitors of mutant BRAF, Vemurafenib and Dabrafenib, were linked to increasing melanoma patients' survival after treatment and were approved by the FDA in 2011 [39]. A particular MEK inhibitor called Trametinib, which acts downstream of BRAF, has also been shown to enhance survival in patients with BRAF mutations compared to chemotherapy [40]. Regarding the effectiveness of kinase inhibitors in treating melanoma, resistance to drugs is a significant issue, and there are few benefits to long-term treatment. Several factors underlying resistance to targeted medicines and strategies for overcoming such

resistance have been proposed. However, research suggests that combining targeted medicine with immunotherapy may result in a more effective treatment response than stand-alone therapy [41].

1.11 Problem statement

Melanoma cancer is the most significant one among the several forms of skin cancer because of its susceptibility to metastasis to another region of the body. It causes significant sickness and death, so a huge amount of studies focus on the treatment of melanoma.

Since statin has been linked to improved cardiovascular health by lowering cholesterol and up regulation of NFκB, we postulate that NFκB will be the option in upregulating mir-126 and its target genes in melanoma cancer. Because of its importance as a diagnostic and therapeutic intervention, MMP-9 could be a viable component in the management of skin cancer.

There is still a discussion of the relationship between statin and its effect on melanoma cells through the microRNA technique, so there is a gap or lack of definitive evidence. This encourages us to perform many studies on statin drug and their effect on melanoma

1.12 Study significance

Since melanoma is the most deadly form of skin cancer, accounting for 80% of cases [42], there is a growing need for novel therapies for patients whose melanoma has spread to other parts of their body. A poor response rate and significant side effects represent the current therapy generation. However, recently, novel drug classes that work by focusing on certain growth factor receptors and the intracellular signaling pathways that connect them have been created. So, developing a more effective drug to treat skin cancer is important.

1.13 Study objectives

1. Simvastatin may help prevent melanoma cancer by controlling the expression of microRNAs.

2. Study the influence of statin through mir-126 and its target gene on melanoma.

3. Study the effect of modulation of NFκB factor by statin on decreasing growth and metastasis of melanoma.

4. Suggests that mir126 is overexpressed by NFkB, which has a role in the regulation of genes involved in melanoma cancer, like MMP-9.

5. Detect the MMP-9 activity on the melanoma cell line.

6. Analyses the effect of simvastatin on melanoma B16F10 and MMP-9

1.14 Research questions

1. Does statin drug increase the expression of mir-126 to inhibit the expression of MMP-9 and prevent melanoma cancer growth and metastasis?

2. How can statin drug influence mir-126?

3. Modulate NFκB expression or function affect melanoma cell growth and metastasis through mir-126?

1.15 Hypothesis

Statin may raise mir-126 by modifying NFκB expression or function to suppress MMP-9, an oncogene associated with melanoma cancer.

1.16 Literature review

Many studies have been performed to determine if there are potential associations between statin and the ability to influence multiple genes in cancer through various mechanisms. Some have reported a positive effect, and others found a negative effect.

1.16.1 Statins

Well-known cholesterol-lowering drugs have emerged as one of the most commonly recommended drug globally due to their efficiency and safety since 1987. This is approved for hyperlipidemia reduction and maintains and inhibits cardiovascular diseases. Statins bind and prevent the HMG-COA reductase enzyme that stimulates the formation of cholesterol and isoprenoid via the mevalonate pathway [43].

Statin is recognized as the first-line therapy for hypercholesterolemia by international guidelines due to its important capacity to reduce cholesterol blood level [44], which acts as a hydroxymethyl glutaryl coenzyme A reductase (HMG-CoA) inhibitor, one of the most regularly prescribe families of medication in the world, its consist of seven medications pitavastatin, , rosuvastatin, atorvastatin, simvastatin, pravastatin, lovastatin, and fluvastatin [45].

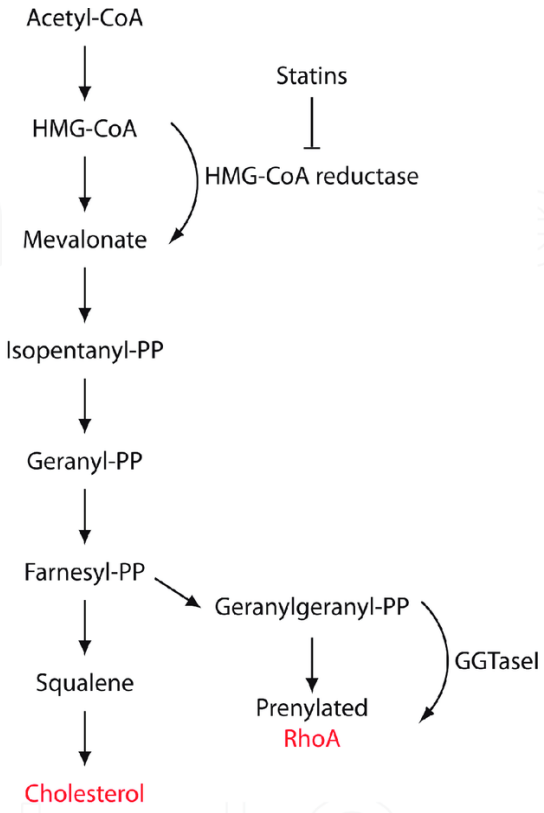
1.16.1.1 The mevalonate pathway

High blood cholesterol is an important risk factor for heart disease, and statins are a frequent medicine used to decrease high levels of cholesterol and LDL(low-density lipoprotein) cholesterol levels [46] by lowering hepatic cholesterol synthesis, which results in higher LDL receptor turnover, greater hepatic LDL-cholesterol absorption, and eventually lower plasma LDL-cholesterol levels [46].

HMG-CoA is responsible for converting HMG-CoA to mevalonate, which is the rate-limiting step of the mevalonate pathway. This pathway generates many end products, which are significant for many cellular functions. For example, to become functionally active, proteins have to go through prenylation (farynesylation and geranylgeranylation) to associate with the plasma membrane. HMG-CoA reductase inhibitors block the rate-limiting step of the mevalonate pathway, resulting in lower levels of mevalonate and its downstream products, which may have major effects on many essential cellular functions [47].

Figure 2

The Mevalonate Pathway



Several studies have revealed other features of statins and their potential effect on other diseases rather than lowering cholesterol, such as decreased inflammatory and thrombogenic responses, the stabilization of atherosclerotic plaque, enhanced endothelial dysfunction, and effects on various types of cancers. At the same time, some studies conclude that statins have no effect on different types of cancer or suggest a low cancer rate among statin users [48, 49]. Others conclude that statins may increase the risk of certain cancers [50, 51].

The major reason for such contentious study results is related to the statins type, lipophilic or hydrophilic statins, which have a different effect on the extrahepatic tissues [51]. This distinction between the two types leads to disparities in the findings of the studies on the effect of statins on cancers. Several studies show that lipophilic statins have anticancer effects, not hydrophilic [52].

1.16.1.2 Pharmacokinetics of statins

Clinical pharmacokinetic features of statins vary, including bioavailability, half-life, lipophilicity, and maximum plasma concentration. Most of the statins are metabolized by the liver before reaching the systemic circulation, which causes their relatively low systemic bioavailability. The lipophilicity (hydrophilic and hydrophobic) does not always provide the same effects. For example, in gynecological tumor cells, the anti-proliferative effect appears to be restricted to lipophilic statin like the ovarian cancer cell line, which is affected by lipophilic satin such as simvastatin and lovastatin. In contrast, the hydrophilic pravastatin has no effect. Hydrophilic statin must be transported into the cell. In contrast, lipophilic statins enter cells simply by diffusion [53]. Atorvastatin, Fluvastatin, Simvastatin, Lovastatin, and Pitavastatin are lipophilic statins, whereas Rosuvastatin and Pravastatin are hydrophilic statins [53].

Statin as an antioxidant agent

Numerous studies conducted in recent years have demonstrated the etiologic involvement of free radicals in both cancer and noncancerous disorders in human [50].

At the site of inflammation, activated phagocytes like monocytes, macrophages and neutrophil release significant free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), this formation of ROS has been found to be caused by the activation of the Nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase enzyme throughout phagocytosis [43]. Recently, a direct relationship between NADPH oxidase and lipopolysaccharides (LPS) induced innate immunity as well as inflammation. This inflammatory process that produces free radicals can oxidize low-density lipoprotein (LDL) cholesterol, thereby accelerating the atherogenesis process. These free radicals can also play an important role in several disease, including cancer, therefore it is important to assess the antioxidant properties of statin as a hypocholesterolemic agent. There are various mechanisms by which statin disrupts oxidation, which might reduce the progression of atherosclerosis, decrease circulating oxidized low-density lipoprotein (oxLDL), and prevent macrophages from absorbing them, in addition to their direct antioxidant effects [46]. Statins limit the activity of oxidant enzymes like NADPH oxidase and myeloperoxidase while increasing the activity of antioxidant enzymes including catalase and paraoxonase [56].

1.16.1.3 Statins and cancers

For many years, researchers have been studying the links between prescription statin therapy and cancer-related deaths. Nielsen et al. discovered a reduction in mortality from cancer within statin consumers who were administered a statin prior being diagnosed with cancer [54]. Another finished cohort study in Finland found that each pre-diagnostic and post-diagnostic statin use was related to decreased chance of dying from breast cancer, these results being based on dose [55]. During a 10-year follow-up period, patients with breast cancer who were used simvastatin experienced a markedly enhanced breast cancer-free survival rate, in ten lower recurrences every 100 women. A Danish trial by Ahern et al. looked at utilizing the recurrence of breast cancer as a clinical outcome [56].

1.16.1.4 Simvastatin

Simvastatin (Zocor) is the most commonly prescribed drug of all statins, with over 500000 patients enhancing from treatment. This indicates the clinical tolerability, cost-efficacy of the drug, and its safety for prolonged use. It is the first lipid-lowering drug established for lowering morbidity and mortality in those suffering from coronary heart disease (CHD), according to the publication of the landmark Scandinavian Simvastatin Survival Study (4S) in 1994 [57]. It is classified as a necessary medication by the World Health Organization [57]. It was initially used in Sweden in 1988 [58], and in most countries, simvastatin became a generic medication in 2003 [58].

Lovastatin is a natural compound first isolated from the filamentous fungus Aspergillus terreus and became a major drug to treat hypercholesterolemia. Simvastatin, a semisynthetic compound of lovastatin with one methyl group added to the side chain, has become the most widely prescribed cholesterol-lowering medication [57].

1.16.1.5 Simvastatin in cancer

Many studies have shown that simvastatin has antiproliferative effects on various cancer cell lines. According to these studies, simvastatin prevents cancer cell growth by causing apoptosis and stopping cell cycle progression through multiple cell signaling pathways [59]. Based on current research, the impacts of this drug depend on the cell line, the dosage, and the length of time the cell is exposed to the drug [59].

Many researches show a decrease in the development and cancer incidence death in patients receiving simvastatin medications. Cardwell and colleagues 2015 discovered the link between the use of statins after a lung cancer diagnosis and a reduction in specific mortality in more than 25% of patients [60]. They also pointed out a decrease in breast cancer patient's death rates following simvastatin use in a retrospective cohort study of 17880 patients [61]. A cohort analysis of 2142 patients suffering from pancreatic ductal adenocarcinoma demonstrated a decreased risk of death, with patients receiving simvastatin died regardless of their cholesterol level. Based on these findings, they proposed that simvastatin actions occur via a lipid-independent mechanism [62]. Yang et al. indicated a 20% lower risk of lung cancer in females who used simvastatin for five years [63]. According to Chen et al. simvastatin reduced comorbidities and the risk of this carcinoma in diabetic patients by approximately 63% [64].

