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microRNAs involved in the aggressiveness of malignant melanoma

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Dedicated to My Family

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Chonglin Luo

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Summary

Malignant melanoma is the most severe form of skin cancer being refractory to current therapies at advanced stages (stage III and IV). The survival of individual patients can vary from months to years, but the molecular mechanism behind this broad survival time range is unknown so far. Many signaling pathways have been found to be important in melanoma and recently microRNAs (miRNAs) have emerged to be essential regulators in these pathways. This study aimed at the identification of miRNAs accounting for the aggressiveness of melanoma.

Using bead-based microarray analyses, we performed miRNA expression profiling on a panel of melanoma cell lines derived from metastatic melanoma patients with either long or short survival times. Upon comparison of these two patient groups a number of differentially expressed miRNAs could be identified. However, subsequent real-time quantitative PCR (qPCR) revealed that only one candidate miRNA (miR-101) was confirmed to be down-regulated in cell lines from short term survivors, compared to long term survivors.

We found that miR-101 could directly target microphthalmia-associated transcription factor (MITF) in melanoma cells, leading to a decrease in intracellular MITF protein expression. One of the predicted target sites for miR-101 in the 3' UTR of MITF was confirmed by luciferase reporter assay. Furthermore, we show that the expression of "enhancer of zeste homolog 2" (EZH2), previously reported as miR-101 target in other types of cancer, was down-regulated by miR-101 also in melanoma. Overexpression of miR-101 inhibited proliferation, invasion and migration of melanoma cells, which could be phenocopied by siRNA-mediated knockdown of MITF or EZH2 using specific siRNA.

miR-137 was reported to act as a tumor suppressor in different cancer entities including melanoma. In this study, we showed that low miR-137 expression correlated with poor survival of stage IV melanoma patients. We identified three novel targets (proto-oncogene c-Met, Y box binding protein 1 [YB1] and the ATP-binding cassette, sub-family B, member 5 [ABCB5]) and confirmed two previously reported targets (MITF and EZH2) for miR-137.

Overexpression of miR-137 suppressed invasion of melanoma cells, which could be phenocopied by siRNA-mediated knockdown of the miR-137 targets c-Met, YB1, MITF and EZH2. Furthermore, miR-137 inhibited melanoma cell migration and proliferation likely through the down-regulation of certain proteins (e.g. YB1 and EZH2) whose individual impact on these processes varied among the two cell lines tested. Finally, miR-137 induced apoptosis in melanoma cell lines and decreased B-cell lymphoma 2 (BCL2) protein expression levels.

In conclusion, our study suggests that miR-101 and miR-137 can function as tumor suppressors in melanoma. We show that overexpression of these miRNAs could lower the aggressiveness of melanoma cells by regulation of multiple signaling pathways, offering new options for targeted therapy against melanoma.

Zusammenfassung

Das Maligne Melanom zeichnet sich im fortgeschrittenen Stadium (Stadium III und IV) durch eine ausgeprägte Resistenz gegenüber aktuell angewandten Therapieformen aus und stellt somit die schwerwiegendste Form aller Hautkrebsarten dar. Die Überlebensdauer der Patienten beträgt zwischen einigen Monaten bis mehreren Jahren, allerdings ist der molekulare Mechanismus der für diese große Schwankungsbreite verantwortlich ist, noch ungeklärt. Bisher konnten etliche Signalkaskaden identifiziert werden, die für das Melanomwachstum relevant sind, doch kürzlich wurden microRNAs (miRNAs) als essentielle Regulatoren dieser Signalwege ermittelt. Das Ziel der vorliegenden Arbeit bestand in der Identifizierung von miRNAs, die zur Aggressivität von Melanomen beitragen.

Mit Hilfe von „Bead“-basierten Micro-Array-Analysen konnte ein miRNA Profil für eine Reihe von Melanom-Linien erstellt werden, die aus Tumorpatienten mit metastasierten Melanomen und langer bzw. kurzer Überlebensdauer stammten. Durch den direkten Vergleich dieser beiden Patientengruppen konnte eine Reihe von differenziell exprimierten miRNAs identifiziert werden. Allerdings konnte in der anschließend durchgeführten quantitative Real-Time PCR Analyse nur eine miRNA (miR-101) als eine in Melanom-Linien aus Patienten mit kurzer Überlebensdauer spezifisch herabregulierte miRNA bestätigt werden.

Wir fanden, dass miR-101 auf den „Microphthalmia-assoziierten Transkriptionsfaktor“ (MITF) einwirkt und zu dessen verminderten intrazellulären Proteinexpression führt. Eine der vorhergesagten miR-101-Bindungsstellen im in der 3'UTR der MITF-spezifischen mRNA konnte mit Hilfe von Luziferase Reporter-Tests bestätigt werden. Darüber hinaus konnten wir zeigen, dass das Protein „Enhancer of Zeste Homolog 2“ (EZH2), das bereits in anderen Tumorentitäten als Zielstruktur für miR-101 beschrieben wurde, auch in Melanomen durch miR101 herab reguliert wird. Durch Überexpression von miR-101 wurden Proliferation, Invasion und Migration von Melanomzellen inhibiert, ein Effekt, der auch stellvertretend durch die si-RNA-vermittelte Ausschaltung von MITF oder EZH2 erzielt werden konnte.

miR-137 wurde für verschiedene Tumorarten, einschließlich des Malignen Melanoms, als Tumorsuppressor- microRNA beschrieben. In der vorliegenden Arbeit konnten wir zeigen,

dass eine niedrige miR-137 Expression mit einer verminderten Überlebensrate von Patienten im Stadium IV einher geht. Darüber hinaus wurden drei Proteine identifiziert, deren Expression miR-137-abhängig moduliert wird, nämlich das Proto-Onkogen c-Met, das „Y-Box Binding Protein 1“ (YB-1) und das „ATP-Binding Cassette, Subfamily B, Member 5“-Protein (ABCB5). Die bereits früher beschriebene miR-137-vermittelte Modulation der Expression von MITF und EZH2 konnte im Rahmen der vorliegenden Arbeit ebenfalls bestätigt werden. Durch Überexpression von miR-137 wurde die Invasion von Melanomzellen inhibiert; dieser Effekt konnte durch siRNA-vermittelte Unterdrückung der Expression von c-Met, YB1, MITF und EZH2 imitiert werden. Darüber hinaus inhibierte miR-137 durch Herabregulierung bestimmter Proteine (u. a. YB1 und EZH2) auch die Migration und Proliferation von Melanomzellen. Die targetierten Proteine zeigten hierbei sowohl innerhalb einer Melanomlinie als auch zwischen den beiden getesteten Melanomlinien unterschiedlich stark ausgeprägte Effekte. Darüber hinaus löste miR-137 Apoptose in Melanomlinien aus und führte zu einer verminderten Expression des „B-Cell-Lymphoma 2“ Proteins (BCL-2).

Unsere Studie legt die Schlussfolgerung nahe, dass miR-101 und miR-137 als Tumor-suppressoren in Melanomen fungieren können. Wir zeigen, dass die Überexpression dieser miRNAs die Aggressivität von Melanomzellen durch die Regulation mehrerer Signalwege verringern kann. Hierdurch könnten Optionen für neue Therapieansätze gegen das Maligne Melanom geschaffen werden.

Abbreviations

α -MSH	alpha melanocyte stimulating hormone
ABC	ATP-binding cassette
ABCB5	ATP-binding cassette, sub-family B, member 5
ac-pre-miRNA	AGO2-cleaved precursor miRNA
AGO	argonaute protein
AJCC	American Joint Committee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog
ALM	acral lentiginous melanoma
AP-1	activator protein 1
ATP	adenosine triphosphate
BCL2	B-cell lymphoma 2
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCND1	cell-cycle mediator cyclin D1
CDC42	cell division cycle 42
CDF	Chip Description File
CDK	cyclin-dependent kinase
CDKN1B	cyclin-dependent kinase inhibitor 1B
cDNA	complementary DNA
CDS	coding sequence
c-Fos	FBJ (Finkel-Biskis-Jinkins) murine osteosarcoma viral oncogene homolog
c-Jun	jun proto-oncogene
c-KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
CLL	chronic lymphocytic leukemia
c-Met	proto-oncogene c-Met
CMV	cytomegalovirus
COX2	cyclooxygenase-2
CREB1	cAMP response-element binding protein 1
CtBP1	c-terminal binding protein 1
CTGF	connective tissue growth factor

CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DCT	dopachrome tautomerase
DGCR8	DiGeorge syndrome critical region gene 8
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTIC	dacarbazine
E2F1	E2F transcription factor 1
E2F3	E2F transcription factor 3
ECL	enhanced chemiluminescence
EMT	epithelial-mesenchymal transition
enh	enhancer
EZH2	enhancer of zeste homolog 2
FC	fold change
FCS	fetal calf serum
FDA	US Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FL	female long survivor
FOXO3	forkhead box O3
FS	female short survivor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPR143	G protein-coupled receptor 143
GSK3B	glycogen synthase kinase 3 beta
HCL	hydrogen chloride
HEK	human embryonic kidney
HGF	hepatocyte growth factor
HITS-CLIP	high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation
hluc+	Synthetic firefly luciferase gene
HMGA2	high mobility group AT-hook 2
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
hRluc	synthetic <i>Renilla</i> luciferase gene
HRP	horseradish peroxidase

HSV-TK	herpes simplex virus thymidine kinase
IEP	immediate early promoter
IGF2R	insulin-like growth factor 2 receptor
IgG	immunoglobulin G
KCNMA1	potassium large conductance calcium-activated channel, subfamily M, alpha member 1
kDa	kilodalton
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LB	lysogeny broth
LEU2	leukemia associated gene 2
LMM	lentigo maligna melanoma
LPH	liposome-polycation-hyaluronic acid
MAPK	mitogen-activated protein kinase
MC1R	G protein-coupled melanocortin receptor 1
MCL1	myeloid cell leukemia sequence 1
MDM2	murine double minute 2
MEK	MAPK/ERK kinase
MeV	MultiExperiment Viewer
Mib1	mindbomb homolog 1
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MITF	microphthalmia-associated transcription factor
ML	male long survivors
MLANA	melan-A
MMP7	matrix metalloproteinase 7
MS	male short survivors
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
NaCl	Sodium chloride
NCI	National Cancer Institute
NFAT5	nuclear factor of activated T-cells 5
NHEM	human normal epidermal melanocyte

NM	nodular melanoma
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog
NSCLC	non-small cell lung cancer
nt	nucleotides
ori	origin
PAGE	polyacrylamide gel electrophoresis
PAR-CLIP	Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation
PARP	Poly-(ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDCD1	programmed cell death 1
PDCD4	programmed cell death 4
PI3K	phosphatidylinositol 3 kinase
PIP2	phosphatidylinositol (4, 5)-bisphosphate
PIP3	phosphatidylinositol (3, 4, 5)-trisphosphate
PLZF	promyelocytic leukemia zinc finger
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homologue
qPCR	real-time quantitative PCR
r	Pearson's correlation coefficient
RAS	rat sarcoma viral oncogene homolog
RB1	retinoblastoma 1
RefSeq	Reference Sequence
RNA	ribonucleic acid
RNAPII	RNA polymerase II
RNU6B	small nuclear RNA U6
RPMI	Roswell Park Memorial Institute
rRNA	ribosomal RNA
SCF	stem cell factor
scFv	single-chain antibody fragment
SD	standard deviation

SDS	sodium dodecyl sulfate
SILV	silver homolog (mouse)
siRNA	small interfering RNA
SLIAC	stable isotope labeling by amino acids in cell culture
snRNA	Small nuclear RNA
SSM	Superficial spreading melanoma
SV40	Simian Vacuolating Virus 40
TBS	Tris-buffered saline
TBS-T	TBS with 0.1% Tween-20
TEMED	Tetramethylethylenediamine
TGFBR2	transforming growth factor, beta receptor II
TGFBR2	transforming growth factor, beta receptor II
Tm	melting temperature
TPM1	tropomyosin 1
TRPM1	transient receptor potential cation channel, subfamily M, member 1
TSP1	thrombospondin-1
TYR	tyrosinase
TYRP1	tyrosinase-related protein 1
UCSC	University of California Santa Cruz
UTR	untranslated region
UV	ultraviolet
VEGF	vascular endothelial growth factor
WNT	wingless-type MMTV (mouse mammary tumor virus) integration site family
WNT3A	wingless-type MMTV integration site family, member 3A
WST	water soluble tetrazolium
YB1	Y box binding protein 1
ZEB1	zincfinger E-box binding homeobox 1

1 INTRODUCTION

1.1 Melanoma

Melanoma is the malignant transformation of melanocytes, which mainly reside in the skin and eye and produce the dark pigment, melanin. Although melanoma accounts for less than 5% of all skin cancer cases, it causes more than 70% of all skin cancer deaths (1). Melanoma patients diagnosed at early stages can be cured by surgery. However, patients with metastasis are usually refractory to current therapeutic strategies and the 5-year survival rates for them is only 15% (2).

1.1.1 Progression of melanoma

In 1984, Clark *et al.* proposed a multi-step progression model of melanocyte-to-melanoma transformation based on clinical and histopathological observations (**Figure 1**) (3). This model has been widely accepted in the melanoma research community (2, 4). In normal skin, melanocytes are evenly distributed in the basal level of the epidermis. The initiation step is the proliferation of melanocytes which leads to the benign nevus. The second step is the development of the aberrant growth, which may occur in a preexisting nevus or as new nevus, resulting in the dysplastic nevus. The next step is the radial-growth phase in which cells are able to proliferate intraepidermally. Then, cells may enter the vertical-growth phase during which they acquire the capability to invade throughout the dermis and form an expanded nodule. Cells isolated from this stage are able to grow in soft agar and form tumor when implanted in nude mice. The last step in the model is metastatic melanoma which disseminate malignant cells to other location of skin and to other organs through the vascular and lymphatic systems.

1.1.2 Types of melanoma

Clinically, there are four main types of melanoma (2, 5). Superficial spreading melanoma (SSM) is currently the most common form of melanoma, accounting for 70% of cutaneous melanoma. It undergoes a radial growth phase in which the lesion is limited to the epidermis.

In the later vertical growth phase, the lesion stretches down into the dermis with an increased metastatic potential. Nodular melanoma (NM) is the most aggressive type of melanoma. It lacks a significant radial growth phase and has a predominantly vertical growth phase. It may appear in a place where a previous lesion was not seen. Most of NMs are darkly pigmented; however they can also be light or even non-pigmented. Acral lentiginous melanoma (ALM) is observed on the palms, soles, under the nails and in the oral mucosa. Unlike other types, ALM does not appear to be linked to ultraviolet (UV) exposure. Lentigo maligna melanoma (LMM), also called melanoma in situ, usually arises on sun-damaged skin such as face, neck and forearms of the elderly. It is the least common and the least aggressive type of cutaneous melanoma. The benign precursor of LMM, lentigo maligna, is a flat and large tan macule which may grow slowly for 3 to 15 years. Less than 5% of lentigo maligna will undergo vertical growth and become LMM eventually.

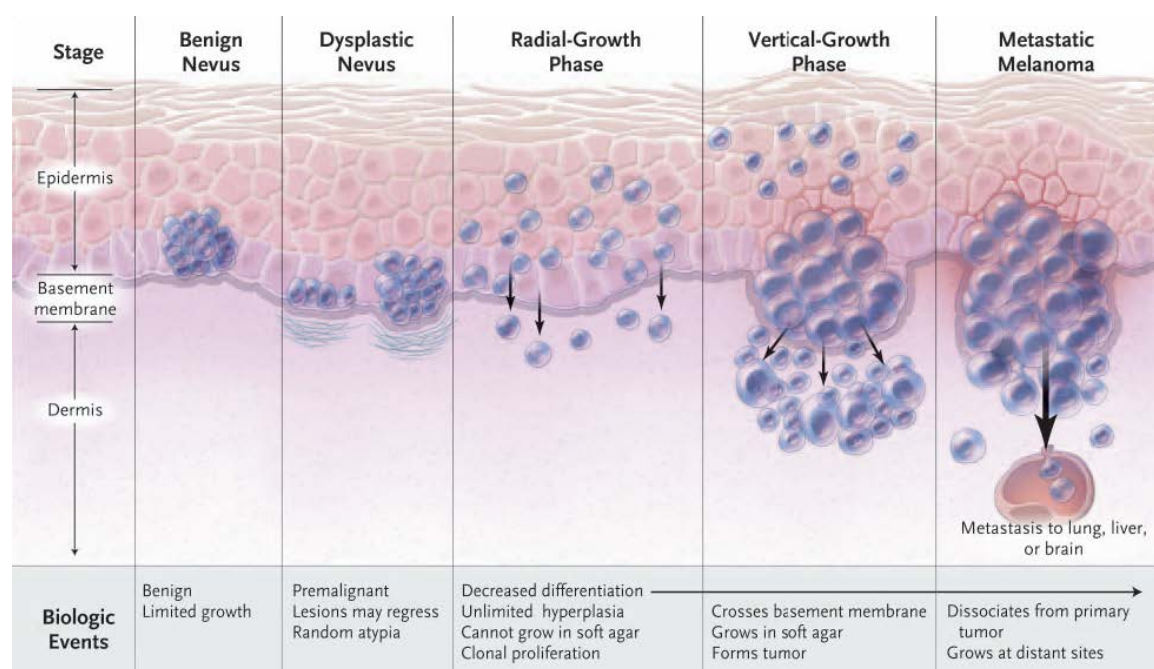


Figure 1 Biological events in Clark's model for melanoma progression. Adapted from Miller *et al.* 2006 (4).

1.1.3 Staging of melanoma

Staging is a method of summarizing how far a cancer has progressed based on the physical examination, imaging tests, biopsies, and other tests. According to AJCC's guidelines (6, 7), melanoma is grouped into five different stages within which subclasses are further categorized. Stage 0 melanoma resides in the epidermis, but has not spread into the dermis.

Stage I and Stage II melanoma are characterized by tumor presence, thickness, mitotic rates, and ulceration status. There is no evidence of regional lymph node or distant metastasis. Stage III melanoma is defined by lymph node metastases and absence of distant metastasis. Stage IV melanoma is characterized by distant metastases and the level of serum lactate dehydrogenase. At this stage, tumor has spread to other organs such as the liver, lung, or brain, to distant areas of the skin, or to distant lymph nodes. The staging system helps doctors to determine patients' prognosis and the appropriate treatments.

1.1.4 Treatment options for melanoma

At early stages, most melanoma can be cured by surgical resection. However, when metastasis is implicated, the disease becomes very aggressive and treatment may include surgery, chemotherapy, radiotherapy or a combination of these methods. Many chemotherapy drugs have been used in the treatment for melanoma. Single-agent Dacarbazine (DTIC) is the only FDA-approved chemotherapeutic agent for the treatment of advanced melanoma, but the response rate is only 10% to 20% and the median response duration is 4 to 6 months. Several adjuvant therapies have also been approved for melanoma (8). Among them, interferon- α and interleuin-2 are widely used in adjuvant immunotherapy for metastatic melanoma, although the response rates are low and toxicity is still a problem (2). In addition, large efforts have been made to stimulate tumor-specific T cell response, including vaccination using specific tumor antigens (e.g. MLANA, SILV and TYR), adoptive T cell transfer and blockage of T cell checkpoint molecules such as cytotoxic T lymphocyte-associated protein 4 (CTLA4) and programmed cell death 1 (PDCD1) (9). Some of these approaches have shown to be promising, but they are still under clinical investigation. Meanwhile, intensive studies have been carried out on crucial molecular signaling pathways involved in melanoma, which have started to elucidate the mechanisms behind this disease and is opening the possibility of targeted therapy.

1.1.5 Important signaling pathways in melanoma

1.1.5.1 Ras/Raf/MEK/MAPK pathway

The Ras/Raf/MEK/MAPK pathway (**Figure 2**), which plays a key regulatory role on melanoma cell proliferation and survival, is constitutively active in most melanoma (2, 4, 10). In melanocytes, this pathway can be activated by paracrine growth factors such as stem cell factor (SCF), hepatocyte growth factor (HGF), and fibroblast growth factor (FGF). In contrast, melanoma cells may produce growth factors, such as HGF and FGF, by themselves and regulate this signaling pathway in an autocrine manner (11). When activated, the growth factor receptors are linked to RAS proteins which then activate RAF proteins that in turn activate MAPK/ERK kinase (MEK). MEK acts on mitogen-activated protein kinase (MAPK, also known as ERK) which phosphorylates many targets in the cytoplasm and interacts with other pathways, such as cell death pathway and MITF pathway. MAPK also translocates into the nucleus and regulates gene expression that promotes proliferation. A more common way to activate this pathway is through gain-of-function mutations in NRAS and BRAF genes. Reports have shown that NRAS is mutated in 15% to 30% of melanomas and BRAF is mutated in 50% to 70% of melanomas (12).

1.1.5.2 PI3K/AKT pathway

Another important pathway in melanoma is PTEN/PI3K/AKT pathway (**Figure 2**) which regulates cell survival, proliferation and motility (2, 4, 10, 13). Typically, growth factors bind to their receptor tyrosine kinases which activate phosphatidylinositol 3 kinase (PI3K), leading to the conversion of the plasma membrane lipid phosphatidylinositol (4, 5)-bisphosphate (PIP₂) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP₃). PIP₃ acts as a second messenger which phosphorylates v-akt murine thymoma viral oncogene homolog (AKT), and in turn, a number of mitogenic processes are activated including inhibition of apoptosis, survival gene transcription, cell cycle progression, protein translation, and proliferation. This pathway is terminated by phosphatase and tensin homologue (PTEN) through dephosphorylation of PIP₃. PTEN mutations have been found in 30% - 50% of melanoma cell lines (13, 14) and PTEN protein levels are low or absent in melanoma (15, 16).

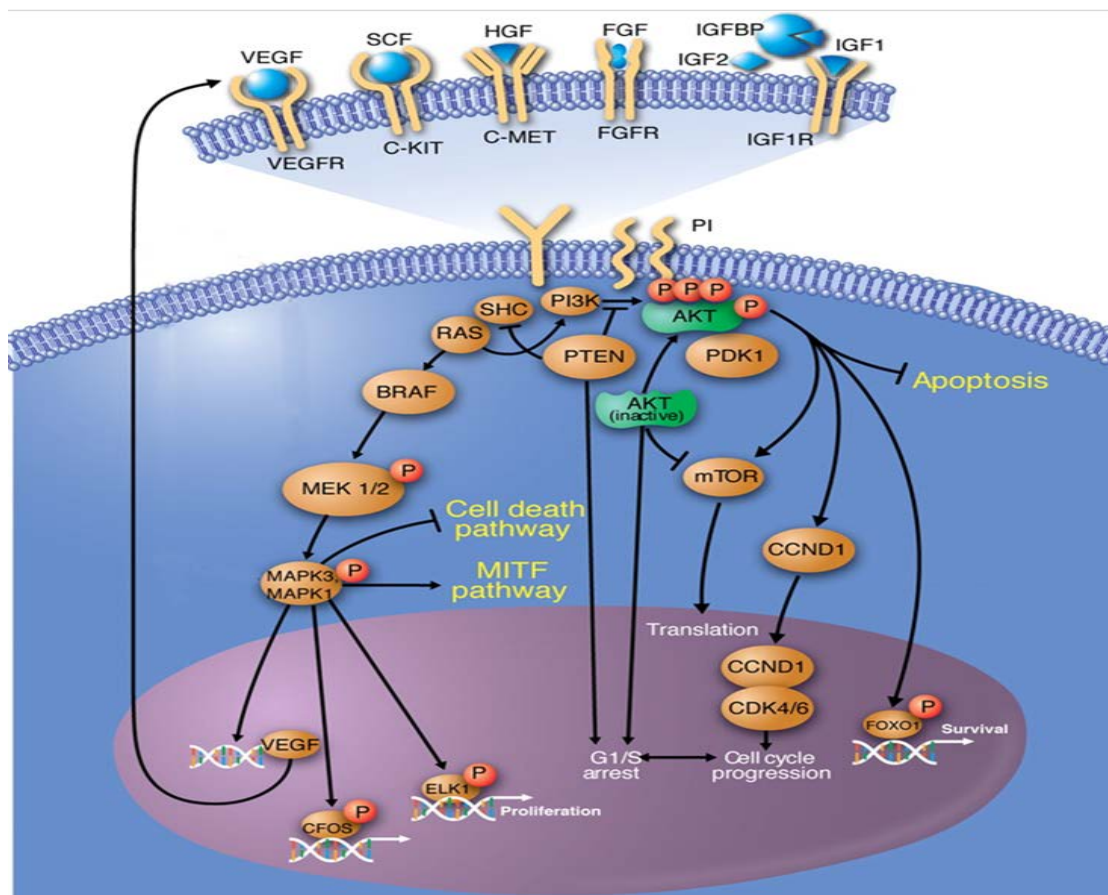


Figure 2 Ras/Raf/MEK/MAPK and PI3K/AKT pathway. VEGF, vascular endothelial growth factor; SCF, stem cell factor; HGF, hepatocyte growth factor; FGF, fibroblast growth factor; c-KIT, v-kit Hardy-Zuckerman 4 feline sarcomaviral oncogene homolog; IGF, insulin-like growth factor; IGFBP, IGF binding protein; P, phosphate; SHC, Src homology 2 domain– containing protein; c-FOS, v-fos FBJ murine osteosarcoma viral oncogene homolog; ELK1, member of ETS oncogene family; PI, phosphatidylinositol; PI3K, phosphatidylinositol 3 kinase; AKT, v-akt murine thymoma viral oncogene homolog; PDK1, phosphoinositide-dependent kinase 1; mTOR, mammalian target of rapamycin; CCND1, cyclin D1; FOXO1, forkhead box O1. Adapted from Sekulic *et al.* (10).