1.16.2 Nuclear factor kappa-light chain enhancer of activated B cells (NFκB)

Cancer is caused by uncontrolled cellular growth of tumor cells as a consequence of a variety of genetic alterations that result in neoplastic transformation and slip away from inhibitory signals, several molecular pathways associated with the initiation, survival, proliferation, invasion, and progression, such as MAPK pathway, PI3K-AKT pathway, MITF pathway and NFκB pathway [65].

1.16.2.1 NFκB pathway

Nuclear factor κB is an essential transcription factor associated with many biological processes such as immune response, stress responses, cell survival, and cell maturation. NFκB activation is essential for protecting organisms from environmental impacts. Abnormal NFκB is frequently observed in a variety of diseases like inflammation and cancers [66]. NFκB is identified in almost all cell types and is associated with activating an unusual number of genes with the response to inflammation [67], infections, and stressors that necessitate rapid gene reprogramming of gene expression [67].

Sen and Baltimore identified the nuclear factor kappa light chain enhancer of activated B cells (NF-κB) transcription factor in 1986 [68]. NF-κB was first identified as a protein that attaches to the immunoglobulin kappa-light-chain enhancer in the nucleus of B cells [68]. However, it was discovered that every cell controls the physiological

processes when reaction with external stimulus, including cytokines, free radicals, stress, ultraviolet radiation, and parts of bacteria and viruses [69].

Many studies have shown that unregulated NF-κB associated with cancer, inflammatory and autoimmune illnesses, septic shock, several infections by viruses, and impaired immune system improvement [69, 70].

The NF-κB transcription factors are dimeric and contain the Rel homology domain (RHD), which has about 300 amino acids. The RHD is found in the amino-terminal regions of the NF-κB proteins and is involved in dimerization, nuclear import, DNA binding, and interactions with a family of inhibitory kB proteins known as IkBs [69].

The mammalian NF-κB family, comprised of five members, involves the RelA, RelB, and c-Rel proteins, which have a trans-activation domain (TAD) in the C-terminus, stimulating gene expression. The NF-κB1 and NF-κB2 proteins are huge precursors, p105 and p100, respectively, that go through proteolytic cleavage to generate the mature NF-κB subunits, p50 and p52 [69, 70]. The p50 and p52 NF-κB subunits cannot initiate transcription since they don't have a transactivation domain (TAD); instead, they can just function as transcriptional activators as they create dimers with other subunits that contain TAD [70]. They can also connect as homodimers and inhibit gene transcription by blocking the TAD dimer from interacting with DNA [70].

The IkBs protein is identified by the presence of 5-7 ankyrin repeats (ANK) responsible for the NF-κB adhesion [71]. The IκBs mask the nuclear localization signal (NLS) of NF-κB proteins and keep them separated and dormant in the cytoplasm, isolating them from their inhibitory properties because they have six or more ankyrin repeats [72]. The IκBs also have a PEST and an N-terminal regulatory domain [72]. There are many IkB proteins, such as IκBα, IκBε, IκBβ, IκBδ, and Bcl-3, as well as ANK domains found in the precursors of p100 and p105, which act as IkB proteins called IκBδ and IκBγ, respectively. The best-known proteins are IκBα and IκBβ, which are found in all tissues; however, IκBε, IκBδ, and Bcl-3 are found in hematopoietic cells [73]. The p50/p65 dimer is the primary target of IκBα, while IκBβ is related to p65/c-Rel dimers [74].

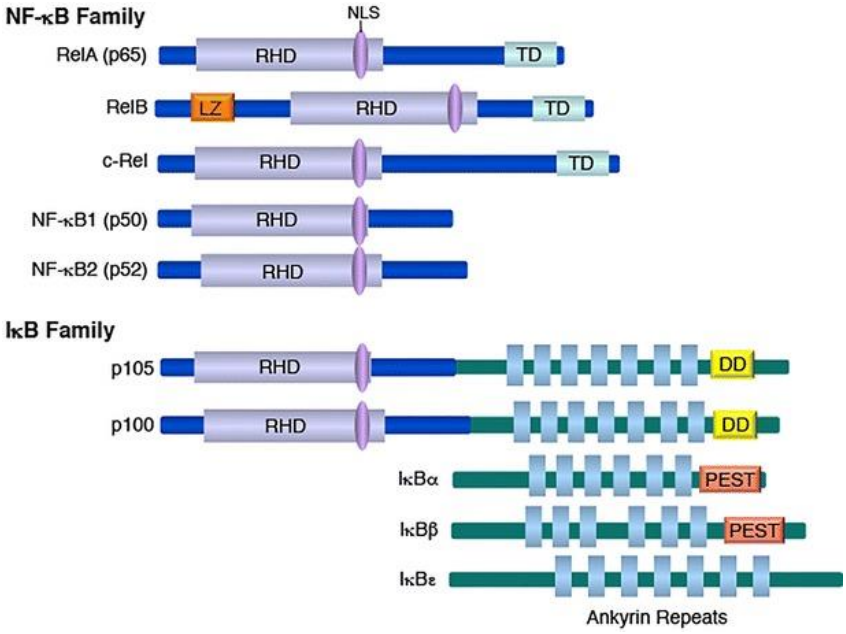
NF-κB is nearly found everywhere. This controls the expression of numerous genes, the vast majority of which are associated with immune and inflammatory reactions. NF-κB plays a critical function in lymphocyte growth and initiation, which required for both innate and adaptive immune reactions. Also, this transcription factor regulates several genes associated with proliferation, cell survival, and differentiation, furthering its role in immunity and inflammation [74].

1.16.2.2 Mechanism of NFκB

In the normal state, NFκB dimers are found in the inactive form duo to their attachment to the inhibitory protein (IkB). Activation of NFκB occurs through the breakdown of this IkB, such as cytokines, hypoxia, and radiation, which result in the transfer of the dimer to the nucleus where they connect to a complementary sequence in the promoter of the DNA sequences, p50/p65 heterodimer is the first discovered dimer and the most common one of dimers. Also several combination have been mentioned either as homodimer including p65/p65, p50/p50, and c-Rel /c-Rel or as heterodimer like p50/c-Rel, RelB/p52, p56/p52, p50/c-Rel [72].

Figure 3

The NF-κB and IκB Families



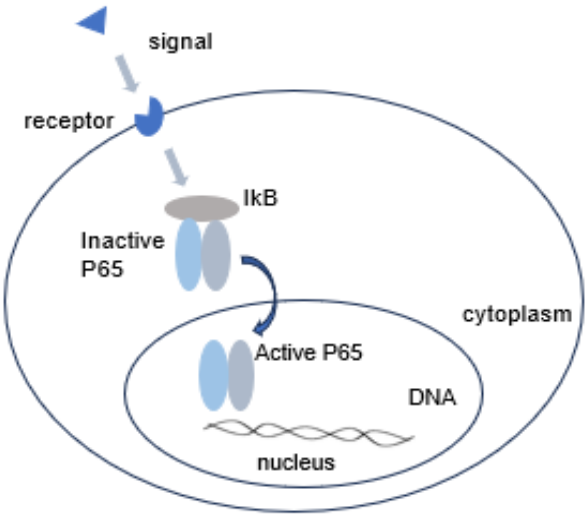
This figure shows the five members of the NF-κB, including the Rel-homology domain (RHD), which regulates the dimerization and DNA binding. The transactivation domain (TAD), which is needed for target gene transcriptional activation and the Lucien zipper (LZ) play a role in target gene transactivation. The IκB family contains the p50 precursor protein p105, the p52 precursor protein p100, IκBα, IκBβ, and IκBε. The ankyrin repeats, which are essential for NF-κB suppression, the p105 and p100s death domain (DD) is essential for their IκB-like functions, the PEST (proline, glutamine, serine, and threonine)-like a sequence of IκBα and IκBβ mediates the turnover of protein [72].

NF-κB has two signaling pathways: the canonical (classical pathway) and the non-canonical (alternative pathway). The canonical pathway represents the greatest abundant signaling associated with activation of NF-κB, which developed through p50, p65, RelB, and c-Rel subunits antigen receptors and inflammatory cytokine stimulates this pathway, the cytoplasmic IB Kinase (IKK) complex, comprising two NF-κB essential modifiers (NEMO) and catalytic alpha and beta subunits, which is recruited in this pathway, the canonical pathway cannot be activated without the IKKB. The canonical NF-κB pathway includes dimers with a p50 subunit, either the p65/p50 heterodimer or p50/c-Rel [75].

The non-canonical pathway, which is primarily participant in immune cell function and bone remodeling, is responsible for the stimulation of p100/RelB dimers [74, 76], works primarily to activate dimers with a p100/p52 subunit, most frequently the p100/RelA heterodimer, via an IKK complex that has two IKK subunits (but no NEMO). In order to partially proteolysis and release of the p52/RelB complex The IKK complex phosphorylates two Serine residues next to the ankyrin repeat C-terminal IkB domain of p100, [74, 77].

Figure 4

Mechanism of NFκB



1.16.2.3 NF-κB and tumors

Many cancers have dysregulated NF-κB, which causes abnormal expression or stimulation of NF-κB transcriptional complexes. Because of its involvement in the regulation of apoptosis, proliferation, and tumor angiogenesis, in addition to malignant cell invasion and metastasis, this transcription factor can act as a new therapeutic strategy in the treatment of many cancers such as melanoma, where NF-κB is overexpressing [78].

1.16.2.4 Simvastatin effect on NF-κB transcription factor:

So many strategies have been used to prevent NF-κB activation. Various compounds have been tested for their ability to inhibit NF-κB. These compounds inhibit NF-κB through different mechanisms like blocking the binding of NF-κB to the DNA, blocking the proteasome from destroying the IB inhibitory subunit, inhibiting the phosphorylation of IκB, reducing NF-κB by gene transfer, blocking nuclear translocation, using antioxidants, and inhibiting direct activation via p50 and p65) [79].

The active form of NFκB is a heterodimer composed of p50 and p65 subunits. Cytoplasmic NFκB binds to the " inhibitor of NFκB "(IKB) which inhibits NFκB transcriptional activity, an extracellular stimuli like pro-inflammatory cytokines and reactive oxygen species (ROS) stimulate the IkB kinase complex (IKK) leading to

phosphorylating IKB and resulting in ubiquitination and degradation of IKB, human RhoA, CDC42, and Rac-1 quickly and effectively activate NFκB transcription through phosphorylating IKB and translocating p50/p50 and p65/p50 dimers to the nucleus, statin by reducing prenylation of such proteins play a significant role in suppressing NFκB action and other inflammatory process [80].

1.16.3 MicroRNAs

Different studies developed a great molecular biology tool that collected massive bioinformatics data on each mRNA and non-coding RNA expression[81]. By a computational technique, they discovered distinct microRNA related to the development of cancer, genes, and protein, so it was possible to find certain microRNA associated with melanoma [81].

Modification of cellular phenotype utilized by a regulatory process of small noncoding micro-RNAs with 22 nucleotides in length [82].

MicroRNAs are a type of short, non-coding RNA that regulates the expression of mRNAs. All cellular processes, fat cell division, and the evolution of cellular morphologies are controlled by miRNA, which usually suppresses gene expression or can stimulate it [83]. MiRNA controls its own mRNA through immediate target recognition, which is accomplished by perfect or imperfect base pair binding. These can either prevent translation or result in the full destruction of the target [84, 85].

MicroRNAs work at the posttranscriptional level by suppressing the target mRNA expression, mainly via interactions with the 3 UTR (untranslated region) [86, 87]. When a miRNA binds to its target mRNA with imperfect base-pairing, it generally mediates translation repression [86]. When a miRNA binds to its target mRNA with perfect base-pairing, it mediates mRNA degradation through the removal of the poly (A) tail of the mRNA. Loss of this poly (A) tail causes the mRNA susceptible to exonucleolytic destruction [86, 87].

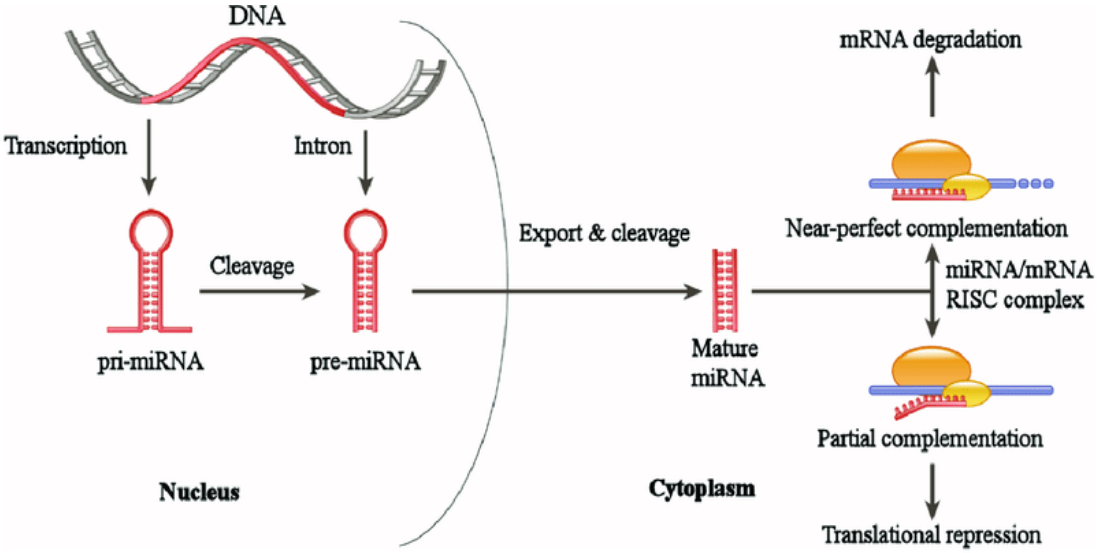
1.16.3.1 Biogenesis of MicroRNA

The biogenesis of microRNA starts in the nucleus; it might be expressed in the same manner as other genes, and it often contains hundreds of bases in length. MiRNAs are transcribed by RNA polymerase II to form a long primary miRNA (pri-miRNA) with a

stem-loop structure [88]. This pri-miRNA undergoes processing before leaving the nucleus. First, this pri-miRNA cleaved to pre-miRNA through a protein complex called DiGeorge syndrome critical region gene 8 (DGCR8), another name for the Pasha, which stands for double-stranded RNA-binding protein and Drosha stands for RNase III enzyme [88]. Pre-miRNA is dissociated with nucleocytoplasmic transporter containing Exportin-5 and transported to the cytoplasm [87, 89], then undergo additional processing by Dicer complex, which removes the stem-loop structure resulting in double-stranded RNA of 20-25 nucleotides called miRNA:miRNA*duplex [90], finally this double strand complex associated with silencing complex called RISC [87, 90], one strand which is the mature RNA guides the RISC complex to the targeted mRNAs and the other strand is degraded [87, 90-92].

Figure 5

Biogenesis of MicroRNAs



In the nucleus, MicroRNA is transcribed to either pri-miRNA or directly to pre-miRNA, then pre-miRNA is transported to the cytoplasm, where it is further cleavage to generate mature miRNA. MiRNA binds to its target mRNAs by forming a complex with miRNA-induced silencing complex (RISC). The translation is repressed if there is partial complementation between miRNA and mRNA. If there is near-perfect complementation between miRNA and mRNA, then the mRNA is degraded. In both ways, there is a decline in the transcription rate [93].

1.16.3.2 MicroRNA in cancer

Tumor growth and progression are commonly viewed as a series of stages marked by the accumulation of genetic alterations over a period of time. Each modification accumulates specific characteristics leading to the malignant condition [94]. Malignant cancer will proliferate uncontrollably, develop resistance to programmed cell death (apoptosis), and gain the potential to invade and metastasis to other tissues [94, 95].

Calin and colleagues in 2002 discovered two microRNA**,** mir-15a and mir-16, in the chromosomal region 13q14. Most chronic lymphoid leukemia (CLL) have this genetic region removed. In over 70% of cases with this loss, the 2 microRNAs were under-expressed [96]. They also discovered that the majority of human microRNAs are found in the genomic area associated with chromosomal abnormalities in human malignancies [96]. Following these preliminary findings, many researches did microRNA expression profiling and reported aberrant miRNA expression as a common event in several cancer types.

1.16.3.3 MicroRNA and transcription factors

MiRNA can behave as an oncogene in cancers or tumor suppressor genes. However, defining the miRNA as an oncogene or tumor suppressor gene may be inaccurate because the function of each miRNA may vary depending on the biological context in which it functions. MiRNA could also be mutated or epigenetically changed, inhibited, or activated by transcription factors, resulting in a change in their expression [97]. Transcription factors (TFs) can attack and control the expression of particular miRNAs. Also, miRNAs can target TFS mRNAs. This feature of miRNA and TF allows cells to build a genomic regulation network wherein negative and positive feedback can function together to effect the genomic nature of the cell [98]. For example, significantly reduced mir-9 expression increased NFκB1 overexpression, improving the NFκB activities and controlling the proliferation of ovarian cancer [99].

1.16.3.4 MicroRNA-126

MIR-126 is a small RNA that acts as a regulator for many cellular processes. It is expressed only in the endothelium. It is also found in the seven introns of the EGFL7 gene, which is located on chromosome 9. There are three isoforms of EGFL7 (EGFL7

isoform A, B, and C), which possess the same open reading frame but are produced by different promoters and use different exons [100]. It affects the expression of different genes through the post-transcriptional process. Based on the kind of cancer, mir-126 has been proven to behave as both a tumor suppressor and an oncogene [101, 102].

Endothelial cells have a high level of expression of mir-126, which is delicately their phenotype. Various physiological and pathological conditions, including angiogenesis, atherosclerosis, and the pro-inflammatory response, influence the expression of mir-126. The lack of vascular integrity and abnormalities in endothelial cell proliferation, migration, and angiogenesis are caused by the deletion of mir-126 [82].

Mir126 inhibits angiogenesis, proliferation, and migrations of endothelial cells while maintaining vascular integrity. Mir126 promotes endothelial cell survival by avoiding apoptosis caused by hypoxia and oxidative stress [83].

1.16.4 Matrix metalloproteinase (MMPs)

Tumor cell invasion and metastasis are multistep methods in which tumor cells leave the primary tumor, penetrate the surrounding tissues and basement membranes (BM), intravasate into the lymphatic or blood, and then adhere and extravasate in external organs to create a secondary tumor [103]. Malignant tumors spread by degrading the extracellular matrix (ECM) and connective tissue surrounding the tumor cells; matrix metalloproteinases (MMPs) break all elements of the ECM and the BM, which are required for cell motility, angiogenesis, and cancer metastasis [103].

The extracellular matrix serves several important functions involved in cellular homeostasis and cell-cell connections. Defects in the extracellular matrix cause structural and morphological changes linked to clinical diseases in many different receptors and chemicals that regulate the extracellular matrix and cell interactions, which regulate cell attachment and alter the expression of genes concerned with differentiation and cell growth[104].

The cancer ECM differs from other tissues in rigidity and content, and it is essential for several stages of tumor development. For example, it can store growth hormones and regulate metastasis by enhancing or inhibiting cell invasion. The ECM is made up of several proteins like glycoprotein, collagen, and proteoglycans, which are generated by

fibroblasts. The growth factor can enhance tumor growth through the production of matrix metalloproteinase family, also known as (MMPs)and disintegrin and metalloproteinase (ADAM) family by tumor cells [105].

Tumor cell invasion and metastasis have been found to involve proteolytic activity to destroy extracellular matrix elements. Hydrolysis of the extracellular matrix seems to enhance tumor cell migration, contributing to cancer's metastatic spread. Matrix metalloproteinases constitute a significant group of proteases linked directly to tumor metastasis and are a family of endopeptidases defined to separate several proteins of the extracellular matrix [106].

The matrix metalloproteinases are a group of proteolytic enzymes that degrade various extracellular matrix elements. MMPs have been involved in tumor invasion, metastasis, and angiogenesis, making them a target for cancer therapy [107].

MMPs are extracellular matrix proteins responsible for the constant alteration of the extracellular matrix that has different substrates, including collagens, proteoglycan, gelatins, and elastin, which have a broad range of action on different proteins. MMPs have been divided into six groups based on structure and function: gelatinases, matrilysins, collagenases, stromelysins, membrane-type MMPs, and other non-classified MMPs [108].

MMPs structure

MMP comprises multiple domains shared by all members of the MMP family, involving prodomain, propeptide, catalytic domain, and hemopexin domain. The propeptide domain typically contains 80 amino acids; the catalytic region has roughly 170 amino acids, and a continuous three histidine necessary for zinc chelation. MMP has a hinge domain with varying lengths and a hemopexin domain of around 200 amino acids, which is necessary for MMPs interactions with other MMPs or with TIMP(tissue inhibitors of metalloproteinases) [108, 109].

There are 23 known human MMPs. They are naturally formed as proenzyme, which are produced inactively by an interaction between the zinc ion and cysteine-sulfhydryl group in the N-terminal domain. The release of this interaction is required for activation, a mechanism known as (cysteine switch) that can take place either intracellular or after secretion through prohormone convertases called (furin), which is temporarily activated. Total activation is accomplished through autocatalytic procedures where the proteinases break down their prodomain, which the enzyme could then degrade and inhibit itself, a regulatory mechanism found in many MMPs [107].

The function of MMPs in metastasis

Metastasis is a multiple-step process that begins with the loss of intracellular interactions and the release of unattached tumor cells via anoikis avoidance, progresses to extracellular matrix destruction and cell migration, entry to the blood vessels or lymphatic system, attachment to the endothelial cells, and then secondary development in other sites [110]. MMPs play dual functions in tumor development and metastasis: first, they encourage tumor growth by destroying the matrix barriers and increasing angiogenesis; second, they can restrict neovascularization [111].

There is much evidence linking MMPs to cancer metastasis; several studies have shown a link between MMP expression and the metastasis of tumors such as colon, prostate, lung, breast, ovarian, and skin carcinomas [112]. The expression of MMP-2 and MMP-3 is linked to esophageal carcinoma metastasis and cell invasion [113], and MMP-1 expression is associated with the metastasis of colorectal cancer [114].

1.16.4.1 Matrix metallopeptidase 9 (MMP-9)

MMP-9, also known as 92-KDa type IV collagenase or gelatinase B, destroys the extracellular matrix in a wide range of physiological processes and tissue remodeling [115]. MMP-9 is increased in pathophysiological situations like developments and wound healing, as well as inflammatory diseases such as arthritis, cancer, hypertension, myocardial infarction, and diabetes [116].

MMP-9 is called gelatinase B duo to its capability to degrade gelatin. It is made up of an NH2-terminal domain, linker domain, catalytic domain, and hemopexin-like domain at the COOH terminus [117]. It also has its own domain called the fibronectin domain,

which is composed of 3 fibronectin type II of 58 amino acids. This domain has a lengthy linker between the catalytic and hemopexin domains (Figure A.1) [118, 119].