1.1.5.3 MITF and β -catenin pathways

MITF is a basic helix–loop–helix leucine zipper dimeric transcription factor, which is the master regulator of melanocyte development and differentiation, and also a key player in melanoma (17). Several pathways regulate MITF expression transcriptionally through the promoter (**Figure 3**). The crucial ones are the melanocortin and β -catenin pathways (4, 10). The alpha melanocyte stimulating hormone (α -MSH) binds to the G protein-coupled melanocortin receptor 1(MC1R), which activate cAMP response-element binding protein 1 (CREB1) through adenylate cyclase. CREB1 binds to MITF promoter and activates the transcription of MITF. Several growth factors such as KIT and HGF activate the MAPK

pathway which mediates the phosphorylation of MITF. Active MITF stimulates the transcription of its downstream targets, including genes involved in melanin synthesis (TYR, TYRP1, and DCT), genes important for survival (BCL2), and a number of melanoma markers (MLANA, SILV, and TRPM1). In the β -catenin pathway, WNT protein binds to the frizzled family receptor to inactivate the kinase GSK3B which phosphorylates β -catenin and targets it for degradation in the proteasome. Thus, WNT signaling results in increased β -catenin levels, which may translocate into the nucleus where it activates the transcription of target genes including MITF, CCND1 and matrix metalloproteinase 7 (MMP7).

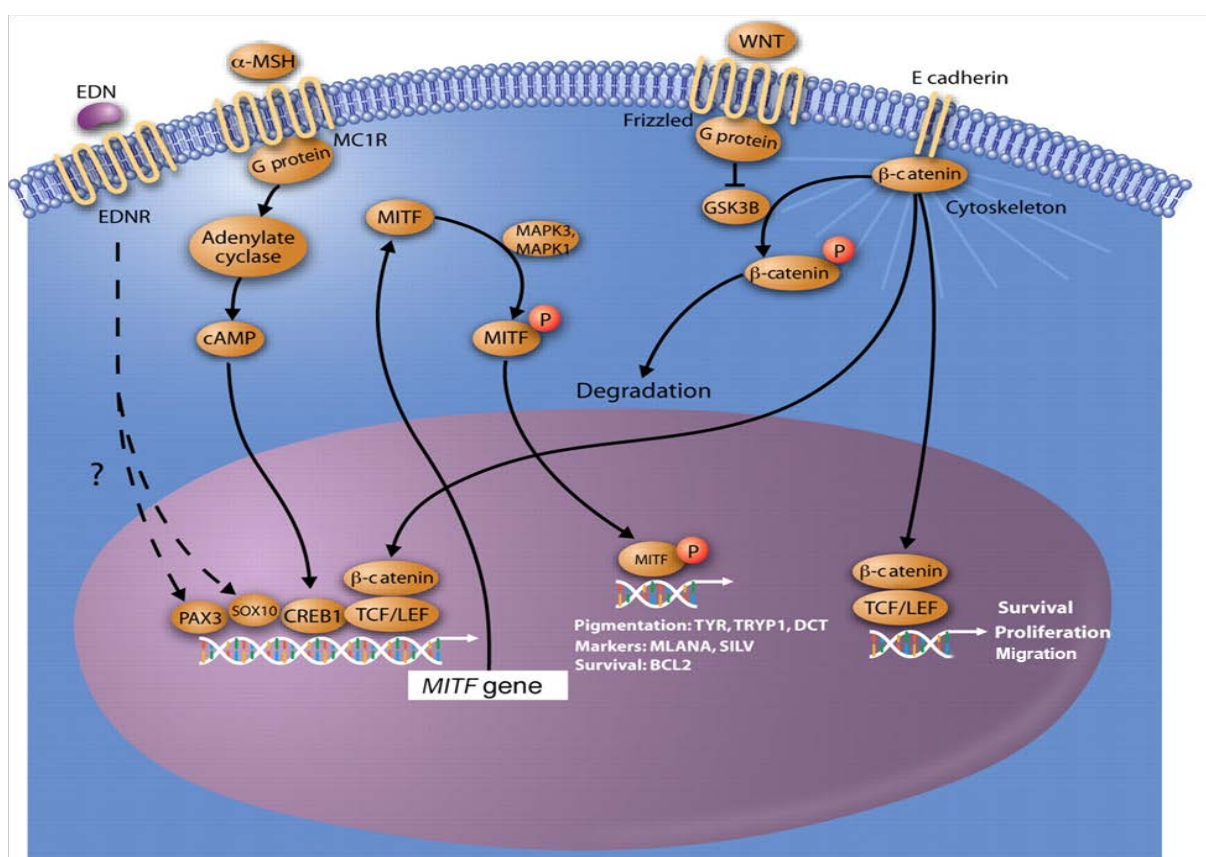


Figure 3 Cooperation of MITF and β -catenin pathways. α -MSH, alpha melanocyte stimulating hormone; PAX3, paired box 3; SOX10, sex-determining region Y-box 10; CREB1, cAMP response-element binding protein 1; TCF, T-cell factor; LEF, lymphoid enhancer-binding factor; GSK3B, glycogen synthase kinase 3 beta; TYR, tyrosinase; TRYP1, tyrosinase-related protein 1; DCT, dopachrome tautomerase; MLANA, melan-A; SILV, silver homolog (mouse); EDN, endothelin; P, phosphate. Adapted from Sekulic *et al.* (10).

In addition to the above-mentioned pathways, many others also play important roles in the development and progression of melanoma. These pathways often interact with one another and thereby form highly complex signaling networks. Enormous efforts have been made to unravel the molecular mechanisms behind this disease. Over the past decade, a new group of

molecules, miRNAs, have emerged to be essential and powerful regulators in most biological processes in cancers, including melanoma (18).

1.2 miRNA

miRNAs are a large family of short (~22 nucleotides [nt]), endogenous and single-stranded RNA molecules that regulate gene expression posttranscriptionally (19, 20). In human, miRNAs are predicted to target at least 30% of all protein coding genes (21). Functional studies show that miRNAs participate in the regulation of mostly every cellular process investigated so far and they are involved in many human diseases (22).

1.2.1 Biogenesis of miRNAs

As shown in **Figure 4**, primary miRNA (pri-miRNA) is transcribed from an independent gene or from an intron of protein-coding gene by RNA polymerase II (RNAPII) in the nucleus (23). A member of RNase III family, Drosha, and a double-stranded RNA binding protein, DGCR8, form a complex that process pri-miRNA into a ~70-nucleotide hairpin precursor (pre-miRNA), which is exported to the cytoplasm by Exportin 5 in a Ran-GTP manner. Some pre-miRNAs are processed from short introns (mirtrons) due to splicing and debranching, therefore bypassing the Drosha-DGCR8 step. In either case, pre-miRNA is cleaved by another RNase III enzyme, Dicer, with the assistance of RNA binding protein TRBP to form a ~70 bp miRNA/miRNA* duplex. In mammal, the 3' arm of some pre-miRNAs with high degree of complementarity along the hairpin stem is cleaved by Argonaute 2 (AGO2) before Dicer-mediated cleavage, thus forming an additional intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA), which facilitates subsequent strand dissociation (24). Then, one strand of the miRNA/miRNA* duplex (guide strand) is preferentially loaded into an miRNA-induced silencing complex (miRISC), while the other strand (passenger strand or miRNA*) is released and degraded. Not all miRNA* are only byproducts and sometimes both strand can be functional. Based on complementarity, miRNA guides the miRISC to the target mRNA, leading to translational repression, mRNA deadenylation, or degradation.

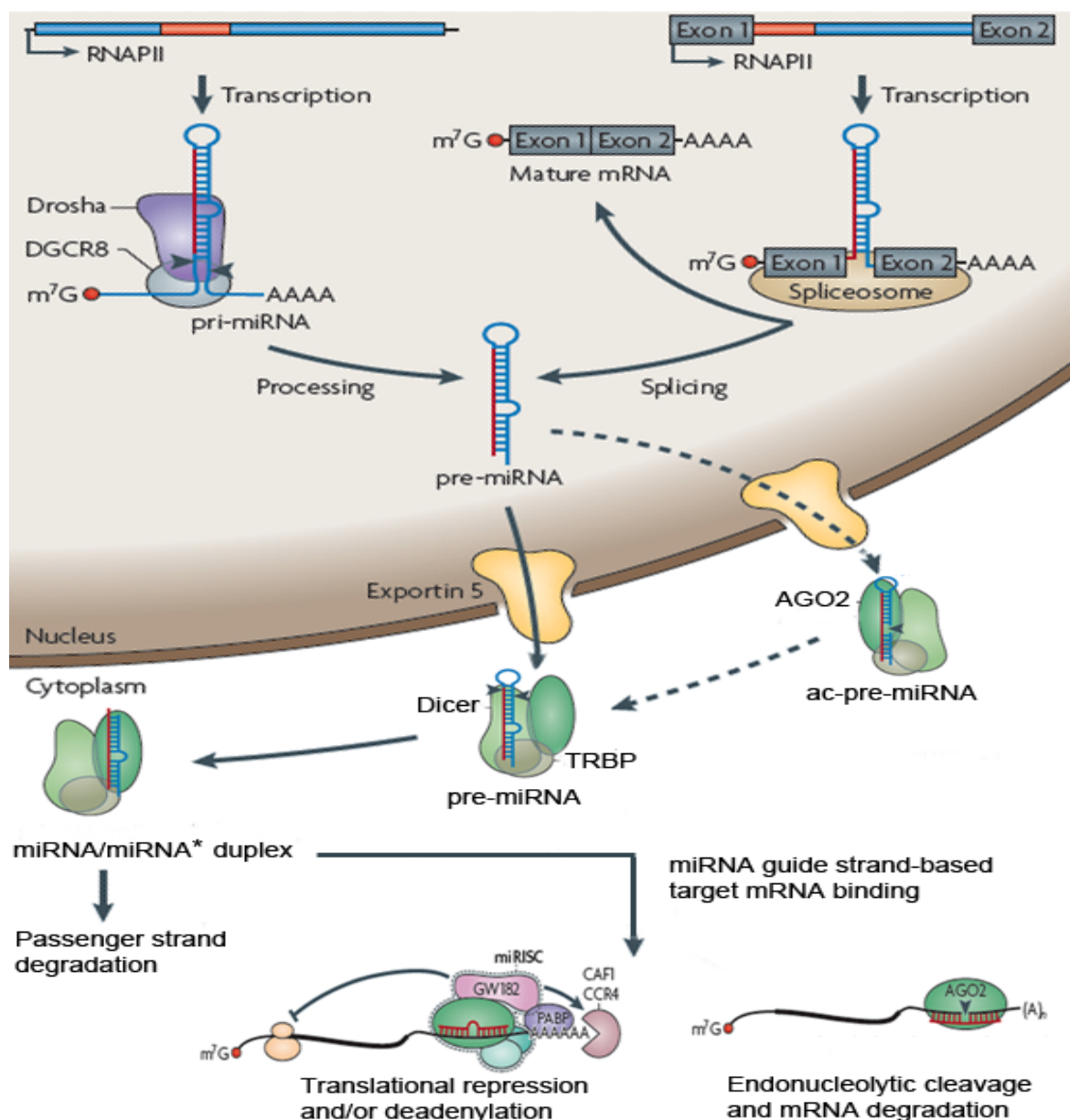


Figure 4 miRNA biogenesis. RNAPII, RNA polymerase II; DGCR8, DiGeorge syndrome critical region gene 8; GW182, glycine-tryptophan protein of 182 kDa; m⁷G, 7-methylguanosine-cap; PABP, poly(A) binding protein, miRISC, miRNA-induced silencing complex; ac-pre-miRNA, AGO2-cleaved precursor miRNA. Adapted from Krol *et al.* (22)

1.2.2 Identification of miRNA targets

Identification of miRNA targets is the key to understand the functions of miRNAs. To this end, several approaches have been invented to screen and to validate miRNA targets (25).

1.2.2.1 Computational prediction of miRNA targets

Basic principles of miRNA target prediction

miRNAs regulate gene expression through binding to the mRNA of their targets. Although the mechanism of targeting is not totally elucidated so far, some important features have emerged on target recognition, which have become the basis of the current prediction algorithms (20). Bioinformatic analysis of the first known miRNA-mRNA interaction showed that complementarity of miRNA seed region (position: 5' nucleotides 2-8) to the 3' UTR of the target mRNA is often important, and this commonly accepted principle have been applied in almost all currently used algorithms (20). Other criteria that are used in target predictions include evolutionary conservation of target site, free energy of the miRNA-mRNA heteroduplex, and mRNA sequence features surrounding the target site (25). Current prediction algorithms put different weight on these parameters.

Examples of prediction algorithms

TargetScan (26) and PicTar (21) are among the most commonly used algorithms. Both of them require stringent seed pairing although the impact of the target nucleotide opposite to the first miRNA nucleotide are different, and both take into account the conservation of target sites which improves their prediction (21, 26-28). TargetScan also considers features in the surrounding sequences of target sites, summed as "context score" in the output of the program, which improves the prediction for non-conserved target sites (29). The miRanda (30) algorithm requires moderately stringent seed pairing and allows G·U wobbles, which is more sensitive in predicting imperfect seed-pairing target sites, and also uses free energy of miRNA-mRNA heteroduplex and evolutionary conservation as criteria (31).

Some other algorithms are generated based on the notion that target site accessibility is critical in miRNA-mRNA interaction (32, 33). Those algorithms consider not only the free energy of miRNA-mRNA heteroduplex, but also the free energy required to unfold the secondary structure surrounding the target site (25). One example is the PITA algorithm, which calculates the combined interaction energy ($\Delta\Delta G$) based on the miRNA-mRNA hybridization energy (ΔG_{duplex}) and the energy cost to make the target site accessible

(ΔG_{open}) (32). PITA calculates $\Delta\Delta G$ for seed match irrespective of conservation and searching for target sites outside the 3' UTR is possible.

Limitations of miRNA target prediction

Although computational methods are strong tools to suggest putative miRNA targets, they are still not satisfactory due to the fact that the mechanisms of miRNA function is not fully understood. A prediction algorithm often offers a list of hundreds of candidate targets for a single miRNA. The lists of predicted targets generated by different algorithms often have limited overlaps (20, 34), which may be attributed to several reasons. First, the seed-pairing rules applied to predict targets vary in different algorithms. For instance, TargetScan rewards an A on position 1, whereas the other algorithm with stringent seed pairing reward a Watson–Crick match on this position (20). Second, the ranking methods are different across those algorithms. Third, some algorithms use different 3' UTR databases. For example, Targetscan uses the NCBI Reference Sequence (RefSeq) to define 3'-UTRs for human genes (29, 35), whereas miRanda uses the University of California Santa Cruz (UCSC) database (31, 36). Multiple lines of evidence suggest that in addition to 3' UTR, miRNA can also regulate gene expression through binding to the 5' UTR or the coding region of mRNA (37-40). However, most commonly used prediction tools, such as TargetScan, Pictar, and miRanda, do not predict target sites outside 3' UTR. Moreover, 3' pairing of the miRNA can supplement seed pairing to enhance target recognition, or in some cases it can even compensate for a mismatch in the seed (20), which raises more challenges for target prediction. Therefore, experimentally screening and validation of miRNA targets are essential in miRNA research.

1.2.2.2 Experimentally identification of miRNA targets

Microarray based Transcriptome analyses

The discovery that miRNAs can down-regulate the mRNA level of their targets paved the road for a number of subsequent studies (41, 42) in which overexpression and/or inhibition of a individual miRNA followed by transcriptome-wide microarray analysis was applied to identify candidate miRNA targets (28, 43, 44). In the initial study conducted by Lim *et al.*, the brain-specific miR-124 or muscle-specific miR-1 was individually transfected into HeLa cells

and microarray analysis was performed for the transfectants (41). In each case, the miRNA shifted the mRNA expression profile towards that of its original tissue. About 100 mRNAs were downregulated and the 3' UTR of these mRNAs had a significant propensity to pair to the 5' region of the miRNA (41). This pioneer study prompted other groups to use similar methods to investigate miRNA targets and functions. To reduce off-targeting effects from miRNA overexpression, some studies used complementary oligonucleotides to specifically inhibit the miRNA and searched for up-regulated mRNAs (43, 44). Nicolas *et al.* applied both overexpression and inhibition of a miR-149 to narrow down the list of putative miRNA targets (45). They found only 49 overlapping mRNAs out of hundreds of mRNAs that were affected by either treatment. Twenty-one of the 49 mRNAs contained complementary sequences to miR-149 seed, and interestingly, none of them were predicted by the commonly used target prediction algorithms, indicating a significant false negative prediction by these algorithms.

While these methods are successful in identifying a number of miRNA targets, they may fail to identify targets that do not undergo mRNA degradation. The microarray analysis itself cannot distinguish between direct targeting and indirect effect, thus bioinformatic approaches are often applied to facilitate target identification. Nevertheless, putative targets should be validated by other experiments.

Proteomic approaches

Proteomic approaches are attractive because they are capable of identifying targets regulated both by transcript destabilization and by translational inhibition. A well-known technology is stable isotope labeling by amino acids in cell culture (SLIAC). In this method, cells with or without miRNA treatment are cultured in medium containing heavy or light isotopes of essential amino acids typically arginine and lysine, and then the two populations of cells are 1:1 pooled followed by mass spectrometry analysis to identify and quantify proteins that were synthesized with heavy or light amino acids. Thereby, proteins that are regulated during treatment of miRNA can be identified (46). Vinther *et al.* reported the first study to utilize SILAC for miRNA target identification. They found 12 out of 504 identified proteins were repressed by miR-1 overexpression in HeLa cells and 8 out of the 12 genes contain miR-1 seed sequences, which is a significant enrichment to the non-regulated genes (46). Following

this study, two large-scale studies using SALIC to identify miRNA targets were carried out (33, 47). Baek *et al.* deleted mir-223 in mouse neutrophils and identified affected proteins (33). Selbach *et al.* pulse-labeled HeLa cells with isotopes to identify newly synthesized proteins that were regulated by miRNAs in HeLa cells (47). Both studies demonstrated that a single miRNA could affect the expression of hundreds of proteins. However, proteomic methods cannot distinguish between direct and indirect targets.

Immunoprecipitation of miRISC components

miRISC is the place where miRNAs bind to their target mRNA and execute their function with the assistance of other proteins, such as Ago proteins. Approaches have been developed to isolate the components of miRISC and identify miRNA targets.

Several studies have identified miRNA targets by immunoprecipitation of miRISCs containing either tagged or endogenous Ago proteins (48, 49). However, pull-down experiments using miRISC proteins do not specify mRNAs to a particular miRNA. To overcome this problem, an approach using tagged miRNA to specifically pull down the target mRNAs was developed by Orom and Lund recently (50). Generally, synthetic miRNA duplex is labeled with biotin at the 3' end of the guide strand and transfected into cells, where the guide strand is loaded into the miRISC and binds to the target mRNA (50). The miRNA-mRNA complexes are captured by streptavidin beads and mRNAs can be purified and analyzed (50). However, it is not clear whether the biotin tag can have an effect on the binding of miRNA and mRNA.

Recently a new technique called high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation (HITS-CLIP), has been developed, which utilize UV irradiation to crosslink RNA and RNA-binding proteins prior to immunoprecipitation, followed by deep sequencing to comprehensively identify the bound RNAs (51). HITS-CLIP is powerful in finding the potential targets mRNAs for miRNAs. However, there are two major disadvantages: the efficiency of UV 254 nm RNA-protein crosslinking is usually low, and the location of the crosslink is not readily identifiable within the sequenced crosslinked fragments (52). To overcome these disadvantages, Hafner *et al.* improved the method by treating the cell with a photoactivatable nucleoside analog 4-thiouridine which could substitute thymidine in transcription and subsequently increase the crosslinking efficiency (53). This method is

termed as photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP). The more attracting feature of PAR-CLIP is that there is a prominent transition from thymidine (T) to cytidine (C) in the crosslinked sites during cDNA synthesis from the isolated RNA fragments. This allows the method to map target sites more precisely.

Validation of putative miRNA targets

Identification of putative miRNA targets using the above-mentioned methods is only the first step. The next step is to specifically validate whether a putative target is a bona fide direct target of a miRNA (25, 34). Usually, qPCR and Western blotting are performed to examine the effect of miRNA (overexpression or knockdown) on the mRNA and protein levels of a target gene. Direct targeting is confirmed by reporter assay in which the expression of a reporter plasmid containing full-length or a fragment of the 3' UTR is regulated by miRNA. Specific target site can be identified by introduction of mutation or deletion in the putative target site in the reporter assay.

1.2.3 miRNA and cancer

The first study that links miRNA to cancer showed frequent down-regulation or deletion of miR-15a and miR-16 most chronic lymphocytic leukemia (CLL) patients (54). Since then, miRNA expression profiling and functional studies of miRNAs in cancer have been growing exponentially, which demonstrate that miRNAs are important players in cancer development and progression.

1.2.3.1 miRNA expression profiling in cancer

In 2005, Jiang *et al.* used a qPCR method to examine the expression of 222 miRNA precursors in 32 human cell lines from lung, breast, colorectal, hematologic, prostate, pancreatic, and head and neck cancers, and they found that the cell lines could be grouped on the basis of their tissue origins using hierarchical clustering analysis (55). Lu *et al.* used a bead-based flow cytometric miRNA expression profiling method to analyze 217 miRNAs from 334 samples of multiple human cancers. The resulting miRNA profiles reflected the developmental lineage and differentiation state of the tumors, which showed the potential of

miRNA profiling in cancer diagnosis (56). In another study, Volinia *et al.* carried out a large-scale microarray analysis of 228 miRNAs in 540 samples including different types of cancer and normal controls (57). They identified a cancer miRNA signature composed by a large portion of overexpressed miRNAs and some of them had shown well characterized cancer association (57). Moreover, many predicted targets of these differentially expressed miRNAs are oncogenes or tumor suppressors including Retinoblastoma 1 (RB1) and transforming growth factor, beta receptor II (TGFB2), which were further validated experimentally (57). The investigations on miRNA profiling paved the way for the later more extensive studies on the functions of miRNAs in cancer.

1.2.3.2 Functions of miRNA in cancer

Cancer cells display dysregulated miRNA expression profiles compared to their normal counterparts. Studies have shown that miRNAs can act as either oncogenes or tumor suppressors depending on their downstream target genes (tumor suppressors or oncogenes) (58).

miRNA as oncogene

A number of miRNAs have been found to be oncogenic. In the miRNA profiling study carried out by Volinia *et al.*, 21 differentially expressed miRNAs were found in at least three different cancers, and on the top of the list was miR-21, which was overexpressed in 6 types of cancer, including breast, colon, lung, pancreas, prostate and stomach cancer (57). miR-21 was also shown to be overexpressed in cholangiocarcinomas, and directly target the tumor suppressor PTEN, whose function is altered in various advanced cancers. Other studies have shown that miR-21 can target the pro-apoptotic factor PDCD4 and the tumor suppressor gene tropomyosin 1 (TPM1) in breast cancer (59, 60), and miR-21 expression can be induced by AP-1 (c-Jun/c-Fos heterodimer) transcription factor which in turn inhibits expression of PTEN and PDCD4 (61).

There is a cluster of miRNAs, miR-17-92 cluster, the members of which are known to act as oncogenes promoting cell proliferation, suppressing apoptosis, and inducing tumor angiogenesis (62). The miR-17-92 cluster is up-regulated in both hematopoietic malignancies

and solid tumors (57, 63, 64). In a human B-cell line, overexpression of c-Myc induced the expression of the miR-17-92 cluster that in turn inhibits the expression of E2F1, which is also a target of c-Myc and induces apoptosis at high level (65). Thus, c-Myc promotes E2F1 transcription and in parallel limits the translation, allowing a tightly controlled proliferative signaling (65). The miR-17-92 cluster can inhibit the expression of the anti-angiogenic factor thrombospondin-1 (TSP1) and the connective tissue growth factor (CTGF), thereby promoting tumor angiogenesis (66).

A number of other oncogenic miRNAs have been shown to play essential roles in cancer, such as miR-200c which targets zincfinger E-box binding homeobox 1 (ZEB1) and facilitates epithelial-mesenchymal transition (EMT) in various cancers (67), miR-10b which promotes tumor invasion and metastasis in breast cancer (68), and miR-146b-5p which contributes to thyroid tumorigenesis (69).

miRNA as tumor suppressor

Meanwhile, a lot of miRNAs have been shown to be tumor suppressors. The observation that the 13q14 region is frequently deleted in the CLLs suggested the existence of a tumor suppressor gene in that locus (54). For a long time, scientists had been searching for a candidate tumor suppressor in this region, but did not identify a suitable protein-coding gene. Calin *et al.* discovered that miR-15a and miR-16-1 are located in the first intron of a non-coding gene called leukemia associated gene 2 (LEU2), which is in the minimally deleted region in the CLLs (70). miR-15a and miR-16 are both deleted or down-regulated in the majority of CLL patients. Further studies from the same laboratory showed that both miRNAs could down-regulate BCL2 expression posttranscriptionally and induce apoptosis in a leukemic cell line model (71). The miR-15a/miR-16-1 deletion was also found in other cancers, such as prostate, breast, ovary, and bladder cancers (72). Another study in prostate cancer showed that miR-15a and miR-16 target CCND1 and WNT3A, which promotes cell survival, proliferation and invasion (73). These findings suggest that miR-15a and miR-16 are bona fide tumor suppressors.

The let-7 family is among the most actively studied tumor suppressive miRNAs. It has been shown that the let-7 family is down-regulated in different cancers such as lung cancer(74),

ovarian cancer (75), and colon cancer (76). Takamizawa *et al.* found that reduced expression of let-7 in human lung cancers was associated with poor postoperative survival (77). Johnson *et al.* showed for the first time that let-7 could target all three human RAS genes (HRAS, KRAS and NRAS) (74), and in another report they found overexpression of let-7 in cancer cell lines altered cell cycle progression and inhibited cell division by targeting multiple genes, which suggested that let-7 could be a master regulator of cell proliferation pathway (78). Akao *et al.* reported that let-7 inhibited protein expression of RAS and c-MYC in colon cancer cells and thereby significantly suppressed cell growth (76). Lee *et al.* found that let-7 repressed expression of the HMGA2 oncogene (79).