MMP9 is located within chromosomal area 20q13.12 in humans, which codes for a protein (707 amino acids) released in the cells' matrix as pro-MMP9, a dormant pro-enzyme. Pro-MMP9 is attenuated to 80 amino acid residues on the N-terminus, where the cysteine switch domain controls the zinc ion, producing the protein catalytic domain and making it inactive. In the extracellular matrix, the pro-MMP9 inactive form is converted to the active form by other proteinases such as MMP3 and MMP [120].

Several studies have shown that MMP9 upregulation is strongly impacted by genetic changes or alterations of the tumor microenvironments; furthermore, epigenetic modifications have been demonstrated to cause MMP-9 to be overexpressed in different cancers, including melanoma [120].

MMP9 may be a suitable marker option that can be easily detected in a melanoma patient's peripheral blood sample; furthermore, MMP9 has been demonstrated to be an indicator of aggressiveness in a number of malignancies, including melanoma [121].

Chapter Two

Materials and Methods

2.1 Study time

Work was carried out from July 2022 to October 2023 after getting agreement from the faculty of graduated studies, the Institutional Review Board (IRB) of An-Najah National University in May 2022, and verbal approval from the An-Najah Laboratory for Research on Cancer and Stem Cells in Nablus. Further research writing, including an introduction and literature review, as well as data analysis and results, has been extended until November 2023.

2.2 Study design and sitting

An experimental laboratory study for cancer and stem cell studies was conducted at the Center of Najah University. The research was carried out in controlled conditions in the lab, and the data acquired support the main idea that a relationship exists between simvastatin and decreased melanoma growth and metastasis.

2.3 Materials and methods

2.3.1 Study Cell lines

High-glucose Dulbecco's Modified Eagle's Medium (DMEM) with phenol red (Fuji Film Wako, Osaka, Japan), L-glutamine, 1% penicillin/streptomycin (P/S) (Nacalai Tesque, Kyoto, Japan), and 10% fetal bovine serum (FBS; G.E. Healthcare, Chicago, IL, USA) were used to maintain melanoma cell lines, including B16F10 [CRL-6475; American Type Culture Collection (ATCC), Manassas, VA, USA] as well as B16F1 (CRL-6323). Moreover, high-glucose DMEM, 10% FBS, and 1% P/S were used to culture mouse embryonic fibroblast-1 (MEF-1) cells. B53 cells were cultured in the following culture medium: RPMI 1640 (4500 mg/L glucose), 2 mM glutamine, and 10% FBS. Human umbilical vein endothelial cells (HUVECs) were cultured at 37°C/5% CO2 on 0.1% gelatin (Wako Pure Chemicals)-coated culture plates (Falcon) in endothelial growth medium-2 (EGM-2; Lonza; cc4176). The murine MS-5 stromal cultures were maintained in Iscove's Modified Dulbecco's Medium (IMDM; Wako, Japan), containing 10% FBS and 1% P/S.

2.3.2 Drug treatment in vitro

Simvastatin was kept at -20 °C after being dissolved in a 10 mM dimethyl sulfoxide (DMSO) stock solution. The cells were cultured normally for 24 hours for the experiments and treated with (5, 10, 25, and 50 µM) simvastatin. At the end of treatment, cells were washed with PBS and examined under the microscope. Recombinant human MMP9 (100 ng/ml; R&D system)

2.3.3 Mice

For the experiments, we used male mice of type C57BL/6 with an age range of 8 to 12 weeks at An-Najah National University in Nablus, Palestine. The Institutional Animal Care and Use Committee of An-Najah National University approved the protocols for the animal procedures.

2.3.4 Wound healing

B16F10 motility will be evaluated by utilizing a wound healing assay, as discussed in an earlier finding. Six-well plates will be seeded with cells at around 80% confluence. After the cells are confluent, the six well-plates will be scratched or wound with two lines of equal width in each well using a 1-mL pipette tip spearhead that is positioned perpendicular to the plate's bottom. If a sterilized pipette tip or others damage confluent cell single layers, full DMEM will be applied after the plate has been gently washed three times with PBS. Using inverted microscopy, the cells that migrate into the cell-free space were counted; five randomly selected fields will be analyzed for each well. Three separate experiments will be carried out.

2.3.5 Cell culture

B16F10 cells will be seeded in triplicate on six-well plates from Thermo Fisher Scientific, Lafayette, CO, USA, at a density of 5×10^6 cells/well. The cells will be treated with or without a specified dosage of simvastatin after 16 hours of overnight cultures, treatments include adding PBS (control) and simvastatin at different concentrations (5, 10, 25, 50 µM). After 24 hours. viable cells will be counted with the trypan blue dead cell exclusion dye (#207-17081, Wako). Finally, cells will be collected using Trizol to extract RNA at a specified interval following treatment.

2.3.6 Melanoma Model in Vivo

On day zero, mice received an injection of B16F10 cells (1×10^6/200 µL/mouse, s.c.) following two PBS washes (90% viability, as determined by trypan blue exclusion) and subsequent inoculation. When mice lost more than twenty percent of their starting body weight, had symptoms of extreme pain, or appeared dormant, they were put to death. The tumor growth was recorded every day. On day twelve, we observed the weighing of the removed tumors.

In order to treat local tumors, intraperitoneal injections of simvastatin (100 mg/kg) or carrier (DMSO/PBS) were administrated daily for five days, beginning on day 5.

2.3.7 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qPCR)

Real-time PCR can measure gene expression and allows for rapid and reliable quantification of mRNA transcription [122], which use commercially available fluorescence detecting thermocycle to amplify specific nucleic-acid sequence and simultaneously measure their concentration such as fluorescence labeled probes like TaqMan or DNA binding dyes such as SYBR Green, target sequence are amplification and quantified through the same PCR equipment [123].

Trizol reagent was used to extract total RNA from cell lines, and the PrimeScript RT Master Mix was applied to reverse-transcribed RNA into complementary DNA (cDNA) in accordance with the manufacturer's instructions. This kit is designed for quick and efficient reverse transcription and includes all reagents needed for cDNA synthesis. The cDNA was kept at –30°C. We used the miRNeasy Mini Kit (Qiagen, Germany) for miRNA extraction. The Mir-XTM miRNA First-Strand Synthesis Kit (cat no. 638313, Takara, Japan) was utilized for reverse transcription in accordance with the manufacturer's instructions.

The Step One Plus Real-Time PCR System (Applied Biosystems, USA) with the FastStart Universal SYBR Green Master was used to measure the mRNA expression levels by qPCR.

Using the 2-ΔΔCt procedure, we can determine the relative mRNA expression for each gene and miRNA. Every quantitative PCR analysis has been carried out three times and twice independently. All qPCR data for gene expression were normalized for b-actin mRNA expression unless otherwise mentioned. b-actin mRNA was one of the first RNAs employed as a reference target, as it was ubiquitously expressed [124]. Mir-126 expression level was standardized to kit MRQ smart using the 2−ΔΔCT method. The following primers in forward and reverse directions were used:

Table 1

The forward and Reverse Primers

Gene	Forward	Reverse
mMMP9	5'-AGACGACATAGACGGCATCC-'3	5'-TCGGCTGTGGTTCAGTTGT-'3
mβ-actin	5′-CTAAGGCCAACCGTGAAAAG-′3	5′-ACCAGAGGCATACAGGGACA-′3
m-P65	5′-CCTCTGCTTCCAGGTGACAG-′3	5′-ATGTGAGAGGACAGGGGTCA-′3
miR-126	5'-CATTATTACTTTTGGTACGCGCTGT-'3	kit MRQ smart

2.3.8 Human MMP9 cloning

We used the genomic DNeasy Extraction Kit (Qiagen, Germany) to isolate the human MMP9 coding sequence from the A549 lung cell line and cloned the sequence into the LV-EF- L3T4-IRES2-EGFP vector.

Table 2

The MMP9 cloning sequence

Restriction enzyme	MMP9 coding sequence
XhoI	5- GGGCTCGAGATGAGCCTCTGGCAGCCC-3
EcoRV	5- CCCGATATCCTAGTCCTCAGGGCACTGCAG–3

The XhoI and SmaI sites found in the eukaryotic expression vector LV-EF- L3T4-IRES2-EGFP were used to insert the purified fragment. Furthermore, cloned plasmids were sequenced using the Sanger technique (An-Najah University, Palestine). In DH5α E.coli cells, Plasmids were amplified.

2.3.9 Lentivirus production for MMP9 overexpression

The three-plasmid system (packaging plasmid, transfer plasmid and enveloped plasmid) was used for the preparation of the Vesicular Stomatitis virus glycoprotein–pseudotyped lentivirus (Salama et al., 2020). The LV-EF-L3T4-IRES2-EGFP included mouse MMP9 or no MMP9 gene.

2.3.10 Plasmid Construction

MiR-126 cloning:

Constructs: Genomic DNA from T71b cells was extracted using the DNeasy Tissue Extraction Kit (Qiagen). The genomic sequence of the primary (pri)-miR-126 segments was amplified by genomic PCR using PrimeSTAR polymerase (Takara). The oligonucleotide sequences used for PCR were GGGGCTCGAGCTGGCTCCTTGCCTGGTGGA (forward) and TTTTCCCGGGTGGCCACTGCCACAGCTGTGGGG (reverse). PCR products were purified using a PCR purification kit (Qiagen) and sequenced. The purified genomic PCR product was cloned into the XhoI-SmaI site of a mammalian Lentiviral expression vector, "LV-EF-L3T4-IRES2-EGFP", and the constructed vectors were sequenced to confirm the insertion of miR-126.

2.3.11 MMP9 ELISA

Enzyme immunoassays (ELAs) track and measure immunologic responses by utilizing the catalytic qualities of enzymes. One heterogeneous ELA method used in clinical analysis is the enzyme-linked immunosorbent test. In this kind of test, one of the reaction components is covalently or nonspecifically bonded to the surface of a solid phase, like plastic bead, magnetic particle, or microtiter well. The connection makes the separation of bound and free-labeled reactants easier [125].

The most widely used ELISA methodology application includes adding an aliquot of sample or calibrator containing the antigen (Ag) to be quantified, and then the solid-phase antibody (Ab) allows binding to it. After washing, an enzyme-labeled antibody is added to generate a solid phase Ab-Ag-Ab enzyme (sandwich complex). The enzyme-substrate is introduced after the unbound antibody has been removed. The product produced depends on the amount of antigen present in the sample [125].

ELISA test was performed to detect MMP-9 levels in B16F10 melanoma. As recommended by the company, the ELISA kit for mice MMP9 (R&D Systems, Minneapolis, MN, USA) was utilized.

2.3.12 "Small interfering ribonucleic acids (siRNA)-based gene knockdown"

SiRNA (small interfering RNA) is commonly used in gene function studies and has significant potential as a technique for testing drug targets and treating disease. It is also highly specific for target gene knockdown [126]. Several studies suggest that siRNA-mediated gene silencing may serve as a reliable and valuable strategy for large-scale screening of gene function as well as drug-target identification and validation [127, 128].

Cells were seeded in a 6-well plate at a concentration of (2 x 10^5 cells/well) for 16 hours before transfection with siRNA against MMP9, P65, or control sequences. Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) was used to transfect SiRNA-targeting genes at a final dosage of (100 nM) into cancerous cells. Following 12 hours, an alternative media was added in place of the transfected media. Thermo Fisher Scientific, Lafayette, CO, USA, provided the siRNAs. Knockdown efficacy was determined by qPCR in cells 12 hours after gene silencing.

Table 3

The siRNAs targeting human MMP-9 and P65

SiRNAs	Synthesized Oligonucleotides Sequences
Si-MMP9	5′-ACCUCCCACUAUGUGUCCCACUAUA-3′
Si-P65	5′-CCA UCA GGG CAG AUC UCA A-3′
Si-Ctrl	5′-GCUCCACAGAGUAUACCUU-3′

2.4 GEPIA2

GEPIA2 is a comprehensive online server and resource for studying the relationship between gene expression and immune infiltrates in various sorts of cancer (http://gepia2.cancer-pku.cn/#index). [129].

2.5 Statistical analysis

At least three trials were conducted for each experiment. Microsoft Excel 2016 for Windows was used for all analyses. Findings are shown as the “mean ± standard error of the mean (SEM).” The examination of the two categories was done using the student t-test. P-values <0.05 were considered to be significant. *, **, and *** correspond to $p < 0.05$, 0.01, and 0.001, respectively.

2.6 Ethical consideration

“The Institutional Review Board” of Palestine’s An-Najah University approved the research, which complied with the standards of Helsinki's criteria. “The National Institutes of Health's Guide” for managing and utilizing Laboratory Animals was adhered to when researching animals and euthanizing animals. “An-Najah University Committee on the Ethics of Animal Experiments” accepted the protocol.

Chapter Three

Results

3.1 Simvastatin inhibits melanoma cell growth and migration.

Several investigations have proven that simvastatin inhibits the growth of different cancer cell lines, including lung, breast, prostate, and hepatic cancer, which inhibit cancer cell growth via multiple cell signaling pathways [59, 130]. Simvastatin has been approved for its anticancer effects through inducing cell cycle arrest, apoptosis, inhibiting angiogenesis, reversing multidrug resistance, inhibiting invasion and metastasis, and inducing tumor differentiation [131].

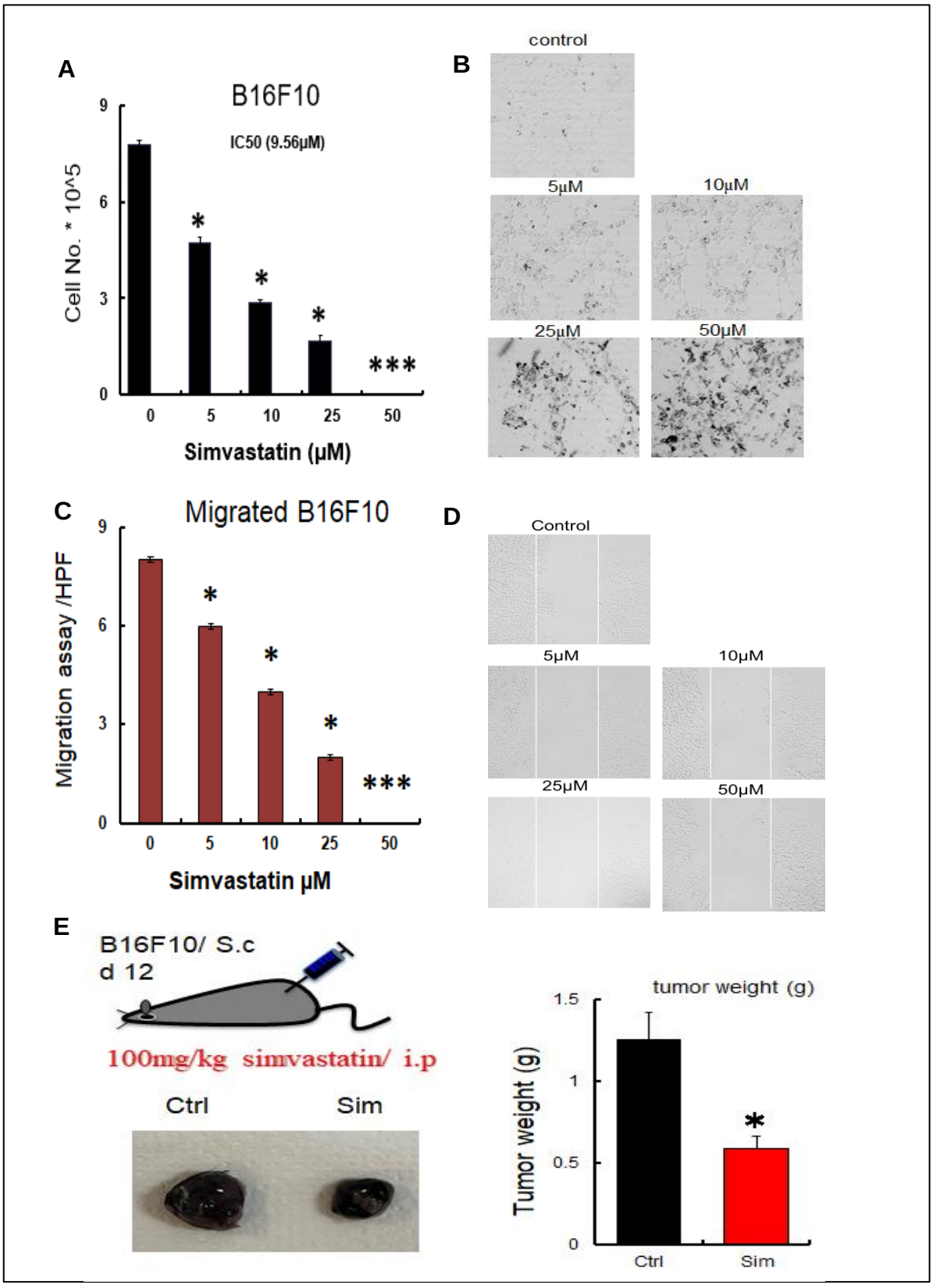
Here, we examined the antiproliferative effect of simvastatin on the melanoma cells. Depending on dose, we found that all melanoma cells treated with simvastatin had significantly lower growth than controls. After 24 hours, viable cells were counted (Figure 6A), and the half-maximal inhibitory concentration (IC50) for B16F10 was determined to be 9.56 µM (Figure 6B).

Then, we investigated how simvastatin affected the B16F10 cell migration in vitro. Simvastatin treatment obviously suppressed B16F10 melanoma cell growth after 24 hours of culture, demonstrating that in the untreated cells, the scratch wound is repopulated after 24 hours. However, in the plates supplied with different concentrations of simvastatin, the cell growth is suppressed, and the scratch stays empty after 24 hours, indicating that simvastatin could limit cancer cell growth and migration. This experiment was repeated three times, and the results were confirmed each time (Figures 6C and D).

To confirm the antiproliferative and anticancer properties of Simvastatin in vivo, subcutaneous injection of B16F10 cells was performed on C57/BL6 mice, and once the small tumor was apparent (via day five), each group of mice was administered 100mg/kg of Simvastatin intraperitoneally (i.p.). The tumor size of mice treated with simvastatin was smaller than that of the control, as demonstrated in (Figure 6E), which supports the anticancer effects of simvastatin.

Figure 6

Simvastatin inhibits melanoma cell growth and migration



B16F10 cells were cultured with different concentrations of simvastatin (A). Macroscopic pictures of B16F10 cells subjected to Simvastatin obtained through inverted microscopy (B). The migrating cells were listed according to high power fields (HPF) in the scraped area (C). Faster wound closure in B16F10 cells treated with Simvastatin (D). C57/BL6 mice received a subcutaneous (s.c.) injection of B16F10 into their right flanks, and beginning on day 5, they were treated with or without simvastatin. The tumor weight was measured 12 days after inoculation (E). Data are expressed as mean ± SEM (unpaired Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2 NFκB Knockdown Inhibits Growth of Melanoma Cells

The NFκB signaling pathway is significant in carcinogenesis [132]. Continuous NFκB activation has been found in different types of malignancies [133], Kwang et al. show that simvastatin has an antiproliferative effect against human myeloid leukemia through downregulation of NFκB [131], and Jung et al. suggest that simvastatin reduces the growth of leukemia cells through NFκB/P65 downregulation, leading to matrix metalloproteinase (MMP9) suppression [134].

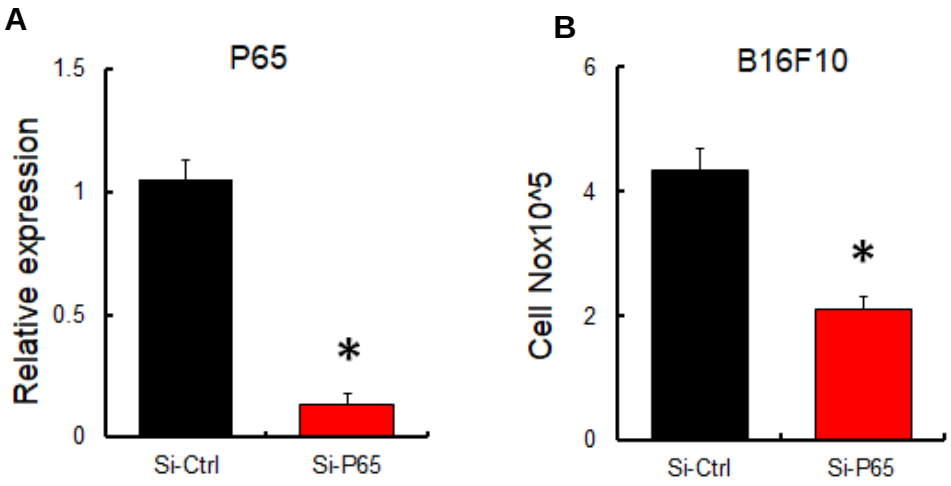
Because NFκB is highly expressed in melanoma, we asked if NFκB is a potential target for melanoma-simvastatin treatment and if NFκB inhibition can reduce melanoma growth.

In this study, we investigate the function of NFκB in B16F10 via RNA interference (RNAi) mechanism to knock down the P65 protein level in melanoma cell lines and mice, and represent its effects on the development and progression of melanoma.

To identify the P65 status in B16F10, the expression of P65 was detected by quantitative polymerase chain reaction (qPCR), as shown in (Figure 7A), which represents that the expression of P65 was significantly decreased in simvastatin-treated cells compared with controls. Also, there is a remarkable decline in the B16F10 cell number compared with controls (Figure 7B), so the knockdown of P65 through the siRNA mechanism significantly affects melanoma growth and development.

Figure 7

NFκB knockdown inhibits growth of melanoma cells



Changes in P65 transcription level determined by qPCR in siRNA gene silenced siP65, and the expression was compared to control (A). SiP65 effect on melanoma cell growth compared to control (B). Data are expressed as mean ± SEM (unpaired Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3 NFκB Inhibition Restores the Expression Of MIR-126 In Melanoma Cells

In recent years, evidence has shown that miRNAs and NFκB play critical roles in tumor growth and progression [135]. MicroRNA has been used as molecular therapeutic targets, cancer diagnostic markers, and prognostic indicators. Restoration of MicroRNA expression level has shown a significant potential for tumor therapy in cellular and animal models. MicroRNA can regulate many target genes responsible for the onset of cancer, progression, development, metastasis, and drug resistance [136]. NFκB can upregulate different microRNAs, which reduce cancer cell proliferation, metastasis, and invasion by targeting various oncogenes [97].

One of the most important microRNAs is MIR-126, which can regulate melanoma cell growth, metastasis, and angiogenesis [101, 102]. We focused on MIR-126 because it was downregulated in several melanoma cell lines at various stages of development compared to normal melanocytes [101].