In 2007, reports from several research groups showed that members of miR-34 family are direct transcriptional targets of p53 tumor suppressor, which connected the p53 pathway with miRNA network for the first time (80-83). Ectopic miR-34 expression induces apoptosis and promotes cell-cycle arrest, while loss of miR-34 can impair p53-mediated cell death (84). Dozens of miR-34 Targets have been identified and many of them are regulators of cell cycle, apoptosis, proliferation, migration and invasion, including c-MYC, E2F3, BCL2, CDK4, CDK6 and c-Met (85). miR-34 expression can be inactivated by promoter hypermethylation in various types of cancer (86).

Another notable tumor suppressive miRNA is miR-101. Varambally *et al.* reported that the genomic locus encoding miR-101 was lost in 37.5% of clinically localized prostate cancer tissues and 66.7% of metastatic tissues (87). The copy number alteration caused a decrease of miR-101 expression during cancer progression paralleled by an increase of EZH2 expression. miR-101 could directly target EZH2 leading to inhibition of cell proliferation, invasion and tumor growth. This targeting was confirmed in different cancers, including bladder transitional cell carcinoma (88), gastric cancer (89), glioblastoma (90) and non-small cell lung cancer (NSCLC) (91). Further studies showed that miR-101 could target multiple oncogenes, including myeloid cell leukemia sequence 1 (MCL1) in hepatocellular carcinoma (92), cyclooxygenase-2 (COX2) in colon cancer (93), and MYCN in neuroblastoma (94).

1.2.3.3 miRNAs in melanoma

miRNA profiling in melanoma

The earliest publication concerning miRNAs in melanoma dates back to 2005 when Lu *et al.* utilized a bead-based miRNA profiling method to analyze 217 miRNAs from 334 samples including several types of tumor tissues and normal tissues (56). In this pioneer study, they found that miRNA profiles were able to classify tumors based on the developmental lineage and differentiation state. There was a general down-regulation of miRNAs in tumor samples compared to normal samples. However, among the large set of samples, there were only three melanoma tissue samples and two melanoma cell lines, which were not grouped together in the clustering analysis. Due to the lack of normal melanocyte controls, comparison of miRNA expression between melanoma and normal melanocytes was not possible from their data.

In the next two years, more melanoma cell lines were included in three studies which analyzed miRNA copy number or expression in panels of different tumor tissues and cell lines (95-97). In 2006, Zhang *et al.* evaluated genome-wide miRNA DNA copy number abnormalities in 227 human ovarian cancer, breast cancer, and melanoma specimens (95). They found that 85.9% (243 of 283) of miRNA genes were located in regions that exhibited DNA copy number abnormalities in melanoma and some of them were specific in this cancer. They confirmed that the miRNA expression was correlated with the copy number of the respective miRNA, which indicated that copy number changes of miRNA genes may account for dysregulation of miRNA.

In 2007, Gaur *et al.* analyzed the expression of 241 miRNAs in normal tissues and a panel of fifty-nine NCI-60 cell lines derived from hematologic, colon, central nervous system, and melanoma tumor (96). The tumor cell lines were clustered in a way that reflected their tissue origin. Fifteen miRNAs (4 up- and 11 down-regulated) were found to be differentially expressed in the eight melanoma cell lines compared to the other cancer cell lines. However, in contrast to the other tumor types, comparison between melanoma and healthy tissue was not performed, because there was no suitable control for melanoma in the panel of normal

tissues used in this study. In another study, Blower *et al.* also performed miRNA profiling for the NCI-60 cell lines and found cell lines from the same origin were clustered together (97). By comparing miRNA expression patterns with compound potency patterns, they suggest that miRNAs may play a role in chemo-resistance.

In 2009, Mueller *et al.* reported the first comprehensive miRNA expression profiling in human normal epidermal melanocytes (NHEMs) and well-characterized melanoma cell lines derived from primary and metastatic tumors (98). They identified 91 differentially expressed miRNAs in primary melanoma cell lines compared to melanocytes, as well as 15 differentially expressed miRNAs in metastatic cell lines compared to primary cell lines and melanocytes. In both comparisons, most of the differentially expressed miRNAs showed up-regulation. Comparison between highly invasive and weakly invasive cell lines revealed 33 differentially expressed miRNAs. Expressions of several candidate miRNAs were confirmed by qPCR in these cell lines as well as in tissue samples. Thus, this study identified miRNAs that might be dysregulated in different steps of melanoma progression such as early progression, invasion and metastasis.

Following the study of Mueller *et al.*, several groups reported their studies on miRNA profiling in melanoma from different aspects. Jukic *et al.* identified miRNAs that were differentially expressed between old patients and young patients and these miRNAs were associated with cell growth, differentiation, migration, invasion, and EMT (99). Segura *et al.* identified a signature of 18 miRNAs whose overexpression was significantly correlated with post-recurrence survival, and a signature of 6 miRNAs which could classify stage III patients into better and worse prognostic categories (100). In a comprehensive study carried out by Philippidou *et al.* (101), miRNA expression profiles for NHEMs and melanoma cell lines were generated using miRNA microarray, real-time miRNA reverse transcription-PCR array, followed by qPCR validation. Tissue samples including benign nevi, primary tumors and metastatic tumors were also analyzed in order to correlate with the expression pattern of cell lines. Discrepancies were found between different detection techniques and also between tissue samples and cell lines. miR-200c was the only miRNA that was commonly down-regulated in melanoma cell lines and tumor tissues robustly detected by the three

different platforms. Subsequently, whole-genome cDNA array was performed on selected melanoma cell lines and melanocytes to identify possible targets of some dysregulated miRNAs and bioinformatic analysis identified a dysregulated gene network centered on MITF, a key regulator in melanocyte differentiation and melanoma development.

Although microarray approaches are widely used in miRNA expression profiling, they are limited to the analysis of known miRNA species. Deep-sequencing is a more advanced technology that is able to identify and quantify not only known miRNAs but also novel miRNAs. Stark *et al.* deep-sequenced 12 small RNA libraries from melanoblasts, melanocytes, congenital nevocytes as well as acral, mucosal, cutaneous and uveal melanoma cells (102). They identified 539 known mature miRNAs along with the prediction of 279 novel miRNA. Their data suggested that some of the potential novel miRNAs were likely to be specific to the melanocytic lineage and might become interesting candidates in melanoma research.

Functions of miRNA in melanoma

Bemis *et al.* reported the first functional study of a single miRNA in melanoma (103). They found that mir-137 gene is located in the chromosomal region 1p22 known to be associated with melanoma susceptibility and computational algorithm predicted MITF as a putative target of miR-137, which was then validated by experiments. Interestingly, they identified a 15-bp variable nucleotide tandem repeat in the 5' pri-mir-137 sequence, which could affect the processing of pri-mir-137 into the mature form and thereby alter its function in melanoma cell lines. Moreover, they found that miR-137 expression was decreased by α -MSH, suggesting that miR-137 might be involved in sun tanning response. After the first report, studies on miRNA functions in melanoma have increased rapidly.

The let-7 family has been identified to act as tumor suppressor through targeting important oncogenes, such as RAS and c-Myc, in various types of cancer (104). The tumor suppressive function was also observed in melanoma. Schultz *et al.* identified 72 differentially expressed miRNAs by miRNA expression profiling in benign melanocytic nevi and primary malignant melanomas (105). Among them, let-7b was significantly down-regulated in melanoma compared with nevi. Overexpression of let-7b in melanoma cells decreased the expression of several cell cycle molecules such as cyclins D1, D3, and A, and CDK 4. In particular, cyclin

D1 was validated as a direct target of let-7b using reporter assays. Overexpression of let-7b inhibited cell cycle progression and anchorage-independent growth of melanoma cells. In another study, Mueller *et al.* found that let-7a was down-regulated in melanoma cell lines compared to melanocytes and the expression of integrin β 3 showed the opposite pattern (106). Integrin β 3 promotes the migration and invasion of melanoma cells and is involved in melanoma progression. Sequence analysis suggested a putative target site for let-7a in the 3' UTR of integrin β 3 which was validated by reporter assay. Overexpression of let-7a decreased integrin β 3 mRNA and protein, and reduced the invasive potential of melanoma cells; while inhibition of let-7b increased the invasion of melanocytes. These studies demonstrated that loss of let-7 family members may contribute to melanoma development and progression through activating various oncogenic processes including pathways acting on proliferation, migration and invasion.

miR-221 and miR-222 are clustered on the X chromosome and are likely transcribed as a common precursor (107). They were found to be overexpressed in different cancers including pancreatic cancer (108), papillary thyroid carcinoma (109), glioblastoma (110), and prostate cancer (111). miR-221/222 were reported to target c-KIT in papillary thyroid carcinoma (109) and erythroleukemic cells (112). The receptor tyrosine kinase c-KIT was found to decrease with melanoma progression (113, 114). Based on these previous findings, Felicetti *et al.* analyzed the expression of miR-221/222 and c-KIT in melanocytes and melanoma cell lines including primary vertical growth phase and metastatic melanomas. They found that miR-221/222 expression was increased with tumor progression and was reversely correlated with c-KIT expression. They identified the promyelocytic leukemia zinc finger (PLZF) transcription factor as a repressor of miR-221/222 by binding to regulatory region of their gene. They proposed that down-regulation of PLZF in melanoma cells unblocks miR-221/222 which in turn represses the expression of cyclin-dependent kinase inhibitor 1B (CDKN1B) and c-KIT, leading to an increase of proliferation and an inhibition of differentiation, respectively. Further *in vivo* experiment showed that overexpression of miR-221/222 promoted tumor growth while inhibition of miR-221/222 by antagomir suppressed tumor growth.

In the paper from Bemis *et al.*, they suggested that, in addition to miR-137, there might be other miRNAs regulating MITF (103). This was proven by the study from Segura *et al.* who reported that miR-182 targets MITF directly (115). miR-182 is located in a chromosomal region (7q31-34) frequently amplified in melanoma and is up-regulated in melanoma cell lines and tissues. Overexpression of miR-182 increased the migratory activity of melanoma cells *in vitro* and their metastatic potential *in vivo*. Silencing of miR-182 induced melanoma cell apoptosis and impaired their invasive capacity *in vitro*. Forkhead box O3 (FOXO3) and MITF are miR-182 targets and mediators of miR-182 function. Considering the complexity of MITF regulation and the long 3' UTR of MITF, the interplay between miR-137 and miR-182, and possibly other miRNAs may contribute to the fine tuning of this important transcription factor.

In most of the above-mentioned studies, miRNA expression profiling analyses were performed first to screen for dysregulated miRNA candidates, followed by target identification and functional study. However, profiling data differ substantially between various studies using different platforms and samples (101). Thus, it is difficult to unravel miRNA networks for functions of interest like invasion and migration, as uncertain expression data would have to be combined with uncertain target predictions. In order to obtain candidate miRNAs relevant for defined functions, functional screenings using miRNA libraries have been applied in various types of cancer including lung (116), breast (117), colorectal (118), pancreatic cancer (119), and also for melanoma (120). Levy *et al.* performed a miRNA library screening in A375M melanoma cell line using matrigel invasion assay, and they found that overexpression of miR-211 led to the most significant reduction of cell invasion (120). miR-211 is an intronic miRNA in the TRPM1 gene which is down-regulated in metastatic melanoma and is thought to function as a melanoma tumor suppressor. Further experiments of the group showed a decreased expression of miR-211 along with its host gene TRPM1. Increased expression of miR-211, but not TRPM1, reduced the migration and invasion of highly invasive melanoma cells with low levels of miR-211 and TRPM1. Using gene network analysis, they identified three central node genes (insulin-like growth factor 2 receptor [IGF2R], transforming growth factor, beta receptor II [TGFB2] and nuclear factor of activated T-cells 5 [NFAT5]) which were validated as miR-211 targets. Knockdown of each target gene phenocopied the effects of miR-211 overexpression on melanoma invasiveness. This study proposed that miR-211

acts as a tumor suppressor whose expression might be blocked via suppression of the TRPM1 locus during melanoma progression. This notion was further confirmed by another study reported by Mazar *et al.* (121) in the same month. Their data showed that miR-211 could inhibit both proliferation and invasion of melanoma cells, and they identified KCNMA1 (potassium large conductance calcium-activated channel, subfamily M, alpha member 1) as a target of miR-211.

miRNA expression can be regulated by different mechanisms: alteration of the miRNA biogenesis machinery, chromosomal abnormalities, mutations, polymorphisms and epigenetic regulation (122). Weber *et al.* analyzed the genomic sequences of 332 miRNAs in the miRBase (Version 8.0) and found that 155 of them (47%) were associated with CpG islands, indicating possible epigenetic modulations for these miRNAs (123). Several lines of evidence have proven that cancer cells can down-regulate tumor suppressive miRNAs by hypermethylation of their promoter regions (124-127). Recently, Perera's group has made efforts on identifying epigenetically regulated miRNAs in melanoma (128, 129). By treating melanoma cells with DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine, they found a list of up-regulated miRNAs and miR-375 and miR-34b were among the ones that had the strongest effects. Further analysis showed that the CpG islands up-stream of miR-375 and miR-34b genes were unmethylated or poorly methylated in keratinocytes and melanocytes, whereas the methylation of these CpG islands was increased with the stage in melanoma cell lines. Both miR-375 and miR-34b were able to inhibit the proliferation, invasion and migration of melanoma cells. Their studies suggested that miR-375 and miR-34b might be silenced by hypermethylation leading to the impairment of their tumor suppressive functions during the progression of melanoma.

miRNAs as prognostic markers in melanoma

Since miRNAs are important regulators of many cellular processes which affect the aggressiveness of melanoma, they may also serve as prognostic markers for melanoma patients like the protein coding genes do. As mentioned above, Segura *et al.* analyzed 59 formalin-fixed paraffin-embedded (FFPE) melanoma metastatic samples using miRNA arrays containing 911 probes. They identified a signature of 18 miRNAs whose overexpression was

significantly correlated with longer post-recurrence survival (>18 months), and a signature of 6 miRNAs which could categorize stage III patients into better and worse prognostic groups (100). Satzger and colleagues tested miR-15b expression in 128 FFPE primary melanoma samples and found that high expression of miR-15b was significantly associated with poor overall and recurrence free survival (130). Caramuta *et al.* reported that low expression of miR-191 and high expression of miR-193b were associated with poor survival in their data set composed of 16 regional lymph node metastatic samples (131). Interestingly, in the aforementioned study by Segura *et al.*, high miR-193b was associated with longer post-recurrence survival. In another two studies from Chen *et al.*, miR-193 was found to act as a tumor suppressor and down-regulate CCND1 and MCL1 (132, 133). Between these studies, there seem to be discrepancies which may be solved by further studies with independent cohort of samples and identification of more miR-193b targets.

1.3 Aim of this study

One of the interesting and yet unsolved questions in the melanoma research field is why melanomas progress extremely fast once they have become metastatic. In our database, we observed that patients with advanced melanoma often have very poor prognosis. However, survival times of individual patients can vary significantly, from months to years, indicating that the aggressiveness of melanoma in these patients may be different. Enormous efforts have been made to understand the molecular mechanisms involved in the aggressiveness of metastatic melanoma. As summarized above, miRNAs have recently emerged as key players in the development and progression of cancer, including melanoma.

The aim of this study was to:

1. identify miRNAs that may account for the aggressiveness of melanoma by miRNA expression profiling of melanoma cell lines derived from metastatic melanoma patients with long or short survival times, respectively;
2. seek for novel miRNA targets and to explore the function of the identified miRNAs in melanoma.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Cells

HEK293T	ATCC, Rockville, MD
Sk-Mel-23	Memorial Sloan-Kettering Cancer Center, New York, NY
Other Melanoma cell lines (Table 1)	Clinical Cooperation Unit of Dermato-Oncology, DKFZ, Heidelberg, Germany
NHEMs from adult skin	PromoCell, Heidelberg, Germany
NHEMs from juvenile foreskin	Isolated from juvenile foreskin
Juvenile foreskin	Department of Surgery, University Hospital Heidelberg, Heidelberg, Germany
TOP10 <i>E. coli</i> competent cells	Invitrogen, Carlsbad, CA

Table 1 Information of melanoma cell lines

Sample	Age (yr)	Sex	Stage at biopsy	Survival ^a (mo)	Group	1st round miRNA chip ^b	1st round qPCR	2nd round miRNA chip	2nd round qPCR	Gene Chip ^c
Ma-Mel-12	48.5	M	IV	5.9	Long			x	x	MA
Ma-Mel-26a	73.0	M	III	3.3	Long	x, P	x	x	x	MA
Ma-Mel-36	27.2	M	IV	6.0	Long	x, P	x	x	x	MA
Ma-Mel-51	47.7	M	IV	3.1	Long	x, P	x	x	x	HD
Ma-Mel-57	78.2	M	IV	5.0	Long	x, P	x	x	x	HD
Ma-Mel-59a	52.3	M	IV	6.7	Long	x, P	x	x	x	MA
Ma-Mel-71	45.6	M	IV	5.0	Long	x, P	x	x	x	MA
Ma-Mel-73a	74.6	M	IV	3.9	Long	x, P	x	x	x	MA
Ma-Mel-75	59.8	M	III	6.6	Long	x, P	x	x	x	MA
Ma-Mel-134	67.7	M	III	4.7	Long	x, P	x	x	x	HD
Ma-Mel-38	63.4	F	IV	8.0	Long	P	x	x	x	HD
Ma-Mel-63a	44.2	F	IV	4.7	Long	P	x	x	x	MA
Ma-Mel-66b	65.6	F	IV	6.7	Long			x	x	MA
Ma-Mel-76	63.4	F	IV	6.7	Long	P	x	x	x	MA
Ma-Mel-80d	63.6	F	IV	6.8	Long			x	x	HD
Ma-Mel-101	33.6	F	IV	8.4	Long	P	x	x	x	HD
Ma-Mel-103a	47.4	F	IV	10.0	Long	P	x	x	x	HD
Ma-Mel-1	64.9	M	IV	27.9	Short			x	x	HD

Sample	Age (yr)	Sex	Stage at biopsy	Survival ^a (mo)	Group	1st round miRNA chip ^b	1st round qPCR	2nd round miRNA chip	2nd round qPCR	Gene Chip ^c
Ma-Mel-8a	56.3	M	III	53.4	Short	x, P	x	x	x	HD
Ma-Mel-13	49.5	M	IV	45.8	Short	x, P	x	x	x	MA
Ma-Mel-53	69.1	M	IV	53.9	Short	P	x	x	x	MA
Ma-Mel-61a	28.7	M	IV	35.6	Short	x, P	x	x	x	MA
Ma-Mel-61e ^d	30.5	M	IV	35.6	Short			x	x	HD
Ma-Mel-79b	40.4	M	IV	33.8	Short	x, P	x	x	x	MA
Ma-Mel-119 ^e	73.5	M	IV	67.8	Short	x, P				
Ma-Mel-122	47.5	M	IV	49.5	Short	P	x	x	x	HD
Ma-Mel-5	49.4	F	IV	35.1	Short	P	x	x	x	HD
Ma-Mel-19	62.3	F	IV	146.7	Short	P	x	x	x	HD
Ma-Mel-20	56.0	F	IV	31.8	Short	P	x	x	x	HD
Ma-Mel-35	72.4	F	IV	62.0	Short	P	x	x	x	MA
Ma-Mel-37b	59.1	F	IV	108.1	Short	P	x	x	x	HD
Ma-Mel-39a	57.2	F	IV	38.6	Short	P	x	x	x	MA
Ma-Mel-67	51.1	F	IV	40.3	Short	P	x	x	x	MA
Ma-Mel-86b	36.3	F	IV	65.1	Short	P	x	x	x	MA
Ma-Mel-128a	56.3	F	IV	25.4	Short	P	x	x	x	HD

a. Survival time was calculated as overall survival time after diagnosis of stage IV melanoma.

b. Samples used in the respective experiment are indicated with "x". Samples pooled in the 1st round miRNA chip analysis are indicated with "P".

c. Gene chip data was from our own experiment (HD) or from Prof. Dr. Schadendorf (MA) published in (134, 135).

d. Ma-Mel-61a and -61e cell lines were derived from different metastasis of the same patient. Only Ma-Mel-61e was included in the subsequent t-test in order to insure independent samples.

e. Ma-Mel-119 cell line was contaminated with fibroblasts and was excluded from further experiments.

2.1.2 Cell culture media and supplements

PRMI 1640

PAA Laboratories, Pasching, Austria

Fetal Calf Serum (FCS)

PAA Laboratories, Pasching, Austria

Melanocyte Growth Medium M2

PromoCell, Heidelberg, Germany

2.1.3 Kits

miRNeasy Mini Kit

Qiagen, Hilden, Germany

TaqMan MicroRNA Reverse Transcription

Applied Biosystems, Foster City, CA

TaqMan MicroRNA Assays

Applied Biosystems, Foster City, CA

TaqMan Gene Expression Assays

Applied Biosystems, Foster City, CA

TaqMan Universal PCR Master Mixture

Applied Biosystems, Foster City, CA

Transcriptor First Strand cDNA Synthesis

Roche Applied Science, Mannheim, Germany

Phusion High-Fidelity PCR	Finnzymes, Espoo, Finland
Rapid DNA Ligation	Roche Applied Science, Mannheim, Germany
QuikChange Lightning Site-Directed Mutagenesis	Stratagene, La Jolla, CA
Dual-Luciferase Reporter Assay System	Promega, Madison, WI
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAprep Spin Miniprep Kit	Qiagen, Hilden, Germany
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden, Germany
ECL Plus Western Blotting Detection System	GE Healthcare, Buckinghamshire
Effectene Transfection Reagent Kit	Qiagen, Hilden, Germany

2.1.4 Microarrays

Affymetrix HG-U133 plus 2.0 chip	Affymetrix, Santa Clara, CA
Human miRNA Expression Profiling V2 Panel	Illumina, San Diego, CA

2.1.5 Enzymes

Restriction Enzymes (EcoRI, XhoI, NotI)	New England Biolabs, Beverly, MA
Pow SuperYield DNA Polymerase	Roche Applied Science, Mannheim, Germany

2.1.6 Plasmids

pCMX-PL1	a gift from Dr. Armin Pscherer, Molecular Genetics, DKFZ, Heidelberg, Germany
psiCHECK2	Promega, Madison, WI

2.1.7 Oligonucleotides

DNA primers (Table 2)	Eurofins MWG GmbH, Ebersberg, Germany
miRNA mimics	Dharmacon, Lafayette, CO
siRNAs	Dharmacon, Lafayette, CO

Table 2 DNA primers for cloning

Name	Sequence (5' – 3')
mir101-1-F	ATTGAATTCGAGCCTTCAGAGAGAGTAATGC
mir101-1-R	CATCTCGAGTTCACCTTCTTTCTTCCTGG
MITF-R-S	ATACTCGAGCGAATCCTCCCTGCACTGC
MITF-R-A	AATGCGGCCGCATGTGAAAACCAAATGCTTTAATG
MITF-S1M2-F	TCTCTTGCGGATGCTTGGTACTTAAATGTTAATAATAGTCACCTGC
MITF-S1M2-R	GCAGGTGACTATTATTAACATTTAAGTACCAAGCATCCGCAAGAGA
MITF-S1M3-F	GTCTCTTGCGGATGCTTGGTACACCAATGTTAATAATAGTCACCTGC
MITF-S1M3-R	GCAGGTGACTATTATTAACATTGGTGTACCAAGCATCCGCAAGAGAC
MITF-S2M2-F	CAAAGTTGTTGAATTTATTTTTAAGAAAGAAATACTTAATTGGGAAGT TACTGTTACTTGATAACAATG
MITF-S2M2-R	CATTGTTATCAAGTAACAGTAACTTCCCAATTAAGTATTTCTTTCTTAA AAATAAATTCAACAAGTTTG
MITF-S2M3-F	TCAGCAAAGTTGTTGAATTTATTTTTAAGAAAGAAATACACAATTGGG AAGTTACTGTTACTTGATAAC
MITF-S2M3-R	GTTATCAAGTAACAGTAACTTCCCAATTGTGTATTTCTTTCTTAAAAAT AAATTCAACAAGTTTGCTG
c-MET-R-A	TATGCGGCCGCTTGCATCACTTTACTTTAATTGC
c-MET-R-S	ATACTCGAGTGCTAGTACTATGTCAAAGCAAC
YB1-R-S	AATCTCGAGAATGCCGGCTTACCATCTCT
YB1-R-A	ATGCGGCCGCCTTTATTAACAGGTGCTTGCAG
ABCB5-R-S	TAACTCGAGTGCTGTTGAGGTAGCACATATTT
ABCB5-R-A	TAAGCGGCCGCTGATTCTCAAGTCCCAATCTT

2.1.8 Antibodies

anti- β -actin monoclonal (#691001)	MP Biomedicals, Solon, OH
anti-MITF monoclonal (M6065)	Sigma, Saint Louis, MO
anti-c-Met monoclonal (#3127)	Cell Signaling Technology, Beverly, MA
anti-YB1 polyclonal (# 2749)	Cell Signaling Technology, Beverly, MA
anti-ABCB5 polyclonal (600-401-A77)	Rockland Immunochemicals, Gilbertsville, PA
anti-EZH2 monoclonal (#3147)	Cell Signaling Technology, Beverly, MA
anti-BCL2 monoclonal (#2870)	Cell Signaling Technology, Beverly, MA
anti-PARP polyclonal (#9542)	Cell Signaling Technology, Beverly, MA

Goat-anti-mouse IgG-HRP (sc2005)	Santa Cruz Biotechnology, Santa Cruz, CA
Goat-anti-rabbit IgG-HRP (sc2004)	Santa Cruz Biotechnology, Santa Cruz, CA

2.1.9 Reagents

Gene Ruler 100 bp DNA Ladder	Fermentas, St Leon-Rot, Germany
O'GeneRuler 1 kb DNA Ladder	Fermentas, St Leon-Rot, Germany
Precision Plus Protein Standards	Bio-Rad, Richmond, CA
Trypsin/EDTA 10x	PAA Laboratories GmbH, Pasching, Austria
DharmaFect Duo Reagent	Dharmacon, Lafayette, CO
DharmaFect 1 Reagent	Dharmacon, Lafayette, CO
Cell Lysis Buffer	Cell Signaling Technology, Beverly, MA
Bio-Rad Protein Assay reagent	Bio-Rad, Richmond, CA
BD Matrigel Basement Membrane Matrix	BD Biosciences, Bedford, MA
Cell Proliferation Reagent WST-1	Roche Applied Science, Mannheim, Germany

2.1.10 Chemicals

Trypsin/EDTA 10x	PAA Laboratories GmbH, Pasching, Austria
Phosphate Buffered Saline (PBS)	Biochrom AG, Berlin, Germany
Dimethyl sulfoxide (DMSO)	Applichem, Damstadt, Germany
Agrose	Sigma, Saint Louis, MO
Tris Base	Sigma, Saint Louis, MO
Ammonium persulfate	Sigma, Saint Louis, MO
Tetramethylethylenediamine (TEMED)	Bio-Rad, Richmond, CA
Sodium dodecyl sulfate (SDS)	Sigma, Saint Louis, MO
Glycine	GERBU Biotechnik, Gaiberg, Germany
Tween 20	GERBU Biotechnik, Gaiberg, Germany
Non-fat milk powder	Carl Roth GmbH, Karlsruhe, Germany
Bovine Serum Albumin (BSA)	Sigma, Saint Louis, MO
Methanol	Sigma, Saint Louis, MO
Ethanol	Sigma, Saint Louis, MO
β -Mercatoethanol	Sigma, Saint Louis, MO
Paraformaldehyde	Sigma, Saint Louis, MO
Crystal violet	Sigma, Saint Louis, MO

HGF	Sigma, Saint Louis, MO
Tryptone	Sigma, Saint Louis, MO
Yeast extrac	GERBU Biotechnik, Gaiberg, Germany
Sodium chloride (NaCl)	Sigma, Saint Louis, MO
Arga	Sigma, Saint Louis, MO

2.1.11 Consumables

Pipette tip (10, 20, 100, 200, 1000 µl)	Starlab, Milton Keynes, United Kingdom
Combitip (2.5, 5 mL)	Eppendorf, Hamburg, Germany
Serological Pipette (5, 10, 25 mL)	Greiner, Frickenhausen, Germany
Safe-Lock tube (0.5 mL, 1.5 mL)	Eppendorf, Hamburg, Germany
PCR strip tube	Greiner, Frickenhausen, Germany
MicroAmp Optical 96-Well Plate	Applied Biosystems, Foster City, CA
MicroAmp Optical Adhesive Film	Applied Biosystems, Foster City, CA
Falcon Round-Bottom Tube (14 mL)	BD Biosciences, Bedford, MA
Centrifuge tube (15 mL, 50 mL)	Greiner, Frickenhausen, Germany
tissue culture flask (75 cm ²)	TPP, Trasadingen, Switzerland
Cell Culture Test Plate (flat bottom, 12-, 24-, and 96-well)	TPP, Trasadingen, Switzerland
Petri Dishe	Greiner, Frickenhausen, Germany
Cryotube	Greiner, Frickenhausen, Germany
Culture-Insert	Ibidi, Munich, Germany
24-well Transwell plate	Corning Incorporated, Lowell, MA
Nitrocellulose Membrane	Whatman, Dassel, Germany

2.1.12 Equipments

ABI 7300 Real-Time PCR System	Applied Biosystems, Foster City, CA
BeadArray Reader	Illumina, San Diego, CA
Biofuge Fresco Centrifuge	Heraeus, Hanau, Germany
Biological Safety Cabinet	Heraeus, Hanau, Germany
BioPhotometer	Eppendorf, Hamburg, Germany
BioRad Mini-Gel apparatus	Bio-Rad, Richmond, CA
CASY1 Cell Counter	Schaerfe System, Reutlingen, Germany

CO ₂ -Incubator	Binder, Tuttlingen, Germany
Gene Array Scanner	Agilent Technologies, Palo Alto, CA
GeneChip Fluidics Station 400	Affymetrix, Santa Clara, CA
Innova 4230 Incubator Shaker	New Brunswick Scientific, Edison, NJ
Leica DM1L Microscope	Leica, Wetzlar, Germany
Microbiological Incubator	Heraeus, Hanau, Germany
MP220 pH Meter	Mettler Toledo, Columbus, OH
Multichannel Pipette	Eppendorf, Hamburg, Germany
Multifuge X3 FR centrifuge	Heraeus, Hanau, Germany
Multipipette	Eppendorf, Hamburg, Germany
Pipetboy	Brand, Wertheim, Germany
Pipette (P2, P10, P100, P200, P100)	Gilson, Bad Camberg, Germany
Power PAC 300 power supplier	Bio-Rad, Richmond, CA
Refrigerator	Liebherr, Ochsenhausen, Germany
Roller-mixer	Karl Hecht GmbH, Sondheim, Germany
Sorvall RT7 Centrifuge	Sorvall, Newton, CT
SpectraFluor Plus fluorometer	Tecan Deutschland, Crailsheim, Germany
Thermomixer	Eppendorf, Hamburg, Germany
Veriti 96-Well Thermal Cycler	Applied Biosystems, Foster City, CA

2.1.13 Softwares

Microsoft Office 2007	Microsoft, Redmond, USA
ImageJ	National Institutes of Health, Bethesda, MD
Graphpad Prism 4	GraphPad Software Inc., San Diego, USA
Leica Application Suite	Leica, Wetzlar, Germany
MultiExperiment Viewer (MeV) 4.4	Institute of Genomic Research, Rockville, MD
Chipster	CSC - IT Center for Science Ltd, Espoo, Finland
Beadstudio Data Analysis Software	Illumina, San Diego, CA

2.2 Methods

2.2.1 Preparation of buffers and media

50 x Tris-acetate-EDTA (TAE) buffer, pH 8.0, 1 L

Tris base	242 g
Acetic acid	57.1 mL
500mM EDTA solution	100 mL
H ₂ O	adjust the final volume to 1 L

1 x PBS, pH 7.4, 1 L

PBS powder	9.55 g
H ₂ O	adjust the final volume to 1 L

10 x SDS-PAGE Running Buffer, 1 L

Tris base	30 g
10 % SDS solution	100 mL
Glycin	144 g
H ₂ O	adjust the final volume to 1 L

1 x Tris-buffered saline (TBS), pH 7.6, 1 L

Tris base	2.42 g
Acetic acid	57.1 mL
500mM EDTA solution	100 mL
H ₂ O	adjust the final volume to 1 L

TBS-T

1 x TBS, with 0.1 % (v/v) Tween 20

Transfer Buffer, pH 8.5, 1 L

Tris base	3 g
Glycin	17.5 g
Methanol	200 mL
H ₂ O	adjust the final volume to 1 L

Stripping buffer, pH 6.8, 100 mL

0.5 M Tris-HCL (pH 6.8)	6.25 mL
10 % (w/v) SDS solution	20 mL
β-Mercaptoethanol	700 μL
H ₂ O	adjust the final volume to 100 mL

Cell freezing medium

RPMI 1640	50 % (v/v)
FCS	40 % (v/v)

DMSO	10 % (v/v)
LB medium, pH 7.5, 1 L	
Tryptone	10 g
Yeast extrac	5 g
NaCl	10g
H ₂ O	adjust the final volume to 1 L

2.2.2 Cell culture

Melanoma cell lines in this study were established from metastasis of patients with stage III or IV melanoma (**Table 1**). Biopsies were obtained from either a solid metastatic lesions or a malignant effusion after a patient's informed consent. Melanoma cell lines and HEK293T cells were cultured in RPMI 1640 medium supplemented with 10% FCS. Normal Human Epidermal Melanocytes (NHEMs) were cultured in Melanocyte Growth Medium M2. All cell lines were maintained at 37 °C in a humidified 5% CO₂ incubator.

2.2.3 Total RNA isolation

Total RNAs including small RNAs from either untransfected or transfected cells were isolated using miRNeasy Mini Kit according to the manufacturer's protocol. RNA concentrations were determined by BioPhotometer.

2.2.4 Microarray

2.2.4.1 Affymetrix Gene Chip

The whole procedure was carried out by the Core Facility of DKFZ according to the manufacturer's recommendations. Briefly, double stranded cDNA synthesis was carried out using 3 µl total RNA and oligo-dT primer, and the cDNAs were used as templates for *in vitro* transcription of biotin-labeled RNA. The resulting cRNAs were purified, fragmented and hybridized to Affymetrix HG-U133 plus 2.0 chips. Then the chips were washed and stained by GeneChip Fluidics Station 400, and scanned by Gene Array Scanner.

2.2.4.2 Illumina MicroRNA Expression Profiling Assay

The whole procedure was carried out by the Core Facility of DKFZ according to the manufacturer's recommendations. Briefly, miRNAs were polyadenylated, reverse transcribed into cDNAs using a biotin-labeled oligo-dT primer with a universal sequence at the 5'-end, attached to a solid phase, and annealed to miRNA-specific oligos which comprise three parts: another 5' universal PCR priming sequence, an address sequence for capturing the product on the array, and the 3' miRNA-specific sequence. The miRNA-specific oligos were extended, eluted and subjected to PCR fluorescent universal primers. Then, single stranded PCR products were prepared and hybridized to capture probes immobilized on beads in the arrays. In the testing (first) round, we used Human miRNA Expression Profiling V1 Panel which was deployed on a 96-sample Universal Array Matrix. In the 2nd round, we used Human miRNA Expression Profiling V2 Panel which was deployed on a 12-sample Universal BeadChip. Arrays were scanned by the BeadArray Reader and intensity per bead type (miRNA) was extracted.

2.2.4.3 Microarray data analysis

Affymetrix gene chip

Raw data of 16 cell lines was from our own experiment and raw data of the other 18 cell lines were provided by Dr. Schadendorf (134) (**Table 1**). All raw data were imported into Chipster software (CSC - IT Center for Science Ltd, Espoo, Finland), normalized using RMA method (136), and reannotated using the Chip Description File (CDF) for Affymetrix HG-U133 plus 2.0 chip (137).

Illumina miRNA chip

Illumina miRNA chip data was processed using Beadstudio Data Analysis Software, including the calculation of detection p-value and quantile normalization (138). In the first round of miRNA expression profiling we calculated the absolute difference between the expression values of the two groups divided by the sum of their standard deviations (SD_Diff), to identify differentially expressed miRNAs between groups. Thus, higher SD_Diff means more

significant difference. In the second round, quantile normalized data was imported into MeV 4.4 software (139). miRNAs with a average signal of less than 500 were excluded. Hierarchical clustering based on average linkage and euclidean distance (140) was performed on all melanoma and melanocyte samples. T-test was used to find differentially expressed miRNAs between groups. Bonferroni correction of p-value (141) was performed in the comparison between melanoma cell lines and NHEMs to reduce the number of significantly differentially expressed miRNAs, but not in the comparison between long survival and short survival groups due to the very small number of differentially expressed miRNAs.

2.2.5 Reverse transcription and qPCR

For quantitation of miRNAs, reverse transcription and qPCR were performed using TaqMan MicroRNA Reverse Transcription Kit and TaqMan miRNA assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Briefly, 40 ng of total RNA was reverse transcribed in a 15 μ L reaction using specific stem-loop primers for mature miRNAs. Two μ L of cDNA was used for the PCR amplification in 20 μ L reaction using the TaqMan Universal PCR Master Mixture kit. For quantitation of MITF, c-Met, YB1 and ABCB5, reverse transcription and PCR were carried out using Transcriptor First Strand cDNA Synthesis Kit and Taqman Gene Expression Assays. Briefly, 500 ng of total RNA was reverse transcribed in a 20 μ L reaction using oligo (dT)₁₈ as primer. cDNA was 1:5 diluted and 2 μ L was used for the PCR in 20 μ L reaction using the TaqMan Universal PCR Master Mixture kit. PCR was performed in triplicates. The thermal conditions are summarized in **Table 3**. Small nuclear RNA U6 (RNU6B) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous control for TaqMan miRNA assay and gene expression assay, respectively. Relative expression of the tested genes was calculated by ΔC_t -method according to the manufacturer's recommendation.

Table 3 Thermal conditions of reverse transcription and qPCR.

miRNA Reverse Transcription		
Temperature (°C)	Time (min)	Cycles
16	30	1
42	30	1
85	5	1
4	∞	1
Total RNA Reverse Transcription		
Temperature (°C)	Time (min)	Cycles
65	10	1
immediately on ice	5	1
50	60	1
85	5	1
4	∞	1
qPCR		
Temperature (°C)	Time	Cycles
95	10 min	1
95	15 sec	40
60	60 sec	

2.2.6 Construction of plasmids

2.2.6.1 PCR amplification of miR-101 precursor and 3'UTRs of target genes

Sequence flanking 67-bp up-stream and 56-bp down-stream of miR-101-1 precursor was amplified from genomic DNA of Sk-Mel-23 using a specific primer pair with restriction sites EcoRI (forward primer) and XhoI (reverse primer). Full-length 3'UTRs of target genes were amplified from genomic DNA of Sk-Mel-23 in case the 3'UTR is in one exon (MITF, c-Mer and ABCB5), or from mRNA of Ma-Mel-86b in case the 3'UTR spans more than one exon (YB1), using specific primer pairs with restriction sites XhoI (forward primer) and NotI (reverse primer). PCR amplicons were gel purified using QIAquick Gel Extraction Kit.

2.2.6.2 Restriction digestion, ligation and transformation

pCMX-PL1 plasmid and the PCR amplicon of miR-101 precursor was double-digested by EcoRI and XhoI and ligated to generate pCMX-PL1-mir-101 plasmid. psiCHECK2 plasmid and the PCR amplicons of target genes were double-digested by XhoI and NotI, and ligated to

generate psiCHECK2-MITF, -c-Met, -ABCB5 and -YB1. The ligation products were transformed into TOP10 *E. coli* competent cells.

2.2.6.3 Colony PCR check and MiniPrep

Colonies were picked up and subjected to PCR using the corresponding primer pairs. Positive colonies are inoculated into LB medium and plasmid was isolated using QIAprep Spin Miniprep Kit according to the manufacturer's protocol.

2.2.6.4 Sequencing and MaxiPrep

Positive plasmids were sequenced (GATC-Biotech, Constance, Germany) and correct ones were purified using QIAGEN Plasmid Maxi Kit according to the manufacturer's protocol.

2.2.7 Transfection

2.2.7.1 Co-transfection for dual-luciferase reporter assay

Appropriate amount of cells were seeded into 96-well plates to achieve 40% - 60% confluency before transfection the next day. 100 ng of pCMX-PL1-miR-101 and 100 ng psiCHECK2-MITF-3'UTR were co-transfected into HEK293T or Melanoma cell lines using Effectene Transfection Reagent. One hundred nM of miR-137 mimic and 100 ng of reporter plasmid of target genes (psiCHECK2-c-Met-3'UTR, -YB1-3'UTR and -ABCB5-3'UTR) were co-transfected into Ma-Mel-86b cells using DharmaFect Duo reagent.

2.2.7.2 Transfection of miRNA mimics or siRNAs for functional assays

Appropriate amount of melanoma cells were seeded into 12-well, 24-well or 96-well plates to achieve 40% - 60% confluence before transfection the next day. 100 nM of miRIDIAN miRNA mimics or ON-TARGETplus SMARTpool siRNAs was transfected into the cells using DharmaFect 1 reagent. Medium was changed within 24 hr after transfection.

2.2.8 Dual-luciferase reporter assay

Cells were cultured in 96-well plates. Twenty-four or 48 hr after transfection, medium was removed and 20 μ L/well of Passive Lysis Buffer was added to lyse the cells followed by 15-min incubation at room temperature. One hundred μ L/well of Luciferase Assay Reagent II was added to quantify firefly luciferase activity and then 100 μ L/well of Stop & Glo Reagent was added to quench firefly luciferase activity and activate Renilla luciferase. Renilla/firefly luciferase ratio was calculated and further normalized to the negative control.

2.2.9 Western blot

Cells were lysed by Cell Lysis Buffer and protein concentrations were determined by Bradford method using Bio-Rad Protein Assay reagent on BioPhotometer. 10 μ g of total protein was separated by 10% or 12% polyacrylamide gel, and electro-transferred onto nitrocellulose membranes. After blocking with 5% of non-fat milk (BSA was used in place of non-fat milk for the detection of MITF) in TBS-T (Tris Buffered Saline with 0.1% Tween-20), the membranes were incubated with primary antibodies at 4°C overnight, followed by 3 times of 10-minute wash with TBS. Then, the membranes were incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG secondary antibody at room temperature for 1 h, followed by 3 times of 10-minute wash with TBS. Blots were exposed to an X-ray film using enhanced chemiluminescence (ECL) system. Densitometric quantification of specific bands was performed using ImageJ software.

2.2.10 Matrigel invasion assay

Invasion assay was performed using Matrigel (10 μ g matrigel / well) coated 24-well transwell plate. Briefly, cells at 72 hr post transfection were seeded into the upper insert with serum-free medium and the lower chamber was filled with RPMI 1640 medium containing 10% FCS or 10 % FSC + 50 ng/mL HGF as chemo-attractant. Cells were kept at 37 °C in a humidified 5% CO₂ incubator. After 24 hr (Ma-Mel-86b) or 48 hr (Ma-Mel-79b), the non-invading cells on the upper side of the insert were removed gently by cotton swab, and the invasive cells on the lower side of the insert, were stained with 0.1 % crystal violet and photographed under a microscope. Cells were counted using ImageJ software.

2.2.11 Wound healing migration assay

Migration of melanoma cells was tested in wound healing assay using culture-insert. First, each insert was attached onto the bottom of one well in a 24-well plate. Cells at 72 hr post transfection were trypsinized, resuspended in RPMI 1640 medium, seeded into both chambers (70 μ l / chamber) of a culture-insert, and incubated overnight for cell adherence. The culture-insert provided two cell culture reservoirs, which were separated by a ~500 μ m thick wall. After removing the culture-inserts on the second day, a “wound” of ~500 μ m was formed between the two cell patches. Then, 500 μ l medium were added into each well. Photos of the remaining gaps (“wound”) were taken under microscope every 24 hr until 48 hr. Widths of wounds were measured at three positions for each replicate by Leica Application Suite.

2.2.12 Proliferation assay

Cell proliferation was determined by a colorimetric WST-1 assay which was based on the reduction of the tetrazolium salt WST-1 into formazan by cellular enzymes. Briefly, 10 μ L of WST-1 reagent was added to the transfected cells cultured in 100 μ L / well medium in a 96-well plate and incubated for 1 h at 37 °C. The absorbance of formazan, which was in proportion with the number of viable cells, was measured at 450 nm (610 nm as reference). The assay was performed 24, 48, 72 and 96 hr after transfection.

3 RESULTS

Although metastatic melanoma patients have very poor prognosis in general, the survival times of individual patients can be distinct, ranging from month to years, which may reflect the different aggressiveness of melanoma among patients. Dysregulation of miRNAs have been found to be involved in the development and progression of cancer, including melanoma. In this study, we hypothesize that there are miRNAs which may contribute to the aggressiveness of melanoma and thereby affect the survival of patients. We selected melanoma cell lines derived from the metastases of patients with either short survival (<10 months) or long survival (> 25 months), as well as NHEMs from healthy donors. We expected to identify differentially expressed miRNAs between the short and long survival groups, as well as between melanoma cell lines and NHEMs, using microarray miRNA profiling. Expression of these miRNAs was further analyzed by qPCR. Selected candidate miRNAs were then subjected to functional assays to test whether they could impact on the aggressiveness of melanoma cells.

3.1 miRNA expression profiling for melanoma cell lines and NHEMs

Microarray technology has been widely applied on miRNA expression profiling. In this study, we used this method to identify miRNAs that are dysregulated in melanoma and may account for the aggressiveness of this disease.

3.1.1 First round

When we started this study, the Core Facility of the DKFZ was establishing the Illumina miRNA expression profiling platform and we joined the testing phase using some of our RNA samples from melanoma cell lines.

3.1.1.1 miRNA expression profiling

In the first round, we used the Illumina MicroRNA Expression Profiling Version 1 panel which was deployed on a 96-sample Universal Array Matrix. The 96 samples were assigned to several research groups and we could test only 16 samples (indicated in **Table 1**). As we did not know whether the gender of patients could have an impact on their respective miRNA profiles, we tested individual melanoma cell lines only from male patients with short survival (MS group, n=9) and long survival (ML group, n=5). We also tested 2 pooled RNA samples of cell lines from short survivors (P-short) and long survivors (P-long) including males and females. As described in the Methods section, we used SD_Diff as a criterion to judge whether the expression of a miRNA was different between groups or samples. A number of differentially expressed miRNAs were found in the comparison between MS group and ML group (SD_Diff > 6) or between P-short and P-long (SD_Diff > 7.5) (**Table 4**). Eleven miRNAs (mir-101, -138, -17*, -19a, -211, -301, -33a, -33b, -363, -507 and -551b) were selected to be further validated by qPCR.

Table 4 Differentially expressed miRNAs in the first round miRNA expression profiling

MS group vs. ML group (SD_Diff > 6)		P-short vs. P-long (SD_Diff > 7.5)	
miRNA	FC	miRNA	FC
miR-224	-5.6	miR-551b	-17.4
miR-452	-3.4	miR-301	-6.2
miR-31	-2.9	miR-19b	-5.3
miR-182	-2.7	miR-101	-4.4
miR-183	-2.5	miR-214	-4.0
miR-17*	-1.9	miR-199a	-3.6
miR-33b	-1.9	miR-550	-3.3
miR-19a	-1.8	miR-33a	-3.2
miR-139	1.8	miR-507	-2.8
miR-148a	1.8	miR-138	-2.6
miR-519e	1.9	miR-130a	-2.1
miR-135b	2.0	miR-143	-2.0
miR-18b	2.1	miR-130b	-1.5
miR-363	2.2	miR-204	-1.4
miR-211	2.3	miR-135b	-1.0
miR-187	3.7	miR-199a*	1.1
		miR-374	1.1
		miR-186	1.4

MS group vs. ML group (SD_Diff > 6)		P-short vs. P-long (SD_Diff > 7.5)	
miRNA	FC	miRNA	FC
		miR-211	1.9
		miR-346	1.9
		miR-504	2.4
		miR-421	2.4

SD_Diff, the absolute difference between the expression values of the two groups divided by the sum of their standard deviations. FC, fold change; Positive value, up-regulation; Negative value, down-regulation; MS, male short survivors; ML, male long survivors; P-short, pooled short survivors; P-long, pooled long survivors.

3.1.1.2 qPCR validation of selected miRNAs

qPCR was performed to test the expression of the 11 selected miRNAs in a larger number of melanoma cell lines (14 from short survivors and 15 from long survivors as indicated in **Table 1**). These cell lines included the 14 cell lines from male patients which were used in the previous miRNA microarray analysis, and more cell lines from male and female patients. Pearson's correlation coefficients (r) were calculated between the miRNA microarray data and the qPCR data in the 14 cell lines shared in both methods (**Table 5**). All miRNAs showed positive correlations. Some miRNAs showed high correlations, such as miR-138 ($r=0.81$), miR-211 ($r=0.89$) and miR-551b ($r=0.93$); some miRNAs showed moderate correlations, such as miR-301 ($r=0.40$), miR-33a ($r=0.48$) and miR-363 ($r=0.51$). When we compared the expression of the 11 miRNAs in same the cell lines of the MS group ($n=9$) and the ML group ($n=5$) as used in the microarray analysis, we found that three miRNAs (miR-138, miR-17*, and miR-19a) showed significant differences between the two groups (t-test, $p<0.05$) (**Table 5**). However, when we compared the expression of the 11 miRNAs in a larger number of cell lines from short survivors ($n=14$) and long survivors ($n=15$), we could not find any of the 11 miRNAs to be differentially expressed between the two groups (t-test, $p<0.05$) (**Figure 5**). Only miR-101 expression showed a trend of higher expression in long survivors compared to short survivors (t-test, $p=0.11$).

Thus, the differentially expressed miRNAs (long survivor vs. short survivors) identified by microarray in a small number of cell lines from male patients could not be validated by qPCR in a larger number of cell lines from a combination of male and female patients. This is likely due to several reasons: (i) the method (SD_Diff) used to select the differentially expressed

miRNAs from the microarray data was not based on t-test; (ii) one of the cell lines (Ma-Mel-119) used in the microarray analysis turned out to be contaminated by fibroblast and could not represent miRNA expression profile of the original cell line; (iii) the differences found between small groups in the microarray analysis could be diminished when comparing larger groups in the qPCR analysis and thereby not significant.

Table 5 Statistical analyses for the expression of selected miRNAs

miRNA	Pearson's correlation coefficient between qPCR and microarray^a	p-value of t-test between MS and ML group^b
mir-101	0.63	0.536
mir-138	0.81	0.005
mir-17*	0.62	0.023
miR-19a	0.62	0.008
mir-211	0.89	0.225
mir-301	0.40	0.665
mir-33a	0.48	0.439
mir-33b	0.54	0.144
mir-363	0.51	0.286
mir-507	0.56	0.528
mir-551b	0.93	0.181

a. Pearson's correlation coefficient (r) between the miRNA microarray data and the qPCR data in the 14 cell lines shared in both methods.

b. T-test was performed to compare the qPCR-measured expression of the selected miRNAs in the cell lines from the MS group ($n=9$) and ML group ($n=5$) which were used in both microarray and qPCR analyses.