Our findings from restored melanoma expression in vitro and in vivo represent that MIR-126 downregulation is connected to tumor growth and its neoplastic role. We focused on MIR126 in a panel of cancer cell lines, including (the stromal cell line MS-5, mouse embryonic fibroblasts MEF, B16F1, B16F10, and B53). (Figure 8A) shows a significant decrease in MIR-126, which was detectable in the aggressive B16F10 cells compared to another type of cell, which was determined via quantitative polymerase chain reaction (qPCR). This result is consistent with earlier research demonstrating that the higher metastatic melanoma has lower MIR-126 expression [137]. Also, the expression level of MIR-126 in tumor cells (T) from Skin Cutaneous Melanoma (SKCM) patients was lower compared with the normal control (N) using Gene Expression Profiling Interactive Analysis (GEPIA2) [138] (Figure 8B). Indicating a physiological link between this microRNA and melanoma progression.

We next investigated the mechanism by which NFκB-MIR-126 affects melanoma cell growth and metastasis. The extracellular matrix (ECM) degrading proteases include matrix metalloproteinase-9 (MMP-9), which has a significant role during melanoma growth, migration, adhesion [139], and metastasis [140]. Because MMP-9 is a downstream target gene of MIR-126 [83], we asked if the NFκB-MIR-126 pathway affects the expression of MMP-9 in the melanoma B16F10 cell line.

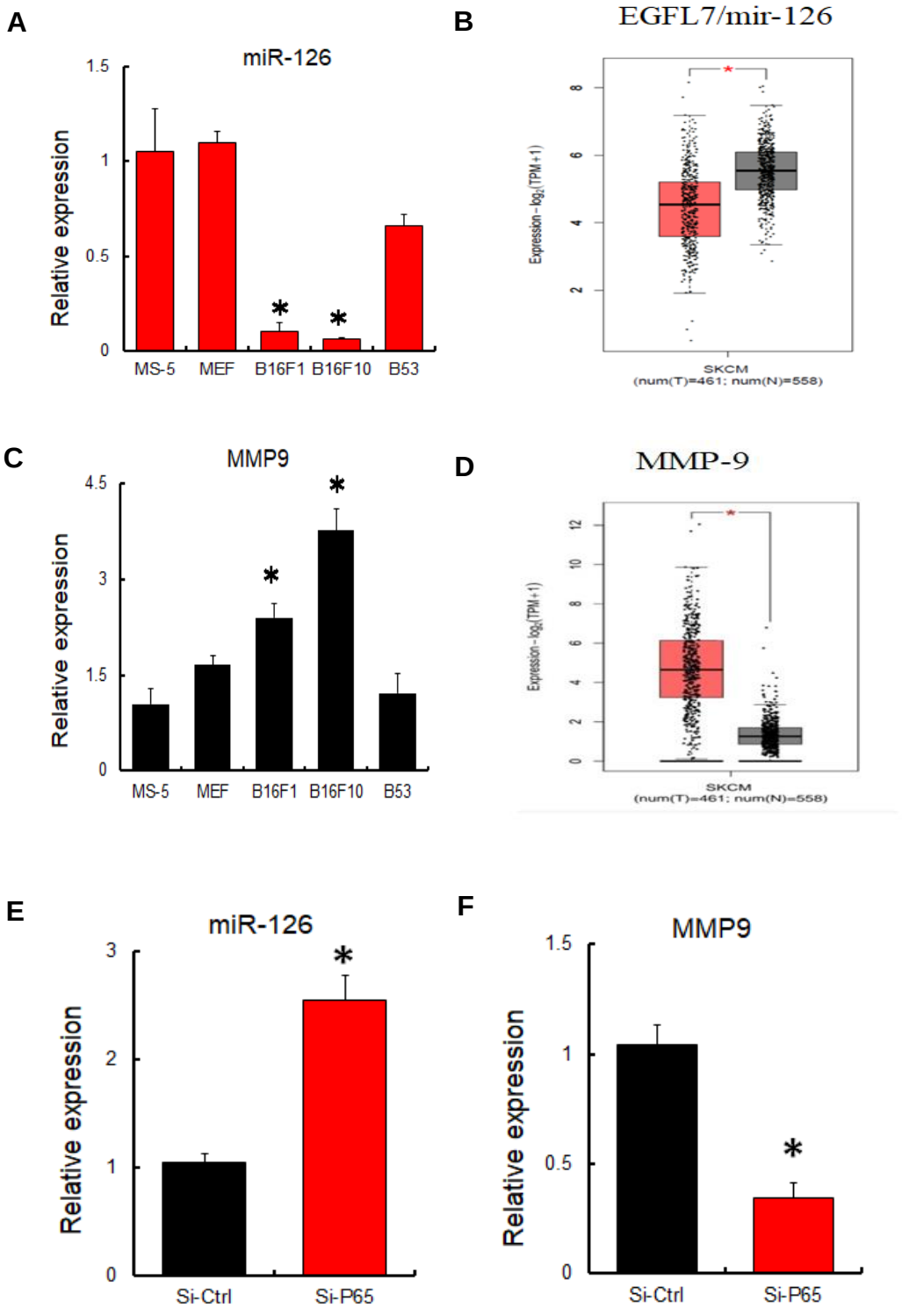
Then, we identified MMP-9 expression in different cell lines, including human and mouse melanoma cells (stromal cell line MS-5, mouse embryonic fibroblasts MEF, B16F1, B16F10, and B53) through qPCR. Low MMP-9 expression was found in mouse embryonic fibroblasts (MEF), Chinese hamster ovary cells (B53), and stromal cell lines (MS-5) compared to melanoma cell lines.

The highly metastatic melanoma B16F10 cells showed higher MMP-9 expression than the less aggressive B16F1 cells (Figure 8C). Confirming earlier reports, cutaneous melanoma has a higher expression of MMP-9 [141]. The GEPIA2 program showed that MMP-9 expression was greater in the tumor (T) than in the nearby normal (N) tissues in patients with skin cutaneous melanoma (SKCM) (Figure 8D).

To investigate the relevance of NFκB, MIR-126, and MMP-9 as a simvastatin target, gene silencing of NFκB (siP65) showed an upregulation level of MIR-126 and a decrease in the expression level of MMP-9 (Figure 8E and F), indicating that NFκB regulates MIR-126 and MMP-9.

Figure 8

NFKB inhibition restores the expression of MIR-126 in melanoma cells



Stromal cell line MS-5, Mouse embryonic fibroblasts MEF, B16F1, B16F10, and B53 were analyzed for mir126 expression by PCR (A). The expression level of MIR-126 in the tumor (T) and normal (N) tissues of patient with skin cutaneous melanoma (SKCM) analyzed by Gene Expression Profiling Interactive Analysis (GEPIA2) program (B). Stromal cell line MS-5, Mouse embryonic fibroblasts MEF, B16F1, B16F10, and B53 were analyzed for MMP-9 expression by PCR (C). The expression level of MMP-9 in skin cutaneous melanoma (SKCM) tumor (T) and normal (N) tissues analyzed by Gene Expression Profiling Interactive Analysis (GEPIA2) program (D). SiP65 effects on MIR126 and MMP9 expression level which compared to controls (E, F). Data are expressed as mean ± SEM (unpaired Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.4 Simvastatin Upregulates MIR-126 and Inhibits MMP-9 in Melanoma Cells

To determine the influence of simvastatin on these two factors, we examine the expression levels of MIR-126 and MMP-9 in response to different concentrations of simvastatin (5, 10, 25, and 50 μM). The qPCR results show that MIR-126 expression increases with increasing concentrations of simvastatin, and MMP-9 expression decreases with increasing concentrations of simvastatin on the B16F10 cell line (Figure 9A and B).

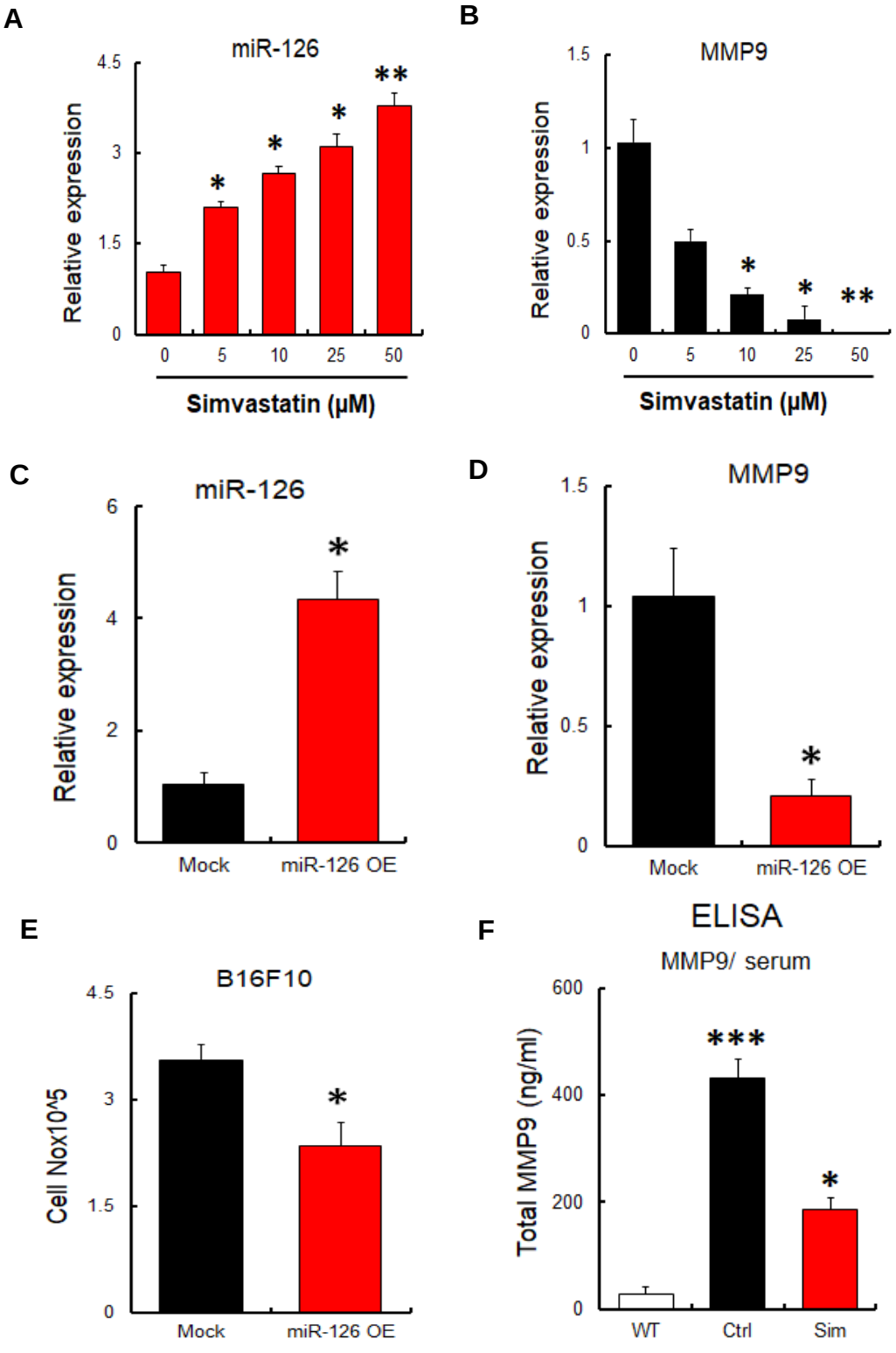
Based on our investigation of MIR126-MMP-9 effects in vitro, we studied whether overexpression of MIR-126 and suppression of MMP-9 cause a reduction in tumor growth and metastasis in vivo.