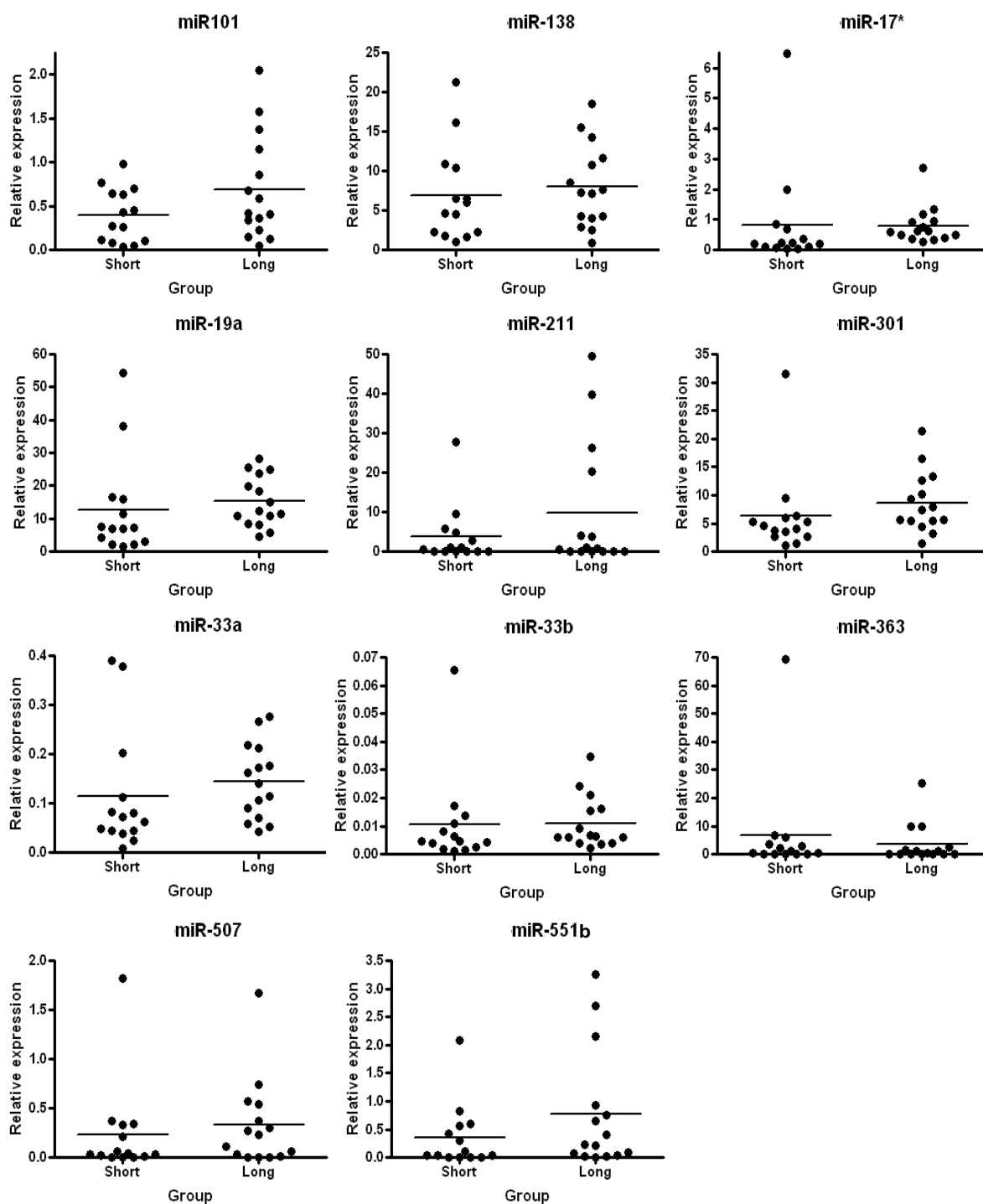


Figure 5 qPCR analysis of miRNA expression in melanoma cell lines. miRNA expression of 14 cell lines from short survivors and 15 cell lines from long survivors was determined by qPCR. Expression was normalized to the internal control RNU6B. The horizontal line shows the average expression level in each group. No significant differences were detected between groups (t-test, $p < 0.05$).

3.1.2 Second round

3.1.2.1 miRNA expression profiling

In the second round, we performed miRNA expression profiling for a large panel of 34 melanoma cell lines (**Table 1**) and 10 NHEMs. The melanoma cell lines included 17 cell lines from short survivors and 17 cell lines from long survivors (Ma-Mel-61a and -61e were derived from different metastases of one patient). The NHEMs included 2 cell lines (M9 and M11) from adult skin and 8 from juvenile foreskin (M2, M4, M6, M7, M8, M10, M12 and M13). The miRNA expression profiling was carried out using the Illumina MicroRNA Expression Profiling Version 2 panel which was deployed on a 12-sample Universal BeadChip.

After data normalization, we performed hierarchical clustering for all samples which showed two distinct clusters (**Figure 6**): one consists of only melanoma cell lines, and the other one consists of all NHEMs and two melanoma cell lines (Ma-Mel-12 and -20). Interestingly, these two cell lines were both highly pigmented as most of the NHEMs were. In the melanoma cluster, there was a small group of 4 cell lines (Ma-Mel-51, -75, -67 and -63) which were also pigmented. Ma-Mel-61a and -61e were clustered close together, reflecting that they were from the same patient. However, separation of short survivors and long survivors was not seen, which indicated that there was no big difference in miRNA expression between the two groups. Of note, melanoma cell lines from male and female, or NHEMs from adult and juvenile were not separated either.

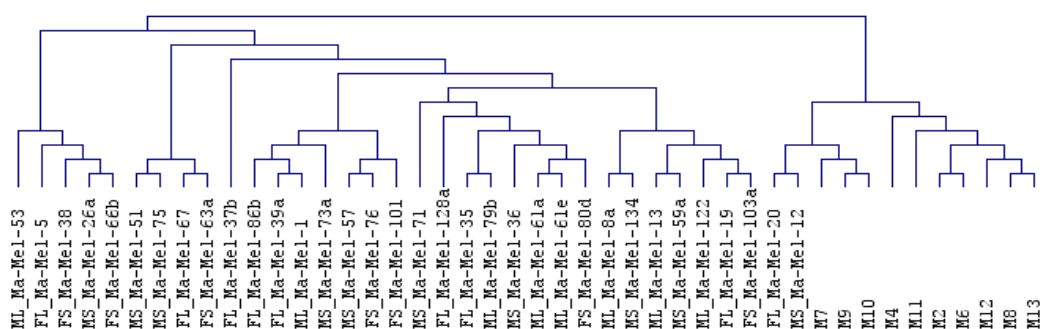


Figure 6 Hierarchical clustering of all melanoma and NHEM cell lines. ML, male long survivor; FL, female long survivor; MS, male short survivor; FS, female short survivor; M, NHEM.

Next, we performed t-test to compare miRNA expression between groups. In order to have independent samples, we excluded Ma-Mel-61a in the t-test. We found 60 differentially

expressed miRNAs between melanoma cell lines and NHEMs (p values were adjusted by Bonferroni correction, $p < 0.001$, **Figure 7**), while only 17 between short survivors and long survivors using even less stringent criteria (unadjusted p-value, $p < 0.05$, **Figure 8**). Hierarchical clustering using these differentially expressed miRNAs was able to separate melanoma cell lines and NHEMs correctly (**Figure 7**) but not short survivors and long survivors due to a few exceptions (**Figure 8**).

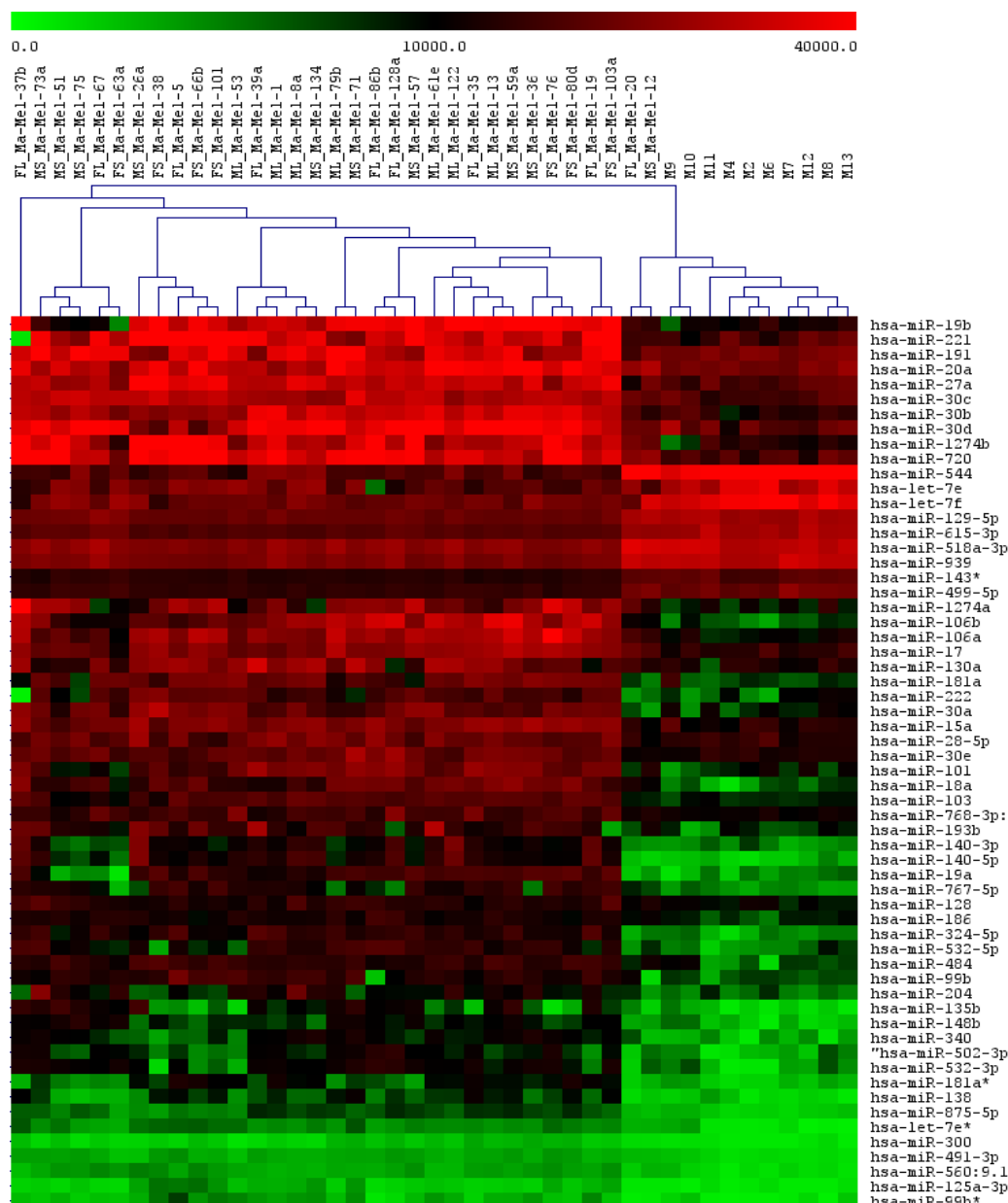


Figure 7 Differentially expressed miRNAs between melanoma and NHEM cell lines. Heat map shows expression levels of miRNAs in the microarray analysis. Column, cell line; row, miRNA. T-test was performed to compare miRNA expression in melanoma and NHEM cell lines. Ma-Mel-61e was excluded in the t-test to insure independent samples in each group. Hierarchical clustering was performed using 60 differentially expressed miRNAs selected by t-test (adjusted Bonferroni correction, $p < 0.001$).

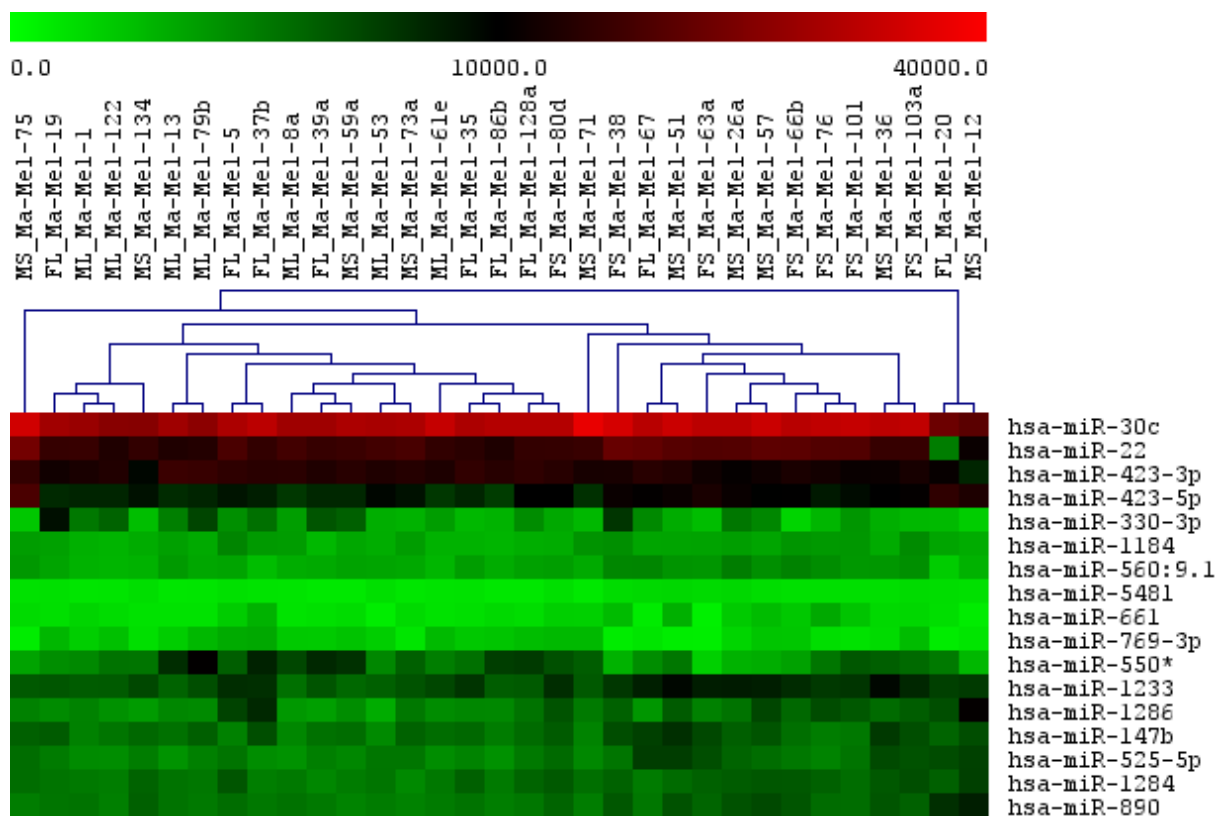


Figure 8 Differentially expressed miRNAs between short survivors and long survivors. Heat map shows expression levels of miRNAs in the microarray analysis. Hierarchical clustering was performed using 17 differentially expressed miRNAs selected by t-test (unadjusted, $p < 0.05$).

3.1.2.2 qPCR validation of the selected miRNAs

We selected 8 miRNAs that were differentially expressed between short and long survivors for further validation using qPCR analysis (**Table 6**). However, none of the 8 miRNAs showed significantly different expression between the two groups (**Figure 9**). The expression of miR-525-5p and -890 were almost undetectable, and the expression of miR-1284 and -1286 were quite low. Poor correlations between chip data and qPCR data were found (**Table 6**), with even negative correlation coefficients for 5 miRNAs (miR-423-5p, -525-5p, 1233, -1284 and -1286). Thus, qPCR analysis failed to validate any of the selected miRNAs in the second round profiling.

In summary, we selected a number of differentially expressed miRNAs between melanoma cell lines from short and long survivors in a first round (with small groups) and a second round (with large groups) microarray analyses. However, qPCR analysis could not confirm the differential expression observed by Illumina microarray analysis.

Table 6 Selected differentially expressed miRNAs between short and long survivors

miRNA	FC ^a	r ^b
miR-22	1.20	0.44
miR-423-3p	-1.15	0.33
miR-423-5p	1.19	-0.22
miR-525-5p	1.17	-0.15
miR-890	1.12	0.15
miR-1233	1.14	-0.23
miR-1284	1.12	-0.17
miR-1286	1.21	-0.13

a. FC, fold change of short survivors to long survivors as analyzed by miRNA microarray.

b. Pearson's correlation coefficient between chip and qPCR data.

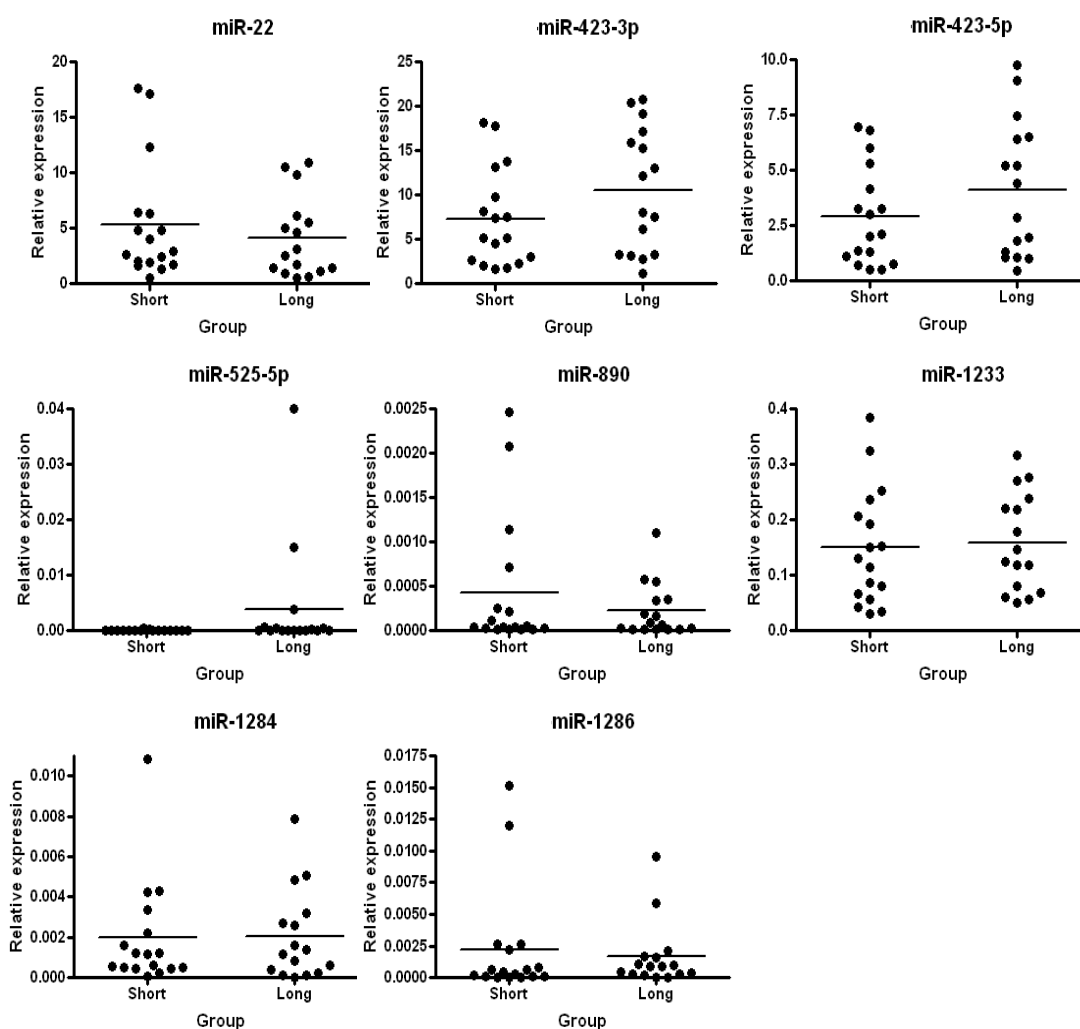


Figure 9 qPCR validation of miRNA expression in melanoma cell lines. miRNA expression was determined in short survivors (n=17) and long survivors (n=16) using Taqman miRNA expression assay. Expression was normalized to the internal control RNU6B. The horizontal line shows the average expression level in each group. No significant differences were detected between groups (t test, $p < 0.05$).

3.2 Investigation of miR-101 function in melanoma cells

In the qPCR analysis for the selected miRNAs from the first round microarray, only miR-101 showed a trend of higher expression in the long survival groups than in the short survival group, though the difference was not significant ($p=0.11$). Given that miR-101 had been reported to be a tumor suppressor in other types of cancer (87, 88) and that miR-101 was predicted to target the important transcription factor MITF, we decided to further analyze miR-101 expression and functions in melanoma cells.

3.2.1 miR-101 is dysregulated in melanoma cells

In order to test whether miR-101 is dysregulated in melanoma cells, we performed qPCR for the 10 NHEMs and 34 melanoma cell lines including the 29 cell lines used in the first round qPCR analysis and 5 additional cell lines. As shown in **Figure 10A**, miR-101 was significantly up-regulated in melanoma cell lines compared to NHEMs ($p=0.010$). Cell lines of long survivors expressed higher miR-101 than those of short survivors ($p=0.028$), or NHEMs ($p=0.010$). Similar results were obtained when only cell lines of stage IV patients were included in the comparisons (**Figure 10B**).

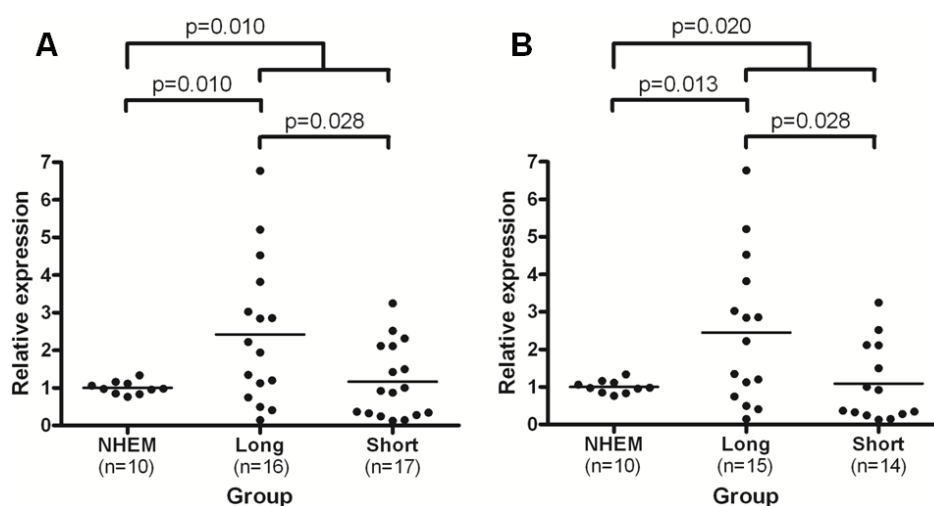


Figure 10 miR-101 is dysregulated in melanoma cells. miR-101 expression was analyzed by Taqman miRNA assay in NHEMs and melanoma cell lines from advanced patients with long survival or short survival. miR-101 expression were normalized to RNU6B and further calibrated to the average of all NHEMs. A. All melanoma cell lines were included. B. Only stage IV cell lines were included. T-test was applied to compare the differences between groups. As Ma-Mel-61a and -61e were from the same patient (long survivor), only Ma-Mel-61a was included in the t-test to insure independent samples.

3.2.2 MITF is a direct target of miR-101

3.2.2.1 Target prediction

Using TargetScan 5.0, MITF was predicted to harbor 2 putative miR-101 binding sites in the 3' UTR (**Figure 11**). One binding site is conserved (site 1, 2934 - 2940 nt), and the other is poorly conserved (site 2, 1338 -1344 nt).

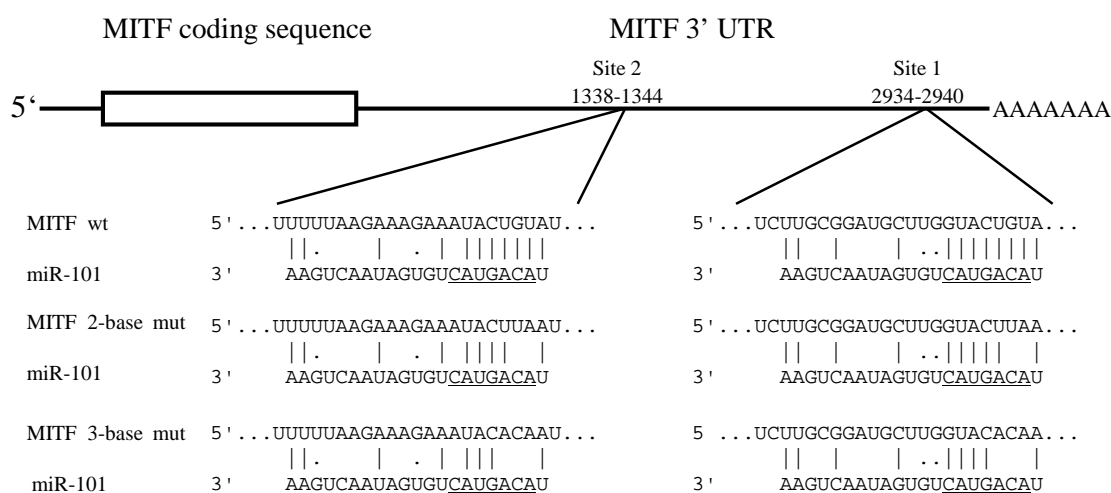


Figure 11 Schematic of the two predicted miR-101 binding sites in the MITF 3'UTR and site mutagenesis design for the reporter assay. Complementarities between miR-101 and target sites are shown. The seed sequence is underlined. WT, wild type; S1M2 or S1M3, site 1 with 2- or 3-base mutation; S2M2 or S2M3, site 2 with 2- or 3-base mutation

3.2.2.2 Construction of a miR-101 expression plasmid and a MITF 3'UTR reporter plasmid

The miR-101 precursor sequence was cloned into pCMX-PL1 to generate pCMX-PL1-mir-101 expression plasmid (**Figure 12**). Full-length MITF 3'UTR was cloned into psiCHECK2 to generate psiCHECK2-MITF-3'UTR reporter plasmid (**Figure 12**), which was subsequently used to generate the reporter plasmids containing mutations in either predicted target site (**Figure 11**).

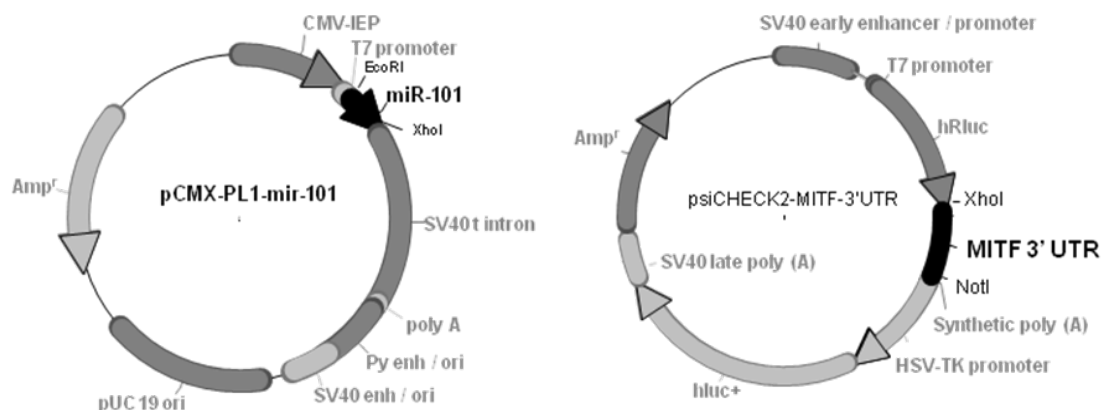


Figure 12 Construction of pCMX-PL1-mir-101 and psiCHECK2-MITF-3'UTR plasmid. miR-101-1 precursor sequence was inserted into pCMX-PL1 plasmid between EcoRI and XhoI sites (left). MITF 3'UTR was inserted into psiCHECK2 plasmid between XhoI and NotI sites downstream of the *Renilla* luciferase gene (right). The *Renilla* luciferase (hRluc) and firefly luciferase (hLuc+) serve as experimental reporter and control reporter, respectively. CMV-IEP, CMV immediate early promoter. Py, polyoma. enh / ori, enhancer / origin. hRluc, synthetic *Renilla* luciferase gene. hLuc+, synthetic firefly luciferase gene. HSV-TK, herpes simplex virus thymidine kinase.

3.2.2.3 Luciferase reporter assay

Co-transfection of psiCHECK2-MITF-3'UTR and pCMX-PL1-mir-101 into HEK293T or melanoma cell lines (Ma-Mel-73a and -86b) strongly decreased the luciferase activities at 24 hr and 48 hr, while co-transfection of psiCHECK2 empty (without cloned 3'UTR) and pCMX-PL1-mir-101 into the same cell lines did not change the luciferase activities (**Figure 13A**). This indicates that miR-101 targets the 3' UTR of MITF directly.

To find out whether the putative binding sites are responsible for the targeting, we introduced a 2- or 3-base mutation in either site. As shown in **Figure 13B**, introduction of 2- and 3-base mutations in site 1 diminished the reduction of luciferase activities caused by miR-101 from 71% to 45% and 37% ($p < 0.001$), respectively. However, introduction of neither 2- nor 3-base mutation in site 2 affected the reduction of luciferase activities. Thus, only the conserved site (site 1) is a real target site for miR-101. Because the mutations in site 1 were unable to impair the targeting of MITF completely, more target sites may exist in the MITF 3'UTR.

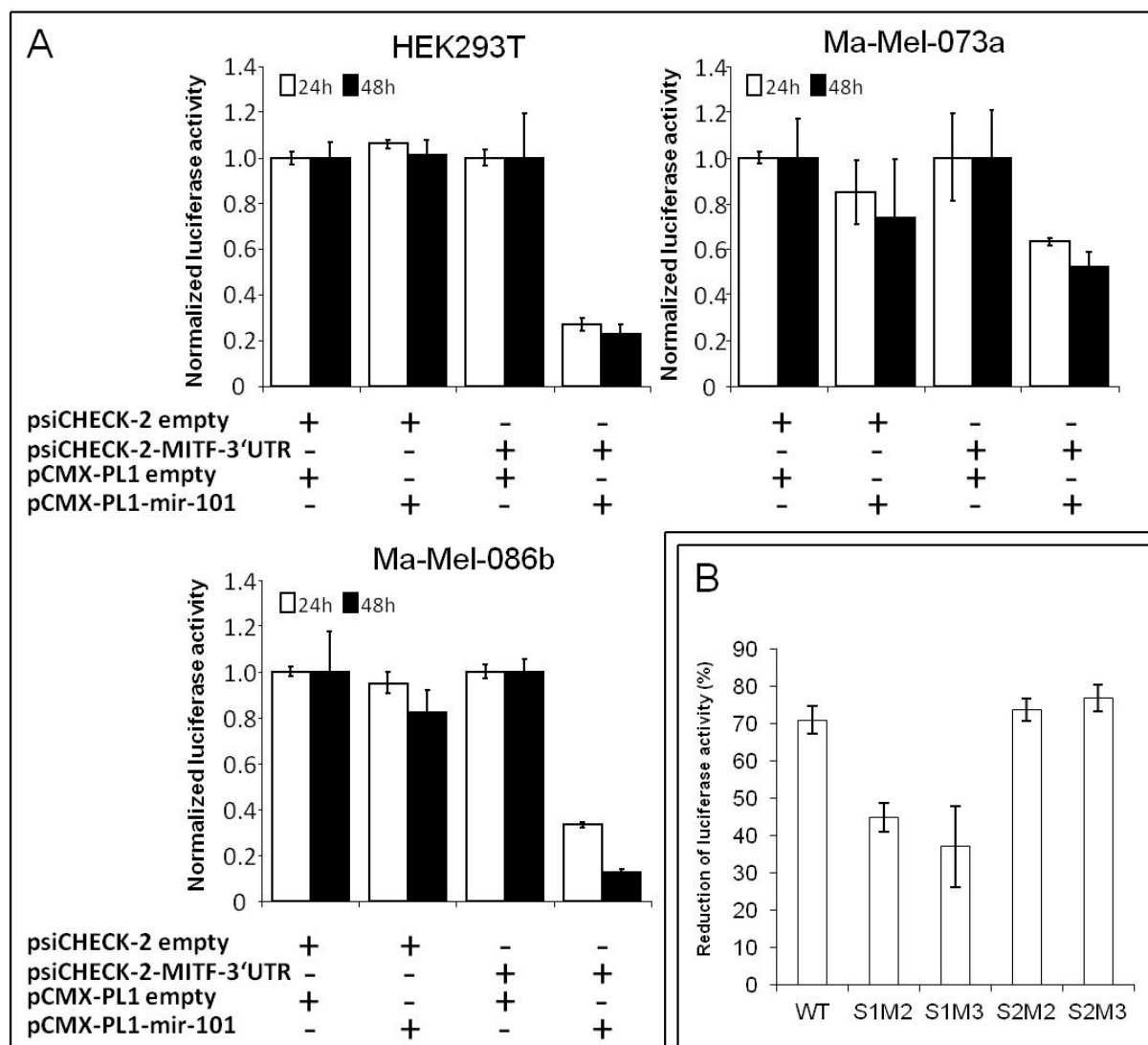


Figure 13 Luciferase reporter assays validate MITF as a direct target of miR-101. **A.** psiCHECK-2-MITF-3'UTR or psiCHECK-2 empty vector was transfected with mir-101 overexpression vector pCMX-PL1-mir-101 or pCMX-PL1 empty vector into HEK293T, Ma-Mel-73a or Ma-Mel-86b cells. Dual-luciferase assay was performed at 24 hr and 48 hr after transfection. *Renilla*/firefly luciferase ratios were calculated, and further normalized to pCMX-PL1 empty which was set to 1. Three independent experiments were performed. Data are shown as mean \pm SD. **B.** pCMX-PL1 empty or pCMX-PL1-mir-101 was co-transfected with psiCHECK-2-MITF3'UTR containing 2-base or 3-base mutation in site 1 or site 2 into Ma-Mel-86b cells. Dual-luciferase assay was performed at 24 hr after transfection. *Renilla*/firefly luciferase ratios were calculated, and further normalized to psiCHECK-2 empty control. Results are expressed as reductions of normalized luciferase activity. Three independent experiments were performed. Data are shown as mean \pm SD. WT, wild type; S1M2 or S1M3, site 1 with 2- or 3-base mutation; S2M2 or S2M3, site 2 with 2- or 3-base mutation

3.2.2.4 miR-101 overexpression down-regulates MITF and EZH2 protein in melanoma cells

To test whether miR-101 is able to down-regulate MITF protein expression, we transfected Ma-Mel-79b and -86b cells with miR-101 mimic and performed Western blot analysis using antibody against MITF. As shown in **Figure 14A**, MITF protein was decreased to 62 % and 56 % in Ma-Mel-79b and -86b cells at 72 hr after transfection of miR-101. We also tested the expression of EZH2, which has been reported to be an oncogene and a target of miR-101 in various types of cancer (87-89, 92, 93). Indeed, overexpression of miR-101 down-regulated EZH2 in the two tested melanoma cell lines (**Figure 14A**). Interestingly, overexpression of miR-101 in both cell lines also caused a cleavage of poly-(ADP-ribose) polymerase (PARP) which is one of the targets of caspase-3 and serves as a indicator for apoptosis (142).

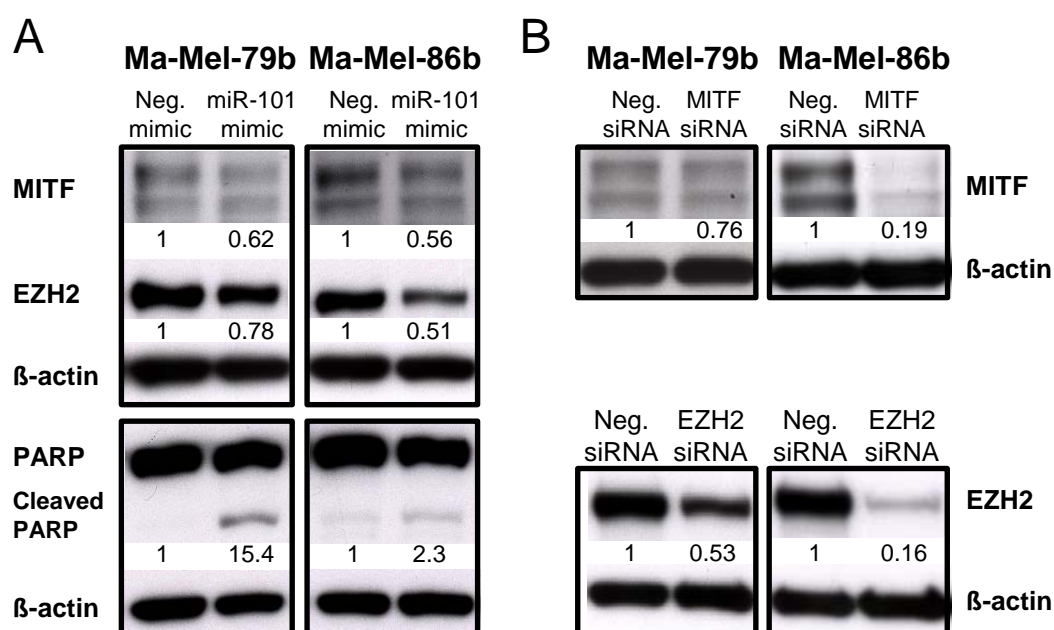


Figure 14 Western blot analysis. A. Ma-Mel-79b and -86b cells were transfected with miR-101 mimic or negative control mimic. The protein levels of MITF, EZH2 and PARP were analyzed. The amount of cleaved PARP is indicative for the induction of apoptosis. B. Ma-Mel-79b and -86b cells were transfected with siRNA against indicated target genes or control siRNA. Seventy-two hours after transfection, protein lysates and total RNAs were prepared, and were subjected to Western blot with indicated antibodies. Relative densitometric quantification is shown below the respective bands.

To examine whether miR-101 could also decrease MITF mRNA, we examined MITF mRNA expression in the transfected cells using qPCR. Surprisingly, MITF mRNA was increased by 48 % and 76 % upon miR-101 overexpression in Ma-Mel-79b and -86b, respectively (**Figure 15**), which seems to be contradictory to our finding that miR-101 overexpression decreased MITF protein levels in both cell lines. It may well be that miR-101 could also down-regulate another target which suppressed MITF transcription or promote MITF mRNA degradation, leading to an increased MITF mRNA level, but the strong suppressive effect of miR-101 on MITF translation resulted in a down-regulation of MITF protein.

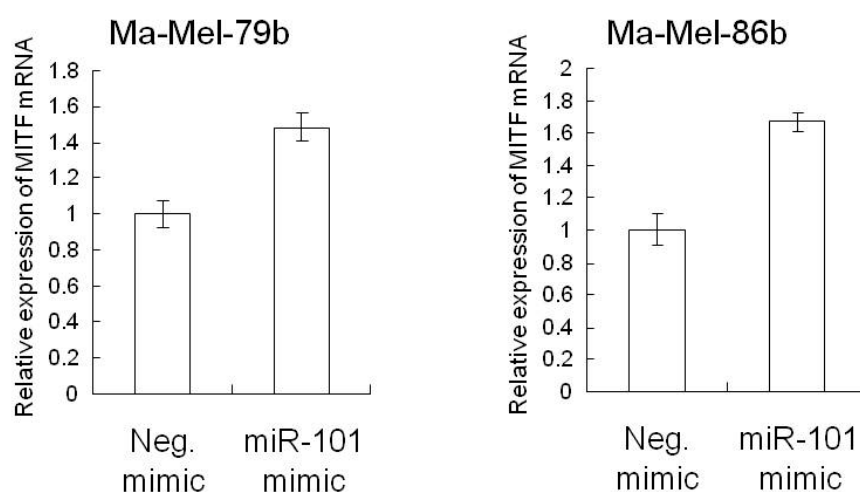


Figure 15 qPCR analysis of MITF mRNA expression in transfected melanoma cells. miR-101 mimic or negative control mimic was transfected into Ma-Mel-79b and -86b cells. Relative expression of MITF to GAPDH was calculated and then normalized to the negative control.

3.2.3 Effect of miR-101 overexpression on melanoma cell proliferation

To test whether miR-101 could affect the proliferation of melanoma cells, we performed proliferation assays for Ma-Mel-79b and -86b cells which were transfected with miR-101 mimic or negative control mimic (**Figure 16**). Twelve percent inhibition of proliferation of Ma-Mel-79b was observed at 48 hr after transfection, and the inhibition was much more pronounced at 72 hr (44%) and 96 hr (48%). However, proliferation of Ma-Mel-86b was not significantly affected by miR-101 overexpression.

In order to find out whether the effect of miR-101 on proliferation could be mediated through its target genes, we examined cells with knockdown of MITF or EZH2 by siRNA in the

proliferation assay. In accordance with the miR-101 overexpression results, knockdown of MITF and EZH2 by siRNA started to inhibit the proliferation of Ma-Mel-79b cells at 72 hr and 48 hr, respectively, while knockdown of MITF and EZH2 showed very little effect on the proliferation of Ma-Mel-86b cells (**Figure 16**). Hence, miR-101 might inhibit melanoma cell proliferation through down-regulation of MITF and EZH2, but the effects may vary in different cell lines.

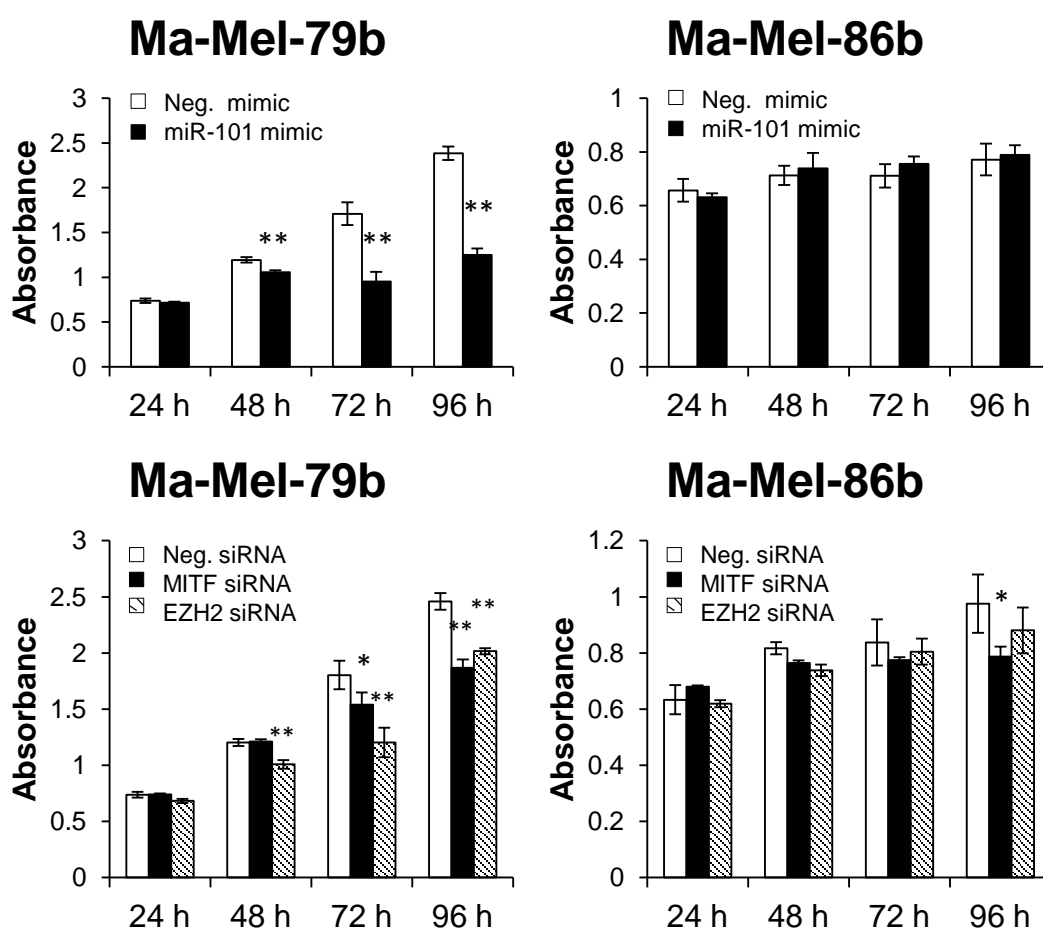


Figure 16 Effect of miR-101 overexpression on melanoma cell proliferation. Ma-Mel-79b and -86b cells were transfected with miR-101 mimic (upper), siRNAs against target genes (lower), or the corresponding negative controls. Cell viability was measured by WST-1 assay every 24 hr after transfection. Results shown are representatives of two independent experiments. Data are presented as mean \pm SD of triplicates. * $p < 0.05$; ** $p < 0.01$.

3.2.4 miR-101 inhibits invasion of melanoma cells

To test whether miR-101 could play a role on melanoma cell invasion, transwell matrigel invasion assay was performed on transfected melanoma cells (**Figure 17**). The invasion of Ma-Mel-79b and -86b cells was reduced to 69% and 68% upon miR-101 overexpression. Knockdown of EZH2 by siRNA decreased the invasion of Ma-Mel-79b and -86b cells to 63% and 65%. Knockdown of MITF by siRNA decreased the invasion of Ma-Mel-86 cells to 31%, but only 71% for Ma-Mel-79b, which might be due to that the siRNA decreased MITF to 19% in Ma-Mel-86b cells, but only 76% in Ma-Mel-79b cells (**Figure 14B**). Thus, miR-101 inhibits the invasion of melanoma cells, which is likely mediated by the down-regulation of miR-101 targets MITF and EZH2.

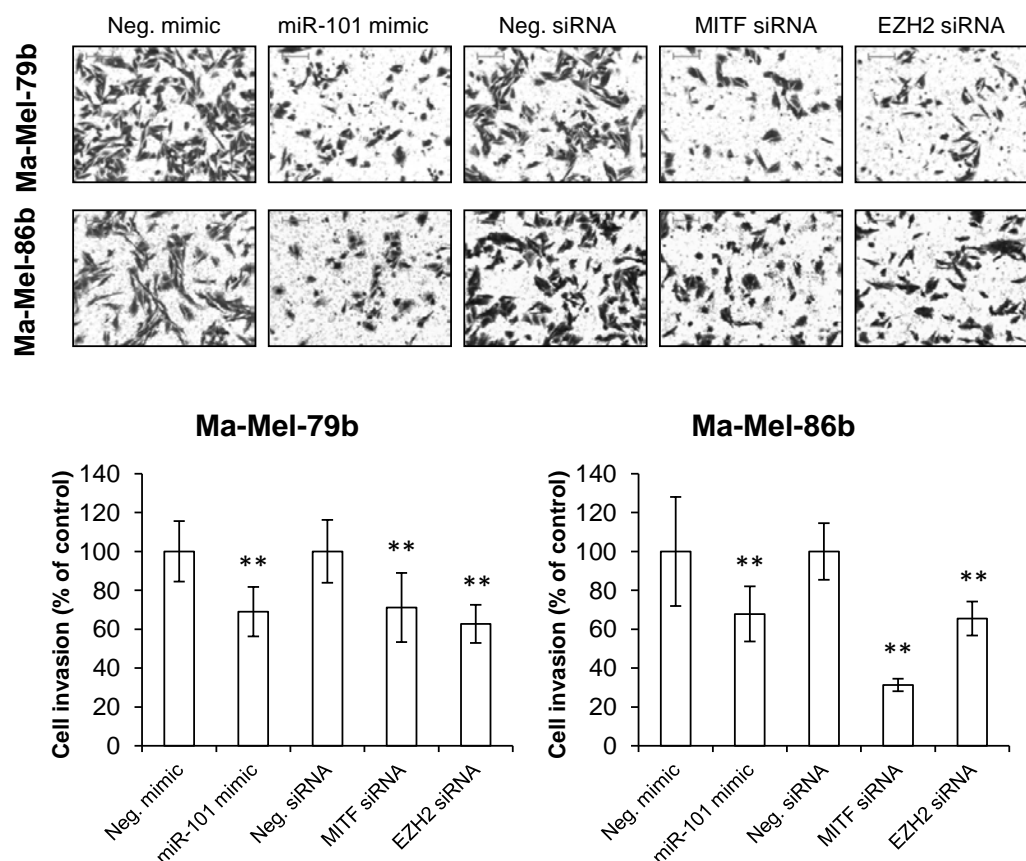


Figure 17 miR-101 inhibits invasion of melanoma cells through down-regulation of MITF and EZH2. Ma-Mel-79b and -86b cells were transfected with miRNA mimic or siRNA as indicated in the figure, and subjected to matrigel invasion assays. Results shown are representatives of at least two independent experiments performed in duplicates. Twelve fields / condition were photographed, and invasive cells were counted and normalized to negative controls which were set to 100 %. Data are shown as mean \pm SD of 12 fields. ** $p < 0.01$. Scale bar, 100 μm .

3.2.5 miR-101 inhibits migration of melanoma cells

To investigate the effect of miR-101 on cell migration, we performed wound healing migration assays on transfected Ma-Mel-79b and -86b cells (**Figure 18**).