On day 12, tumor samples were collected, and gene expression was determined by qPCR. We established B16F10, in which we overexpressed MIR-126 using lentivirus (MIR126 OE). High MIR-126 and decreased MMP-9 were detectable in MIR-126 OE tumors treated with simvastatin compared with mock controls (Figure 9C and D). As was the decrease in B16F10 growth compared with mock control (Figure 9E). Simvastatin treatment reduces MMP-9 expression in mock and mir-126 OE, representing simvastatin-mediated MMP-9 downregulation, including Mir-126 overexpression.

In agreement with these findings, the MMP-9 serum level decreased in tumors injected with MIR-126 OE, as determined by ELISA (Figure 9F). These results indicate that Simvastatin mediates its antiproliferative effect by upregulating Mir-126.

Figure 9

Simvastatin upregulates MIR-126 and inhibits MMP-9 in melanoma cells



MMP-9 and MIR126 expression level according to different concentrations of simvastatin treated cells analyzed by PCR respectively (A, B). MIR126 expression in (MIR126 over Expression) cells compared to mock control by qPCR (C). MMP-9 expression in (MIR126 over Expression) cells compared to mock control by qPCR (D). Comparing the growth of B16F10 cells in (MIR126 over Expression) to mock controls using quantitative PCR (E). MMP9 expression level in MMP9 knockdown B16F10 cells compared to mock control determined by qPCR (F). Data are expressed as mean ± SEM (unpaired Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.5 MMP-9 Downregulation Inhibits Melanoma Growth

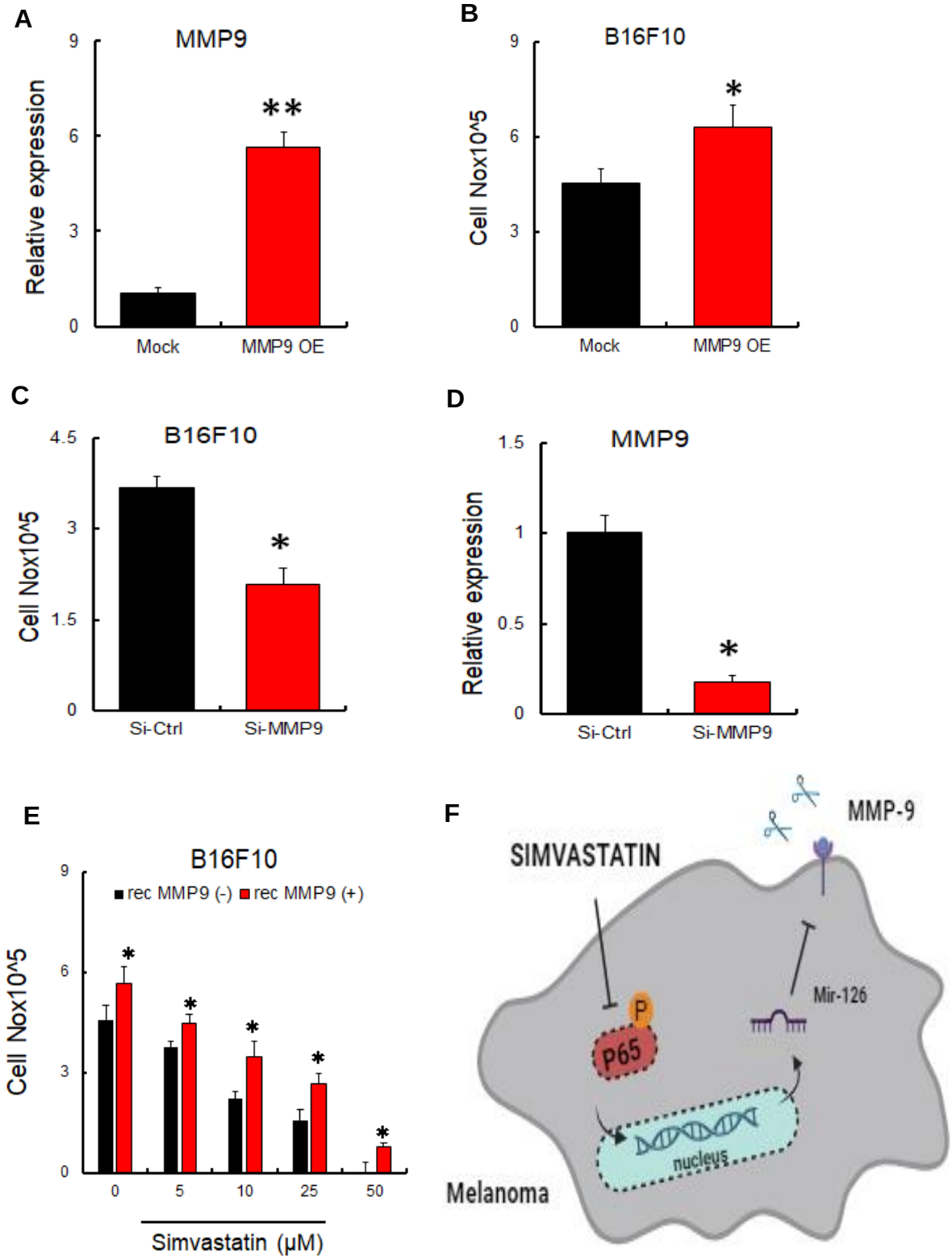
In 2013, Zhao-Yong et al. showed that MMP-9 suppression with siRNA is a potent technique for decreasing B16F10 cell invasion and metastasis in vivo and in vitro [142]. Also, MMP-9 could be a therapeutic target in the treatment of malignant melanoma [142]. According to these data, we created B16F10 cells using lentivirus to overexpress MMP-9 (MMP-9 OE) or gene-silencing MMP-9 (siMMP9) and verified MMP-9 expression by qPCR. Simvastatin treatment of Mock and MMP-9 OE increased MMP-9 overall expression (Figure 10A), according to the role MMP-9 in melanoma migration and growth that MMP-9 OE raised melanoma cell growth (Figure 10B). Simvastatin treatment decreases melanoma cell growth in siMMP9 B16F10 cells (Figure 10C) and decreases the overall expression level of MMP-9 (Figure 10D).

Also to test whether simvastatin can bloke MMP-9, which mediates melanoma cell growth and migration, recombinant (rec) MMP-9 was administrated to B16F10 along with simvastatin. Simvastatin prevented MMP9-induced melanoma growth in a dose-dependent way, as shown in (Figure 10E). These results show that simvastatin's antiproliferative effects were linked to MMP-9 downregulation expression.

Our data indicate that simvastatin has anti-cancer properties with decreasing growth and metastasis of melanoma via NFκB knockdown, which leads to an increase in the level of micro-RNA-126, which targets the MMP-9 oncogene that is responsible of melanoma cell metastasis and growth (Figure 10F).

Figure 10

MMP-9 downregulation inhibits melanoma cell growth



MMP-9 expression level in (MMP-9 over Expression) B16F10 cells compared to mock control through qPCR (A). Effect of (MMP-9 over Expression) on B16F10 cell growth compared to mock control (B). Effect of (MMP-9 Knock Down) on B16F10 cell growth compared to mock control (C). MMP-9 expression level in (MMP-9 Knock Down) B16F10 cells compared to mock (D). B16F10 cells treated with simvastatin were counted after 24 hours of adding the recommended dosage of recombinant MMP-9 (rMMP9) (E). The suggested method by which simvastatin acts on melanoma: simvastatin blocks NFκB/P65, upregulating mir-126, which targets the MMP-9 oncogene, inhibiting growth and metastasis of melanoma (F).

Abbreviations: NFκB/P65, nuclear factor kappa light chain enhancer of activated B cells; mir-126, micro-RNA-126; MMP-9, metalloproteinases-9; KD, knockdown; OE, overexpression. Data are expressed as mean ± SEM (unpaired Student's t-test * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Chapter Four

Discussion

4.1 Discussion

The basic methods for successful cancer treatments are limiting cell growth, blocking oncogenes, destroying proliferating tumor cells, and restoring the expression of tumor suppressor genes. The use of available medicine that reduces tumor growth through a variety of mechanisms, including restoring the expression of epigenetically tumor suppressor genes, is an alternative cancer therapy [143]. From this point, we chose the drug simvastatin and showed its effects on the melanoma B16F10 cell line by restoring the expression of mir-126.

Cutaneous melanoma is a very aggressive type of cancer whose incidence is constantly increasing worldwide [139]. While surgical treatment is usually curative in the early stages, available treatments are currently ineffective after metastatic spread, and patients with advanced disease have a poor prognosis [144, 145], indicating the need for novel and effective treatment.

Several studies have shown that statins' role in the treatment of cardiovascular diseases extends beyond a hypocholesterolemic action [146]. Statins have also been studied for their anti-carcinogenic properties in various models, including colon, rectum, prostate, lung, breast, and skin cancer [147-150]. Other retrospective studies indicated that simvastatin can protect against melanoma development [151, 152]. Also, Jacobs et al. investigated whether simvastatin has a protective effect against melanoma [153].

The present study looked at the effect of simvastatin on cell growth and metastasis in melanoma. We found that simvastatin inhibits the growth and metastasis of the melanoma cell line in vitro and represents its antiproliferative effects in a dose-dependent manner. Mechanistically, simvastatin blocked the transcription factor NFκB, which increased the expression of microRNA-126, which led to the downregulation of the expression of the MMP-9 oncogene, responsible for cancer metastasis.

To assess the growth inhibition caused by Simvastatin treatment, the B16F10 cell line treated with (5,10,25, and 50μM) of Simvastatin for 24 hour showed high growth inhibition in simvastatin-treated cells. Also, we show that simvastatin reduces the tumor

cell migration in a dose-dependent manner. According to the last research, the cytotoxic effects of statins vary depending on the dose, time of cell exposure to the drug, and cell line type [154, 155]. The increased efficiency of Simvastatin treatment against melanoma cell lines may be related to chemical and physical feature variations. Simvastatin is a largely lipophilic statin that can rapidly and deeply penetrate the cell membrane and distribute it to other tissue [156]. Also, the result revealed that melanoma cells are sensitive to simvastatin.

Simvastatin shows the best anticancer effect compared with other statins like rosuvastatin, which show a slight inhibitory effect on melanoma cell growth for 24 hours of treatment compared to simvastatin [59]. According to last researches show that the cytotoxic effects of statins vary depending on the dose, time of cell exposure to the drug and cell line type [51].The increase efficiency of simvastatin treatment against melanoma cell observed may be related to variation in chemical and physical features compared with other statins like rosuvastatin, simvastatin is largely lipophilic and deeply penetrate the cell membrane and distribute to other tissue [53]. This supports the hypothesis that simvastatin, the most commonly used statin, could be a promising therapy with effective growth suppression against melanoma cells [57].

The continuously active NFκB signaling pathway represents a point of confluence for dysregulated cellular pathways in melanoma [133] and has an important role in melanoma development [157], invasion [158], metastasis [159], and angiogenesis [160]. Therefore, NFκB inhibition in cancer cells may be beneficial in cancer prevention and treatment [79]. Kwang et al. show that simvastatin inhibits the chemotherapeutic agents that induced NF-κB activation in human myeloid leukemia cells and only simvastatin produced from fungi reduces the NFκB activation [131]. Hilgendorff et al. investigated the effects of various statins on the polysaccharides that induce the NFκB activation and showed that simvastatin efficiently inhibited NFκB activation [161]. Manu et al. have shown that simvastatin inhibited the proliferation and invasion of human gastric cancer cells by blocking the NF-κB signaling pathway [162]. Also, Kang et al. demonstrated that simvastatin treatment reduces cell growth and proliferation by inhibiting NFκB in the prostate [129]. Melanoma cells generate and express a number of cytokines, including TNF, IL-6, and NFκB, which are key proinflammatory cytokines and cell

proliferation regulators [163]. Vladimir et al. show that simvastatin treatment downregulates NFκB in melanoma [163].