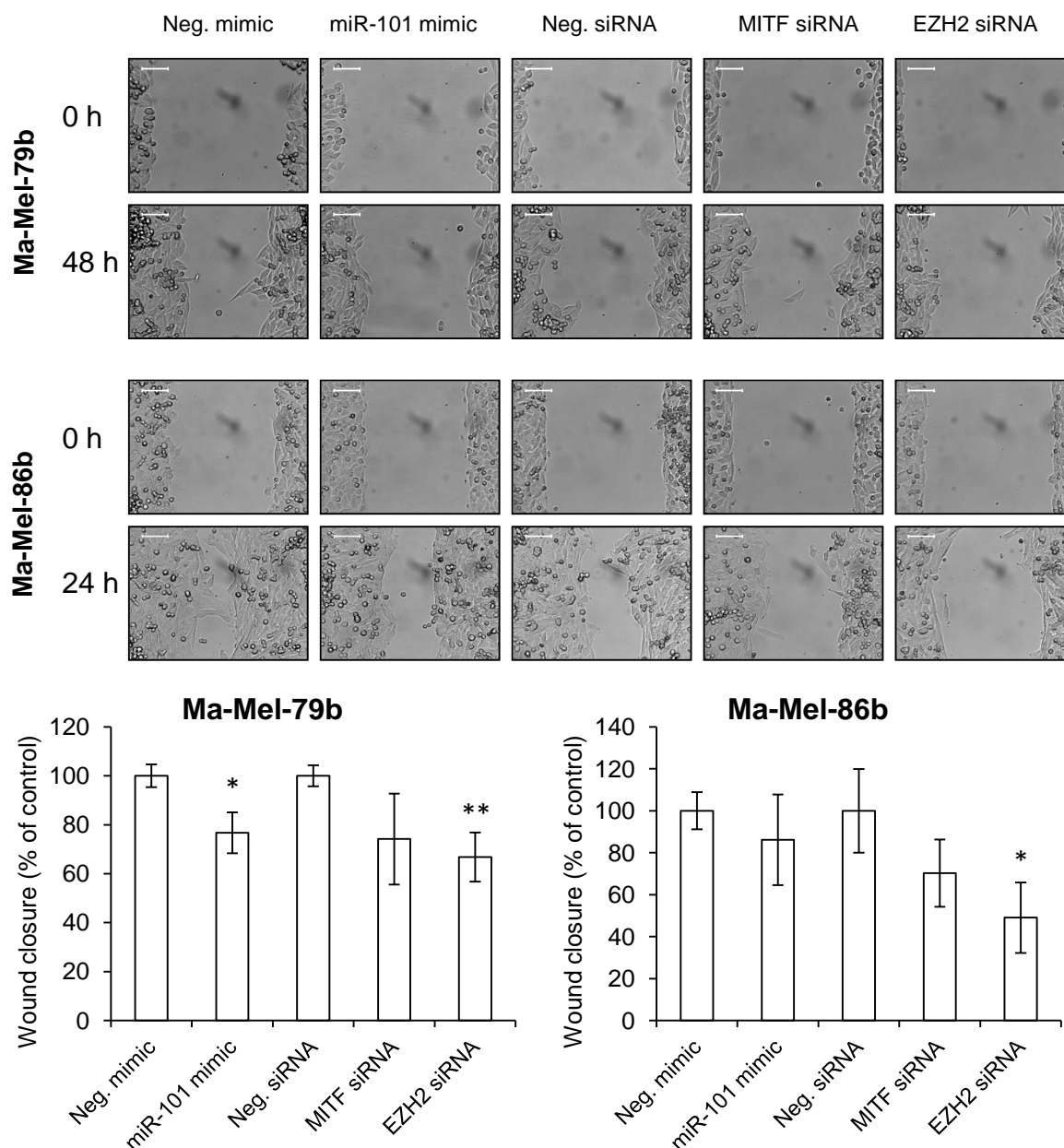


Figure 18 miR-101 inhibits migration of melanoma cells. Ma-Mel-79b and Ma-Mel-86b cells were transfected with miR-101 mimic or negative control mimic, or with siRNAs against target genes or negative control siRNA. Seventy-two hours after transfection, cells were harvested, seeded into each side of an Ibidi culture-insert, and incubated overnight. Cells were photographed immediately after insert removal and 24 hr (Ma-Mel-86b) or 48 hr (Ma-Mel-79b) after removal to monitor migration. Images shown are representatives of two independent experiments performed in duplicates. Wound closure was measured and normalized to negative controls which were set to 100 %. Data are shown as mean \pm SD of 4 wells from two independent experiments. * $p < 0.05$; ** $p < 0.01$. Scale bar, 100 μm .

The migration of Ma-Mel-79b was reduced to 77%, 74% and 67% upon transfection of miR-101, MITF siRNA, and EZH2 siRNA, respectively. However, the migration of Ma-Mel-86b was not significantly inhibited by miR-101, though it was inhibited by knockdown of MITF and EZH2. Therefore, miR-101 can inhibit the migration of melanoma cells, but the effects may vary in different cell lines.

In summary, we found that miR-101 was down-regulated in melanoma cell lines from short survivors compared to those from long survivors. We identified MITF as a novel target for miR-101 and confirmed the previously reported target EZH2 in melanoma cells. Functional assays showed that miR-101 could act as a tumor suppressor in melanoma cells by inhibiting proliferation, invasion and migration, which was likely mediated by the down-regulation of miR-101 targets MITF and EZH2.

3.3 Investigation of miR-137 function in melanoma cells

In 2008, Bemis *et al.* reported the first functional study on a miRNA in melanoma, which demonstrates that miR-137 down-regulates MITF and suggests that miR-137 may be involved in sun tanning response (103). Subsequent studies showed that miR-137 could act as a tumor suppressor in various types of cancer, such as brain tumor, colorectal cancer, head and neck cancer, and gastric cancer (143-146). Although an anti-cancer role of miR-137 has been emerging in melanoma recently (147, 148), a link between miR-137 and patient outcome is still lacking and additional studies are needed to elucidate the function of miR-137 in melanoma. Given that a single miRNA may have hundreds of targets (149), it is valuable to investigate whether other genes are also regulated by miR-137 and how they may contribute to the aggressiveness of melanoma and patient outcome.

3.3.1 Low miR-137 expression is associated with poor survival in stage IV melanoma patients

Using Taqman miRNA assay, we analyzed miR-137 expression of 10 NHEMs and 34 melanoma cell lines derived from metastasis of advanced melanoma patients (stage III and IV) with short or long survival (**Table 1**). Comparisons between groups (NHEMs vs. all melanoma, NHEMs vs. long survival, NHEMs vs. short survival, or long survival vs. short survival) did not show significant differences, due to one cell line (Ma-Mel-134, stage III) in the short survival group which showed extremely high expression and dramatically raised the average expressions of the corresponding groups (**Figure 19A**). When excluding stage III cell lines, we found that miR-137 expression was significantly lower in the short survivor group than in the long survivor group ($p = 0.031$) or in the NHEM group ($p = 0.028$) (**Figure 19B**). There was a trend that miR-137 was down-regulated in stage IV melanoma cell lines compared to NHEMs, although the difference ($p = 0.095$) did not reach a significant level.

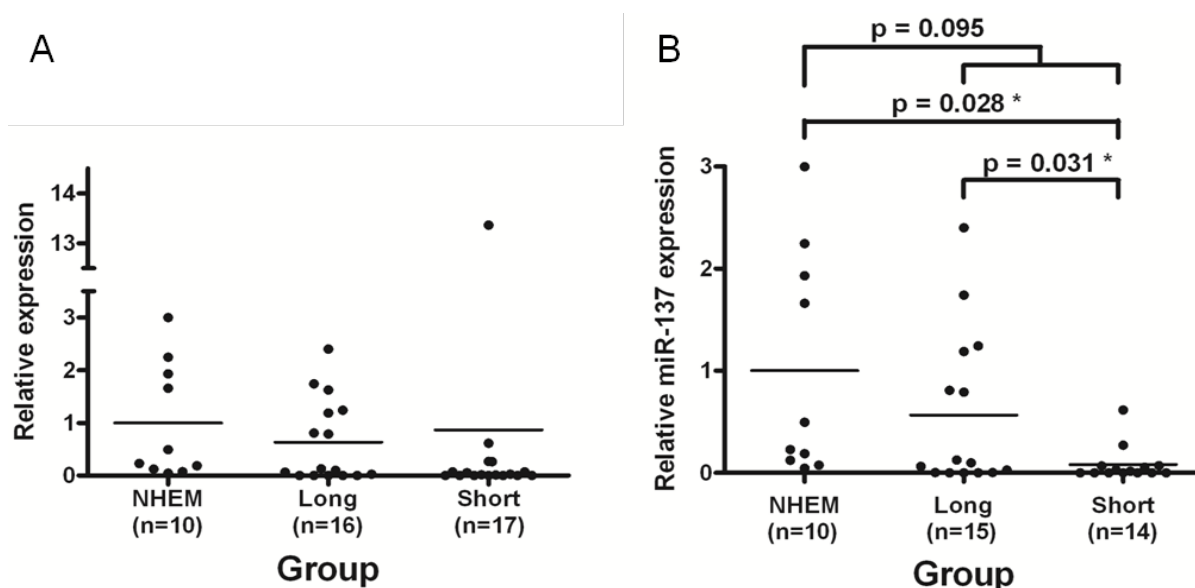


Figure 19 Expression of miR-137 in NHEMs and melanoma cell lines. miR-137 expression was analyzed by Taqman miRNA assay in NHEMs and melanoma cell lines from advanced patients with long survival or short survival. miR-137 expression was normalized to RNU6B and further calibrated to the average of all NHEMs. A. All melanoma cell lines were included. B. Only stage IV cell lines were included. T-test was applied to compare the differences between groups. As Ma-Mel-61a and -61e were from the same patient (long survivor), only Ma-Mel-61a was included in the t-test to insure independent samples. * $p < 0.05$.

3.3.2 miR-137 directly targets c-Met, YB1 and ABCB5

3.3.2.1 Target selection based on microarray data

To look for other candidate targets of miR-137, we did correlation analysis between miR-137 expression and mRNA expression of 17589 different genes, using available miRNA and mRNA microarray data from 34 melanoma cell lines. We found that miR-137 expression was negatively correlated with the mRNA expression of a number of genes. c-Met, YB1 and ABCB5 were selected as candidate targets for miR-137 in this study (**Figure 20**), due to their involvement in melanoma progression and chemo-resistance (150-152).

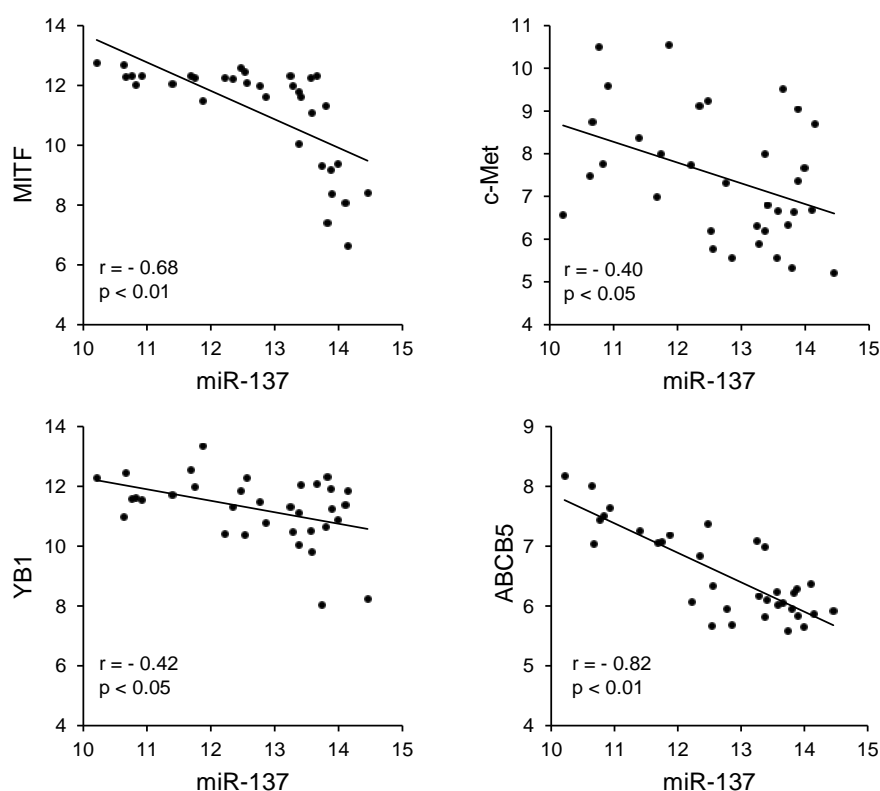


Figure 20 Expression of miR-137 and putative target genes was negatively correlated. miR-137 and target genes' expression levels from 34 different cell lines were obtained from log2 transformed microarray data. Pearson's correlation coefficient (r) and p values were calculated between miR-137 and target gene expression.

3.3.2.2 Construction of luciferase reporter plasmid for target genes

Full-length 3'UTRs of c-Met, YB1, and ABCB5 were cloned into psiCHECK2 to generate psiCHECK2-c-Met/YB1/ABCB5-3'UTR reporter plasmid (**Figure 21**).

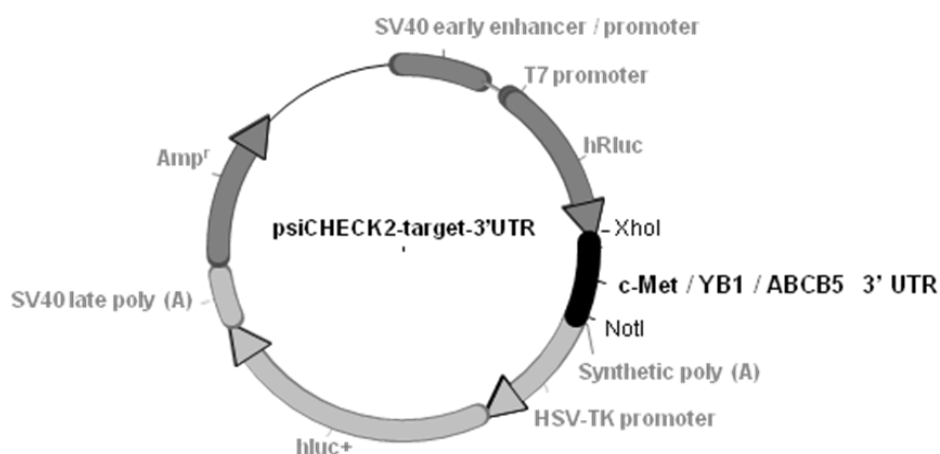


Figure 21 Construction of dual-luciferase reporter plasmid for the 3'UTRs of target genes. The 3'UTR of c-Met, YB1, and ABCB5 were inserted into psiCHECK2 plasmid between XhoI and NotI sites downstream of the *Renilla* luciferase gene. The *Renilla* luciferase (hRluc) and firefly luciferase (hLuc+) serve as experimental reporter and control reporter, respectively. hRluc, Synthetic *Renilla* luciferase gene. hLuc+, Synthetic firefly luciferase gene. HSV-TK, herpes simplex virus thymidine kinase.

3.3.2.3 Luciferase reporter assay

To test whether miR-137 targets c-Met, YB1 and ABCB5 through their 3' UTR, dual luciferase assays were performed using reporter plasmid containing the respective full length 3' UTRs. Ma-Mel-86b cell line was selected for the reporter assay because of very low endogenous miR-137 expression. Luciferase activities were decreased by 51%, 73% and 61%, respectively, when Ma-Mel-86b cells were co-transfected with miR-137 and the corresponding reporter plasmid for c-Met, YB1 and ABCB5 (**Figure 22**).

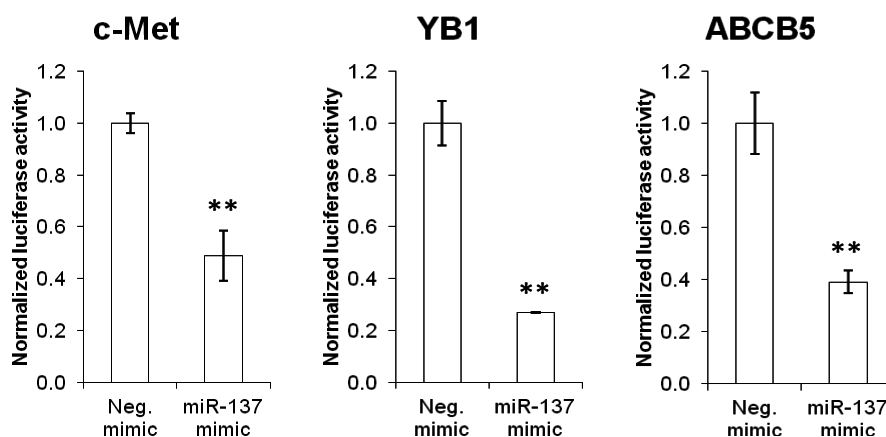


Figure 22 Dual luciferase reporter assay for miR-137 mediated targets repression. Reporter plasmid for different 3'-UTR of target genes (c-Met, YB1 and ABCB5) was co-transfected with negative control mimic, or with miR-137 mimic into Ma-Mel-86b cells. Dual-luciferase assay was performed 48 hr after transfection. *Renilla* / firefly luciferase ratios were calculated, and further normalized to the negative control. Shown are representatives of three independent experiments. Data are presented as mean \pm SD of triplicates. ** $p < 0.01$.

3.3.2.4 Validation of target genes by Western blotting and qPCR

To further evaluate whether miR-137 down-regulates c-Met, YB1 and ABCB5 at protein or mRNA level, or both, we performed Western blot and qPCR to detect the protein and mRNA expression of the three genes for miR-137 transfected Ma-Mel-79b and -86b cells. As shown in **Figure 23** and **Figure 24A**, overexpression of miR-137 decreased the protein and mRNA expression of YB1 and ABCB5 in both cell lines compared to the negative control. miR-137 reduced c-Met protein expression in both cell lines, though c-Met mRNA expression was reduced only in Ma-Mel-79b. Thus, miR-137 may down-regulate both mRNA and protein of c-Met, YB1 and ABCB5, but it can be cell-context dependent.

A few target genes of miR-137 have been reported previously, such as MITF, CDK6 and c-terminal binding protein 1 (CtBP1) in cutaneous or uveal melanoma (103, 147, 148), mindbomb homolog 1 (Mib1) in embryonic neurons (153), Ezh2 in adult neuronal stem cell (154), and cell division cycle 42 (CDC42) in colorectal cancer cell (144). In the present study, we also included MITF and EZH2, because MITF is an important transcriptional factor associated with melanoma cell growth and survival and EZH2 contributes to the development of many solid tumors and its expression was associated with the aggressiveness of cutaneous melanoma (155). Indeed, Western blot showed that both MITF and EZH2 protein

was decreased upon miR-137 transfection in both Ma-Mel-79b and -86b cells (**Figure 23**). Moreover, overexpression of miR-137 also down-regulated BCL2, a downstream target of MITF, in both cell lines (**Figure 23**).

Thus, we identified three new direct targets (c-Met, YB1, and ABCB5) and confirmed two previously reported targets (MITF and EZH2) to be regulated by miR-137 in melanoma.

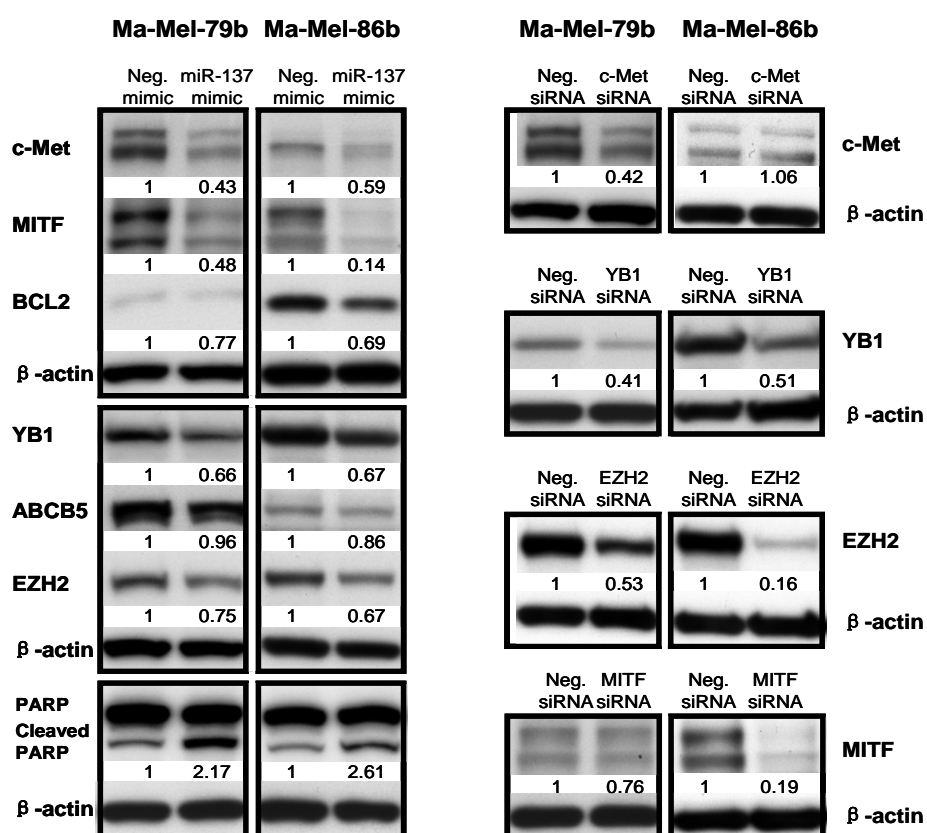


Figure 23 Western blot analysis. Ma-Mel-79b and -86b cells were transfected with miR-137 mimic or negative control mimic, or with siRNAs against indicated target genes or control siRNA. Seventy-two hours after transfection, protein lysates and total RNA were prepared, and were subjected to Western blot with indicated antibodies. Densitometric quantification is shown below the respective band.

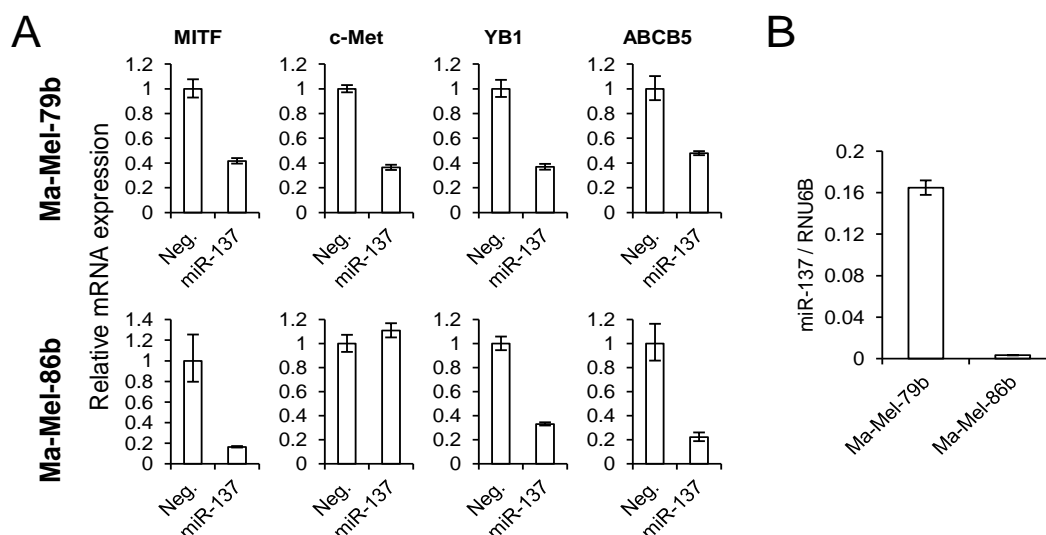


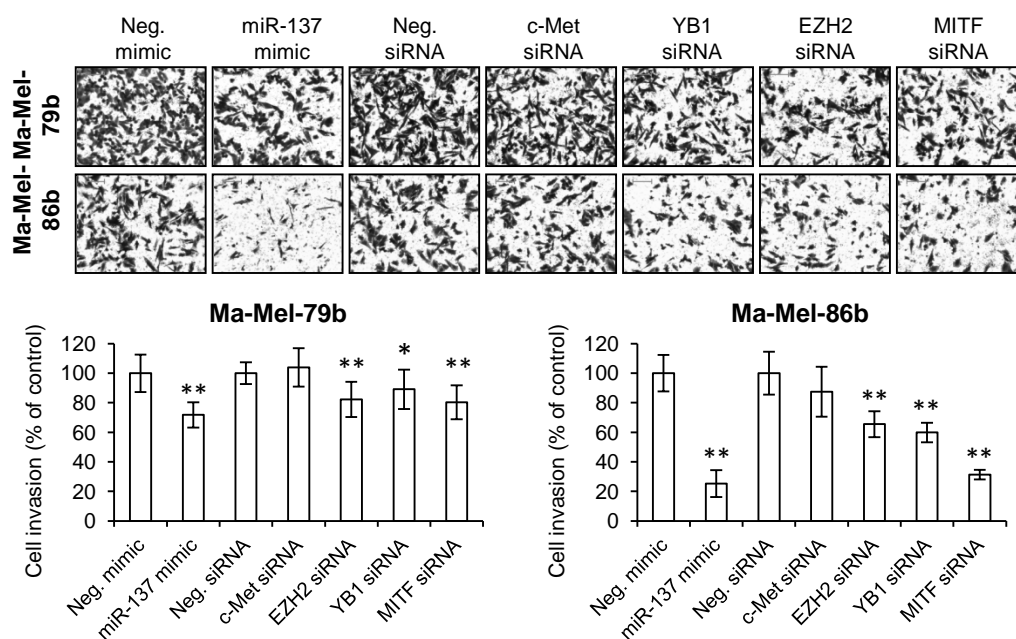
Figure 24 qPCR analysis. A. mRNA levels of c-Met, YB1, ABCB5 and MITF were determined in miR-137 mimic or negative control mimic transfected Ma-Mel-79b and -86b cells. Relative expression of target genes to GAPDH was calculated and then normalized to negative controls. B. Endogenous miR-137 expression was tested in Ma-Mel-79b and -86b cells. miR-137 expression was normalized to RNU6B. Data is shown as mean \pm SD of triplicates.

3.3.3 miR-137 inhibits invasion of melanoma cells through multiple targets

We performed transwell invasion assays to analyze the effect of miR-137 on the invasive behavior of two melanoma cell lines. To validate the importance of the individual miR-137 targets, we also tested whether knockdown of the selected target genes by siRNA could phenocopy the effect of miR-137 overexpression on melanoma cell invasion. **Figure 23** shows that the protein levels of target genes were decreased by siRNA, except c-Met in Ma-Mel-86b. When FCS was used as chemo-attractant, transfection of miR-137 mimic and siRNAs against YB1, EZH2 and MITF significantly inhibited the invasive capacity of both Ma-Mel-79b and -86b cells, while transfection of siRNA against c-Met did not (**Figure 25A**). Notably, such inhibitory effect on cell invasion was much stronger in Ma-Mel-86b cells compared to Ma-Mel-79b cells, which might be explained by the much lower endogenous miR-137 level in Ma-Mel-86b cells than in Ma-Mel-79b cells (**Figure 24B**). When using FCS plus HGF as chemo-attractant, knockdown of c-Met strongly reduced the invasive activity of Ma-Mel-79b cells (**Figure 25B**). This is concordant with the notion that HGF is the only known endogenous ligand of c-Met (156) and most melanoma cells can produce HGF that can

activate c-Met in an autocrine manner (150). On the contrary, knockdown of c-Met failed to inhibit the invasion of Ma-Mel-86b cell (**Figure 25B**) probably due to the fact that the endogenous expression of c-Met in Ma-Mel-86b cells was very low and further reduction did not lead to any effect. Taken together, miR-137 suppressed melanoma cell invasion which could be phenocopied by knockdown of each of the target genes MITF, c-Met, YB1 and EZH2.