In agreement with past studies, our results show that the mechanisms of the antitumor activity of Simvastatin on B16F10 melanoma tumors include inhibitory effects on the expression of NFκB by using RNA interference (RNAi) mechanism to knock down the P65 protein level in melanoma cell line and in the mice, and represents its effects on development and progression of melanoma, that confirmed by quantitative polymerase chain reaction (qPCR) analysis which show that simvastatin treated cells have lower expression of NFκB compared with control and decrease B16F10 cell number compared with control. So, the knockdown of P65 through the siRNA mechanism significantly affects melanoma growth and development.

MicroRNAs have been identified as a significant post-transcriptional regulator. MicroRNA family is a type of Non-coding short RNAs with 20–25 nucleotide length that mainly acts at post-transcriptional levels, which link to sequences present in the 3′-untranslated region (3′-UTR) of target mRNAs in the RNA-induced silencing complex to regulate mRNA degradation or suppress translation. They have been shown to have significant function in a variety of cellular processes involving cancer, in which they can function as both oncogenes and tumor suppressor genes, giving a new level of molecular regulation [86]. MicroRNA regulates biological processes like cell differentiation, apoptosis, cell division, migration, oncogenesis, and metastasis [164, 165].

In our study, we focused on miR-126 because it was found to be downregulated in melanoma cell lines compared with normal human melanocytes at different stages of progression. Alhasan shows that down-regulation of miR-126 has a role in multiple neoplasms, and its restored expression significantly inhibits growth, dissemination, and angiogenesis [166]. Felli et al. show the tumor suppressor function through the marked decrease of miR-126 in metastatic cells, suggesting that restoring their levels could be a possible treatment for advanced melanoma [101]. Our findings indicate that miR-126 downregulation is associated with tumor progression and that their restored expression plays an antineoplastic role in metastatic melanomas in vitro and in vivo. Also, melanoma cells represent a significantly lower expression of mir-126 according to

TIMER program results compared to normal cells. A previous research demonstrated that mir-126 was frequently downregulated in melanoma cells [137, 167].

We demonstrated that B16F10 cells had significantly lower expression levels of Mir-126 compared to other tumor cell lines through qPCR analysis. Simvastatin-treated cells showed an increase in MIR-126 expression level with increasing concentration. We show that enhancing MIR126 expression via the lentivirus overexpression of gene mechanism in melanoma decreased MMP9 production and slowed tumor growth.

These results represent that the NFκB and mir-126 signaling pathways are important mechanisms supporting the anti-cancer activities of simvastatin on melanoma cells; therefore, therapy of simvastatin with NFκB inhibitors prevents the growth of these cells.

Tumor cell invasion and metastasis involve proteolytic activity to destroy the extracellular matrix; the digestion of ECM has seemed to enhance tumor cell migration, which contributes to the metastasis spread of cancer cells [168]. MMPs destroy all ECM components required for cell motility, angiogenesis, and cancer metastasis, such as MMP9, which has an important function in melanoma invasiveness and dissemination via destroying various extracellular components [121]. They also promote invasion and metastasis through tumor cell proliferation and apoptosis regulators in cancers, including melanoma [169]. Invasive melanoma cell lines have been shown to have increased MMP9 expression and activity when compared to non-invasive cell lines [170].

If we were looking for the downstream target genes of mir-126, we found that mir-126 can directly regulate MMP-9, whose abnormal expression has been linked to a range of malignancies, is involved in cell-cell interaction, and can cleave and release a variety of molecules that has a significant function in carcinogenesis. In 2010, Han et al. suppressed Dicer, an important component of microRNA processing, and discovered that disrupting microRNA processing increased the production of MMP-2 and MMP-9, resulting in increased cell line proliferative activity and invasiveness. These findings indicate that MMP-9 and MMP-2 are controlled by microRNAs [171]. Simvastatin can suppress tumor angiogenesis by decreasing the expression of matrix metalloproteinase (MMP9) in endothelial cells, resulting in less invasive cells [172].

Our results show that enhancing the expression miR-126 through lentivirus gene overexpression mechanisms decreases tumor growth and inhibits MMP9 expression levels in melanoma, which have anti-tumor characteristics derived from their restored expression in metastatic melanoma in vitro and in vivo. ELISA analysis showed that MMP9 was significantly downregulated in B16F10 after treatment with simvastatin. Also, we show that mir-126 can regulate its downstream target genes directly as metalloprotease 9 (MMP9). Knockdown of MMP9 via siRNA silencing inhibits melanoma growth, but not in MMP9 (overexpression) OE.

Simvastatin treatment induces NFκB downregulation, leading to mir-126 overexpression. Mir-126 restoration is responsible for downregulating MMP-9. These results reveal the mechanism responsible for the effects of simvastatin on NFκB, mir-126, and MMP-9 expression in human melanoma cells.

4.2 Conclusion

Simvastatin exhibited anti-proliferative characteristics in a dose- and time-dependent manner, preventing the B16F10 melanoma cell line from growing and spreading. This is achieved by blocking the transcription factor NFκB, which in turn upregulates miR-126, confirming its tumor suppressor role in melanoma by targeting the MMP-9 mRNA. These strategies seem to be effective in preventing the B16F10 cell line from growing and spreading. Simvastatin inhibits the migration of tumor cells, as we have further demonstrated. This study provides novel evidence for the anti-tumor effects of statins against melanoma cells by regulating the NF-κB-Mir-126-MMP-9mRNA signaling pathway.

List of Abbreviations

Abbreviation	Meaning
ALM	Acral lentiginous melanoma
ANK domain	Ankryin domain
AJCC	American Joint Committee on Cancer
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extra cellular Matrix
ELISA	enzyme linked immunosorbent assay
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
IC50	half maximal inhibitory concentration
IKK	IkB kinase complex
IkBs	inhibitory kB proteins
LMM	Lentigo Maligna Melanoma
MC1R	Melanocortin 1 receptor
MMP-9	Matrix metallopeptidase 9
MMP-2	Matrix metallopeptidase 2
mRNA	messenger RNA
miRNA	microRNA
miRISC	miRNA-induced silencing complex
NM	Nodular melanoma
NF-κB	Nuclear factor kappa B
NLS	nuclear localization signal
NEMO	NF-kB essential modifier
NADPH	Nicotinamide adenine dinucleotide phosphate
pre-miRNA	precursor-miRNA
pri-miRNA	primary-miRNA
PLS	polysaccharides
Poly A	poly –adenylated
PEST	proline, glutamine, serine, threonine
PCR	polymerase chain reaction

qRT–PCR	quantitative reverse transcription polymerase chain rxn
RNA	Ribonucleic acid
RHD	Rel homology domain
RLN	regional lymph node
SD	standard deviation
SiRNA	small interfering RNA
SLNB	sentinel lymph node biopsy
3′ UTR	three prime untranslated region
SSM	Superficial spreading melanoma
TIMP	tissue inhibitors of MMPs
TAD	transcriptional activation domain
TF	Transcription factor
UVR	Ultraviolet radiation

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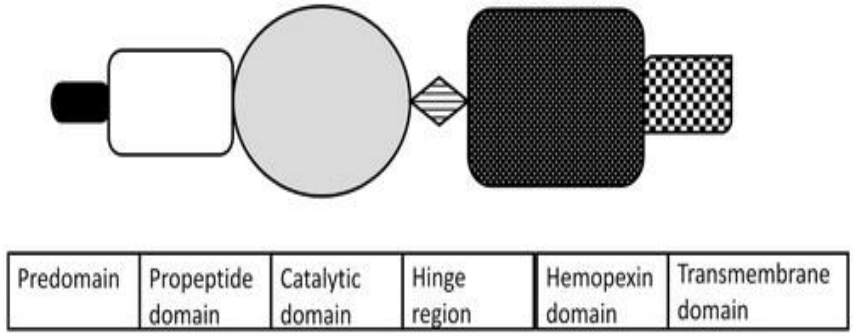
Appendices

Appendix A

Figures of Study

Figure A.1

Structure of MMPs



جـامعـــة النجاح الوطنية

كليـــة الدراســـات العليـــا

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إعداد

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قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في الكيمياء الحيوية السريرية، من كلية الدراسات العليا، في جامعة النجاح الوطنية، نابلس– فلسطين.

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الملخص

الخلفية: الورم الميلانيني هو أخطر أنواع سرطان الجلد، حيث تتزايد معدلات الاصابة بالمرض والوفيات على مستوى العالم. يتمتع الورم الميلانيني بإمكانية انتشار مرتفعة، مما يؤدي الى استجابة محدودة للعلاجات المتاحة بالإضافة الى سوء التشخيص. يتمتع دواء سيمفاستاتين بخصائص مضادة للسرطان يمكن ان تكون بمثابة دواء معاد استخدامه لعلاج سرطان الجلد، هو عضو مهم في عائلة تظهر الدراسات الحديثة دوره في السرطان باعتباره جينا جديدا لقمع الورم لأنه يمكن ان يمنع الخلايا السرطانية من النمو والهجرة عن طريق قمع الجينات التي لها تأثير مباشر.

الاهداف: تهدف هذه الدراسة الى التخفيف والقضاء الى حد ما على سرطان الجلد ،انه بحث كبير وجرئ ولكنه الطريقة الوحيدة لتحقيق ذلك، تم دعم هذا الهدف من خلال استخدام تقنية البحث المتميزة لدينا التي تمكننا من استخدام احدث الافكار لتعزيز اكتشاف علاج جديد، لذلك نقترح استخدام دواء متاح لمنع نمو الورم الخبيث يمكن ان يكون وسيلة جيدة بدلا من اكتشاف علاج جديد للورم الميلانيني، حيث سنقوم بدراسة تأثير دواء السيمفاستاتين على نمو سرطان الجلد والانتشار من خلال تقنية جديدة.

طريقة العمل: أجريت دراسة تجريبية في مختبر جامعة النجاح لأبحاث السرطان والخلايا الجذعية، بحث قمنا بدراسة تأثير دواء السيمفاستاتين على خلايا الورم الميلانيني من خلال فحص مستوى النمو والانتشار لدى هذه الخلايا استجابة لتراكيز مختلفة من السيمفاستاتين ثم فحص مدى هذا التأثير من خلال تثبيط

جينات معينة لها تأثير مباشر على انتشار ونمو الخلايا، وكذلك دراسة هذا التأثير داخل الجسم الحي من خلال استخدام الفئران المخبرية.

النتائج: أظهرت النتائج استجابة خلايا سرطان الجلد الى حد كبير والى اقل التراكيز من دواء السيمفاستاتين، بحيث كان هناك انخفاض في عدد خلايا السرطان وقلت سرعة انتشاره مقارنة بالخلايا التي لم تتعرض لتأثير الدواء، ايضا اظهرت النتائج مدى تأثير السيمفاستاتين على تثبيط الجينات المسؤولة عن انتشار ونمو خلايا الورم الميلانيني.

الخلاصة: حتى الان لم يتم التوصل الى علاج فعال لقتل سرطان الجلد، الا ان هناك جهود مبذولة في ايجاد علاج لتخفيف الاعراض والانتشار، في دراستنا اظهرت النتائج ان دواء السيمفاستاتين يتمتع بخصائص مضادة للتكاثر تحد من نمو ورم خبيث في خلايا سرطان الجلد، بطريقة تعتمد على الجرعة والوقت من خلال التأثير على جينات معينة.

الكلمات المفتاحية: سرطان الجلد، ميكرو ار ان أيه، سيمفاستاتين، انتشار ثانوي للورم، ماتريكس ميتالوبروتياز 9، نوع خلية سرطان الجلد بي 16 أف10.