A FCS as chemoattractant



B FCS + HGF as chemoattractant

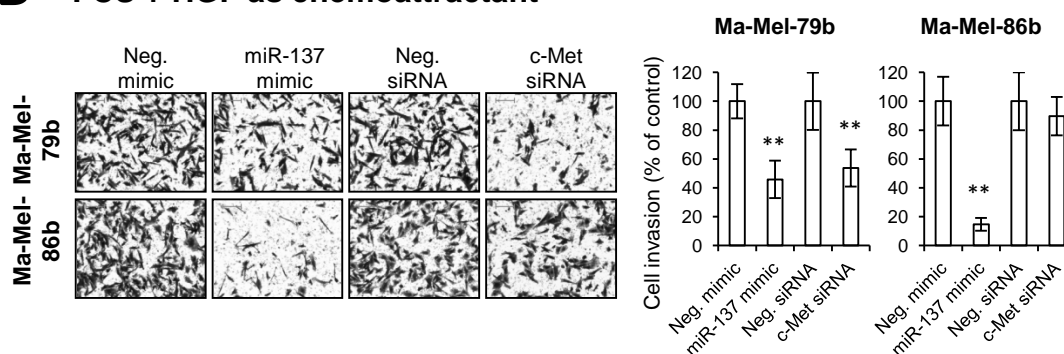


Figure 25 miR-137 inhibits invasion of melanoma cells through down-regulation of multiple targets. Ma-Mel-79b and -86b cells were transfected with miRNA mimic or siRNA as indicated in the figure, and subjected to matrigel invasion assays. A. FCS served as chemoattractant, and invasive cells were stained at 24 hr (Ma-Mel-86b) or 48 hr (Ma-Mel-79b) after seeding. B. FCS and HGF served as chemoattractant, and invasive cells were stained at 24 hr after seeding. Results shown are representatives of at least two independent experiments performed in duplicates. Twelve fields / condition were photographed, and invasive cells were counted and normalized to negative controls which were set to 100 %. Data are shown as mean \pm SD of 12 fields. * $p < 0.05$; ** $p < 0.01$. Scale bar, 100 μ m.

3.3.4 miR-137 inhibits migration of melanoma cells

To test the effect of miR-137 on the migration of melanoma cells, we performed wound healing migration assay with Ma-Mel-79b and -86b cells. As shown in **Figure 26**, overexpression of miR-137 significantly reduced the migratory activities of Ma-Mel-79b and -86b by 36% and 75%, respectively. The migration of both cell lines was suppressed significantly when YB1 and EZH2 were knocked down by siRNA. Knockdown of MITF also inhibited the migration but the difference did not reach a significant level. However, knockdown of c-Met was unable to affect the migration of either cell line. Thus, miR-137 may inhibit melanoma cell migration likely through down-regulation of YB1, EZH2 and MITF.

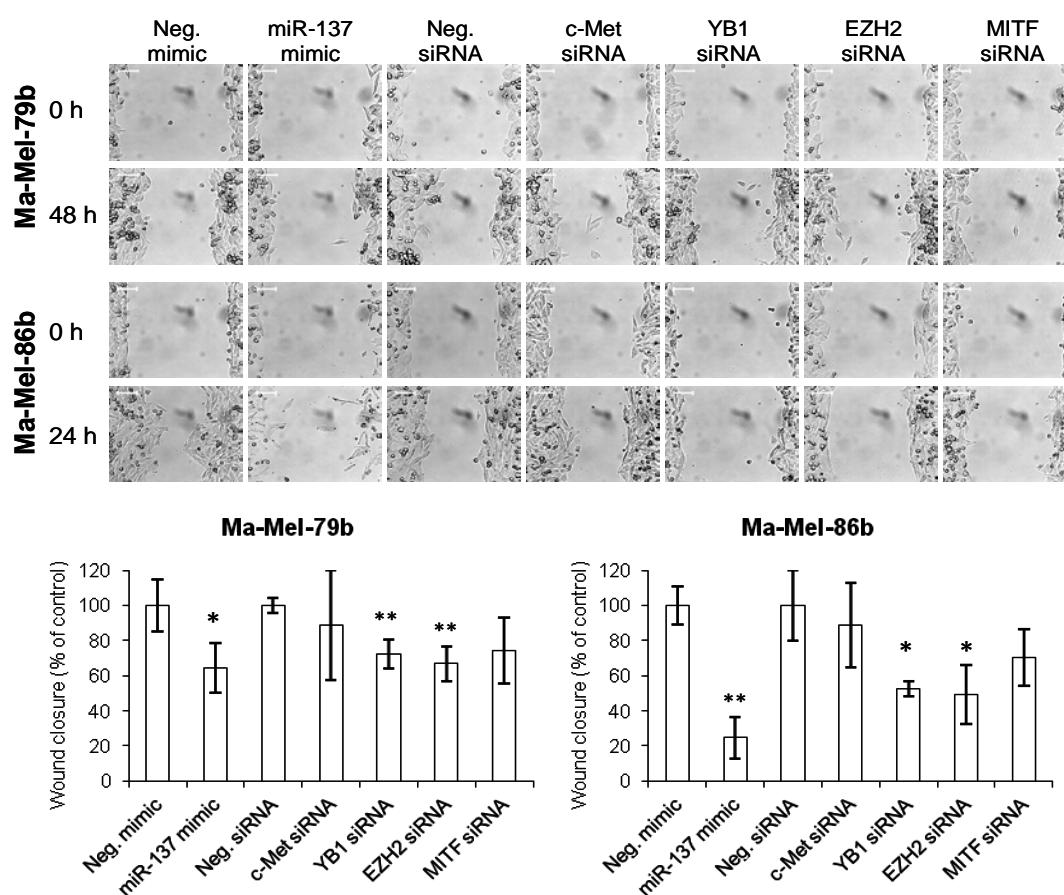


Figure 26 miR-137 inhibits migration of melanoma cells through down-regulation of multiple targets. Ma-Mel-79b and Ma-Mel-86b cells were transfected with miR-137 mimic or negative control mimic, or with siRNAs against indicated target genes or negative control siRNA. Seventy-two hours after transfection, cells were harvested, seeded into each side of an Ibidi culture insert, and incubated overnight. Cells were photographed immediately after insert removal and 24 hr (Ma-Mel-86b) or 48 hr (Ma-Mel-79b) later. Images are representatives of two independent experiments performed in duplicates. Wound closure was measured and normalized to negative controls which were set to 100 %. Data are shown as mean \pm SD of 4 wells from two independent experiments. * $p < 0.05$; ** $p < 0.01$. Scale bar: 100 μ m.

3.3.5 miR-137 suppresses proliferation of melanoma cells

To test whether miR-137 dysregulation may affect the proliferation of melanoma cells, we performed proliferation assay for Ma-Mel-79b and -86b cells that were transfected with miR-137 mimic or negative control mimic. The proliferation of Ma-Mel-79b and -86b cells was suppressed by miR-137 at 96 hr and 72 hr after transfection, respectively (**Figure 27**). Knockdown of MITF and EZH2, but not c-Met or YB1, by siRNA inhibited Ma-Mel-79b proliferation; knockdown of MITF, c-Met and YB1, but not EZH2 inhibited Ma-Mel-86b proliferation (**Figure 27**). Hence, down-regulation of MITF, c-Met, YB1, and EZH2 by miR-137 may contribute to the inhibition of melanoma cell proliferation, but the impact of each target may vary in different cell lines.

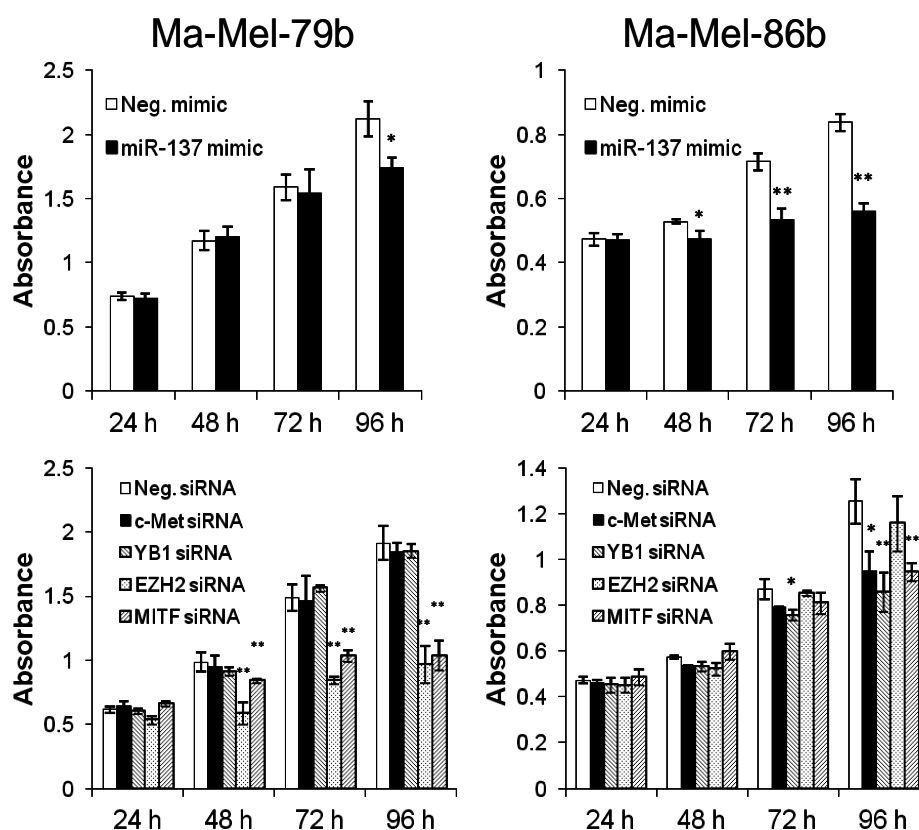


Figure 27 miR-137 inhibits melanoma cell proliferation. Ma-Mel-79b and -86b cells were transfected with miR-137 mimic (upper), siRNAs against target genes (lower), or corresponding negative controls. Cell viability was measured by WST-1 assay every 24 hr until 96 hr after transfection. Results shown are representatives of two independent experiments. Data are presented as mean \pm SD of triplicates. * $p < 0.05$; ** $p < 0.01$.

3.3.6 miR-137 induces apoptosis of melanoma cells

To evaluate whether miR-137 induces apoptosis in melanoma, we performed Western blot to analyze the cleavage of PARP which is a marker for apoptotic cells (142). We found that the 89 kDa cleaved PARP was strongly increased after transfection of miR-137 into both Ma-Mel-79b and -86b cell lines (**Figure 23**). Furthermore, overexpression of miR-137 caused a decrease of the anti-apoptotic protein BCL2 in both cell lines, probably through the down-regulation of MITF (**Figure 23**). This indicates that the intrinsic apoptosis pathway is activated by miR-137.

In summary, we found that low miR-137 expression was associated with poor survival in stage IV melanoma patients. Three novel targets (c-Met, YB1 and ABCB5) were identified and validated for miR-137. Functional assays showed that overexpression of miR-137 could suppress melanoma cell proliferation, invasion and migration likely through the down-regulation of the newly identified targets c-Met and YB1, as well as the previously reported targets EZH2 and MITF. The individual impact of these proteins on migration and proliferation varied among the two cell lines tested. Moreover, overexpression of miR-137 induced apoptosis and decreased BCL2 protein levels in melanoma cells. Thus, miR-137 serves as a tumor suppressor in melanoma.

4 DISCUSSION

4.1 Discrepancy between microarray and qPCR data

In this study, we intended to screen and identify differentially expressed miRNAs between melanoma cell lines from long survival and short survival patients, or between melanoma cell lines and NHEMs, using microarray miRNA profiling, followed by Taqman qPCR validation. For testing purpose, we performed the first round of microarray analysis with a small number of melanoma cell lines including nine from short survivors and five from long survivors. The expression levels of a number of miRNAs were found to be different between the two groups. However, qPCR analysis of 11 selected miRNAs did not show significant differences between the two groups with a larger sample size. Only miR-101 showed a trend of higher expression in long survivors ($p=0.10$). Correlation analysis of miRNA chip and qPCR data was not satisfactory, with Pearson's correlation coefficients being 0.40-0.93. In the second round of miRNA profiling, we included 34 melanoma cell lines and 10 NHEMs. Eight differentially expressed miRNAs between long and short survivors were selected and tested by qPCR. However, none of them showed significant differences between the two groups. Worse still, there was no good correlation between the microarray and qPCR data, with Pearson's correlation coefficients being negative in some cases. Thus, the microarray miRNA profiling in the second round failed to identify differentially expressed miRNAs which were able to be further validated by qPCR.

The discrepancy between microarray and qPCR data may come from the intrinsic technical problems in the microarrays used in our study. Although microarray technology has been successfully utilized to detect and quantitate gene expression, it faces unique challenges when dealing with miRNAs compared to their use in mRNA profiling: the short sequence of miRNAs limits the options for primer design; it is difficult to distinguish the mature miRNAs from their precursors as well as from their family members that may differ by a single nucleotide; mature miRNAs often have quite different melting temperatures (T_m) which make

it difficult to design the same hybridization condition across the entire array for hundreds of miRNAs (157).

Recently, studies have been carried out to compare different miRNA microarray platforms. (157-159). Git *et al.* compared the performances of 6 miRNA microarray platforms (including Illumina) by analyzing three biological samples comprised of normal breast tissue, the luminal breast cancer cell line MCF7, and a breast progenitor cancer cell line PMC42 (157). They found that the overlap of differentially expressed miRNAs identified by the 6 platforms was very low and fold changes of differentially expressed miRNAs obtained from microarray data were compressed compared to those obtained from qPCR data. Pradervand *et al.* analyzed differential miRNA expression in RNA samples from human brain and heart using Agilent, Illumina and Affymetrix microarrays, as well as qPCR (Applied Biosystems) and deep sequencing (Illumina) (158). The Agilent platform showed the best correlation with qPCR ($r=0.9$) and deep sequencing ($r=0.83$), and also the least fold change compression. The Illumina platform showed good correlations with qPCR ($r=0.88$) and deep sequencing ($r=0.8$), but it suffered from a strong fold change compression. They found that differentially expressed miRNAs identified from different platforms varied a lot, and 8 out of 10 selected miRNAs from Agilent and Illumina microarrays showed a Ct value of ≥ 34 in qPCR.

In our study, we also observed that 3 miRNAs (miR-890, -1284 and 1286) which showed moderate expression in Illumina microarray were almost undetectable in qPCR. This may be because the probes on the array had cross-hybridization with other miRNAs or with unprocessed miRNAs (pri- or pre-miRNA) instead of mature miRNAs. Both qPCR (Taqman) and Agilent microarray specifically detect mature miRNAs by stem-loop probes (160). Illumina microarray does not have a special probe design for mature miRNAs, though the vendor claims that the assay design might offer advantages for detecting mature miRNAs more effectively than pre-miRNAs (161). Of note, our microarray data showed that the expression of miR-423-3p was significantly higher in the long survival group than that in the short survival group, while miR-423-5p showed the opposite pattern. However, qPCR failed to confirm the differences. On the contrary, it showed a very strong positive correlation between these two

miRNAs ($r=0.98$), which may reflect the fact that they are from the same pre-miRNA and also indicate that the microarray data may be unreliable.

Another possible reason for the discrepancy between microarray and qPCR data may be the differences in normalization methods. A crucial step of microarray data analysis is the normalization which helps to reduce measurement error and to remove systematic differences between samples that do not have real biological variations (138, 162). Various normalization algorithms, such as cyclic lowess (locally weighted scatterplot smoothing), contrast based methods, quantile normalization, scaling methods and non-linear methods, have been primarily developed for the analysis of large-scale mRNA microarray data (163). Several studies compared the performance of different methods in normalizing miRNA microarray data and quantile normalization turned out to be robust (138, 159, 162). In the original paper that introduced the Illumina miRNA microarray platform, quantile normalization was also chosen as a proper method for data processing (161). The quantile normalization assumes that the distributions of probe intensities on each array are the same (163). This is acceptable if there is no global difference in miRNA expression across samples like the cell lines from long and short survivors in our study. However, quantile normalization may not be a suitable method when the distributions of miRNA expression vary significantly in different samples like the heart and brain, or probably the melanoma cells and melanocytes in our study.

qPCR is considered as the gold standard of DNA or RNA quantification because of its high specificity and sensitivity (164). In miRNA research, miRNA expression is usually validated by qPCR after a selection of candidates from microarray analysis. The normalization methods used for microarray data were not applicable for qPCR, due to limited number of testing miRNAs. Like conventional qPCRs for mRNA expression that use a housekeeping gene as reference, qPCRs for miRNA expression often use a “housekeeping small RNA” as reference (165). U6 snRNA and 5s rRNA are among the most commonly used small RNA references. We also used U6 as a reference RNA in our qPCR analysis. However, several recent studies have shown that expression of U6 and 5s is not always constant in different samples (165-167). Lim *et al.* suggested that a combination of three miRNAs (miR-103, miR-106b and miR-26b), but not U6 or 5s, was suitable for miRNA expression normalization in neuronal

differentiation models (166). Shen *et al.* reported that miR-23a and miR-191 are the optimal reference miRNAs for cervical tissues (167). Peltier *et al.* conducted a comprehensive study on identification of appropriate reference RNAs in a panel of 13 normal tissues and 5 pairs of tumor and normal adjacent tissues (165). miR-191 and miR-103 were found to be the most consistent in their expression across all the samples, whereas U6 and 5s were among the least stable ones. However, melanoma samples were not included in this study. Interestingly, Caramuta *et al.* showed that low expression of miR-191 was associated with poor survival in melanoma patients (131). Thus, further studies are needed to identify suitable references for miRNA quantitation in melanoma.

Other sources of errors may also be implicated in our miRNA profiling experiment, such as differences in RNA sample preparation and performance in microarrays. Based on our results and the results from other groups in the same institute who used the same platform and also had unreliable data, it is more likely that the miRNA microarray platform used in our study did not provide satisfactory sensitivity and specificity in miRNA detection.

4.2 Expression of miR-101 and miR137 is dysregulated in melanoma

Generally, stage IV melanoma patients have very poor prognosis, with median survival of 6 - 10 months and a five-year survival rate of less than 5 percent (168). The survivals of individual patients can vary from months to years, but the molecular mechanism behind is unknown so far. In the past decade, miRNAs have been recognized as important players in cancer and their expression has been associated with the development, progression and prognosis of different types of cancer, including melanoma. miR-101 has been found to be a tumor suppressor in several types of cancer (88-94) and a prognostic marker in endometrial serous adenocarcinomas (169) and in NSCLC (170). Since the first paper on miR-137 and its target MITF in melanoma was published in 2008 (103), there has been an increasing interest in the role of miR-137 in the development and progression of cancers. Several targets of miR-137 have been identified in various types of cancer, which indicate a tumor suppressive

function for miR-137 (143-146). However, so far there has been no published study on the prognostic effect of miR-101 or miR-137 in melanoma. This study is the first one to correlate expression of miR-101 and miR-137 with melanoma patients' outcome. We selected two groups of melanoma cell lines from stage IV patient with distinct survival, and found that lower expression of miR-101 and miR-137 was associated with shorter survival. This is consistent with previous reports and our findings that miR-101 and miR-137 executed their tumor suppressive functions by targeting multiple oncogenes. Thus, down-regulation of miR-101 and miR-137 may contribute to the increased aggressiveness of melanoma cells in stage IV patients. Further studies are warranted to validate the dysregulation of miR-101 and miR-137 expression in a larger population of melanoma patients.

In our study, there was a trend that miR-137 was down-regulated in stage IV melanoma samples compared to NHEMs, which is consistent with the notion that miR-137 is a tumor suppressor. In contrast, miR-101 showed an up-regulation in melanoma samples compared to NHEMs, which seems contradictory to its tumor suppressive effect on melanoma cell in our study. It is noteworthy that miR-101 expression was convergent at relatively low level in NHEMs but divergent in melanoma cell lines. Meanwhile, some melanoma cell lines, most of which were from short survivors, showed extremely low levels of miR-101. It could be possible that miR-101 expression was down-regulated to a very low level to facilitate tumorigenesis at the beginning, but during a certain period of progression, miR-101 expression was reactivated due to the release of such inhibition or induction from other factors. Although most studies reported miR-101 as a tumor suppressor, Sachdeva *et al.* reported that miR-101 was able to promote estrogen-independent growth of breast cancer cells and suppression of MAGI-2 by miR-101 decreased PTEN activity, leading to Akt activation (171). This indicates that miR-101 may also have an oncogenic potential which may favor tumor growth at some point. The confusing expression pattern of miR-101 in our melanoma and NHEM samples may be clarified by the discovery of more miR-101 targets and also upstream regulators.

4.3 Target identification for miR-101 and miR-137

To facilitate target identification for miRNAs, a number of target prediction algorithms have been invented so far. However, current algorithms are not satisfactory due to the fact that only a few principles on miRNA target recognition have been established (25). Thus, experimentation is crucial to identify real miRNA targets. Gene-specific experimental validation with reporter assays, qPCR, and Western blot are commonly used to test individual miRNA-mRNA targeting (34). Using TargetScan 5.0, two miR-101 target sites were predicted at MITF 3' UTR, a conserved one and a poorly conserved one. The reporter assay showed that the conserved site, but not the poorly conserved one, was responsible for targeting. Of note, mutation in the conserved site could not abolish the targeting effect completely, leaving the possibility that there are other target sites for miR-101 in MITF 3' UTR or even in other regions of the mRNA. Several algorithms including miRanda, RNAhybrid (172), and PITA, predicted additional target sites, which should be tested in future.

Although miRNAs were initially reported to repress the translation of target genes without affecting their mRNA level (173), subsequent studies showed that they may also induce mRNA degradation, making global mRNA profiling data useful for screening miRNA targets (41, 42, 174). In the present study, a negative correlation between numerous mRNAs and miR-137 has been found. Notably, MITF and its downstream targets, such as SILV, CDK2, GPR143, MLANA, DCT and TRY (175), were among the most significantly down-regulated genes, which was concordant with the previous report that MITF is a direct target of miR-137 (103). We selected c-Met, YB1 and ABCB5 as candidate targets of miR-137, because they are involved in the malignancy of melanoma and have not been reported to be direct targets of miR-137 so far. We did target site prediction for miR-137 in the 3' UTRs of the selected candidates using 4 different algorithms (TargetScan, PicTar, Miranda, and RNAhybrid). YB1 was predicted by all four algorithms; c-Met was predicted by Miranda and RNAhybrid; ABCB5 was only predicted by RNAhybrid. Our luciferase reporter assays showed direct interactions between miR-137 and all three candidates. Furthermore, mRNA and protein down-regulation upon miR-137 overexpression in two melanoma cell lines was confirmed by qPCR and

Western blot, except that mRNA of c-Met was not decreased in Ma-Mel-86b cells, indicating that translational repression rather than mRNA decay might be dominant in this cell line.

Interestingly, several reports have shown that c-Met is regulated by MITF (176-178). A recent study found that transfection of miR-137 into uveal melanoma cells decreased c-Met expression through the down-regulation of MITF (147). However, our reporter assay showed the possibility of direct interaction between miR-137 and c-Met 3' UTR (**Figure 22**), which also explains why Ma-Mel-86b cells showed a slightly increased c-Met mRNA level (**Figure 24A**) but down-regulated c-Met protein level upon transfection with miR-137 (**Figure 23**).

Altogether, we identified and validated one novel target (MITF) of miR-101 and three novel targets (c-Met, YB1 and ABCB5) of miR-137, and also confirmed the previously reported targets of miR-101 (EZH2) and miR-137 (MITF and EZH2). Interestingly, miR-137 and miR-101 share some targets, namely MITF and EZH2.

4.4 Functional impact of miR-101 and miR-137 through their target genes in melanoma

4.4.1 miR-101 and its target genes

The present study identified and validated MITF as a novel target for miR-101. In melanocytes, MITF is the master regulator of melanocytic differentiation, expression of melanogenic proteins, and cell cycle arrest (17, 179). Although there is little doubt of its crucial role in melanoma biology, the function of MITF is still controversial. It has been reported that MITF is a lineage-specific oncogene in melanoma (180) and is critical in melanoma cell proliferation and survival (17, 179). However, studies also showed that MITF could be down-regulated in advanced melanoma and could suppress melanoma cell proliferation (115, 181, 182). Hoek *et al.* identified two distinct transcriptional signatures (proliferative and invasive) in melanoma cell lines (134), and they have proposed that melanoma cells can switch between proliferative and invasive states *in vivo*, which is likely regulated by local microenvironmental conditions such as hypoxia and inflammation (179). MITF along with other melanocytic genes (e.g. TYR, MLANA, and DCT) were up-regulated in cell lines assigned to the proliferative signature (134).

Gray-Schopfer *et al.* propose that MITF has distinct functions in melanocytic cells at different expression levels and thereby its protein levels must be carefully controlled. High levels of MITF may promote cell cycle arrest and differentiation, while very low levels may cause cell cycle arrest and apoptosis.

In our study, the proliferation of Ma-Mel-79b cells was significantly suppressed upon miR-101 overexpression, which could be phenocopied by siRNA knockdown of MITF. However, overexpression of miR-101 in Ma-Mel-86b cells did not inhibit the proliferation and knockdown of MITF decreased the proliferation only at 96 hr post transfection. Of note, in Ma-Mel-86b cells, there seemed to be a slight increase of proliferation at 48, 72 and 96 hr post transfection of miR-101; and there was also a slight increase of proliferation at 24 hr post transfection of MITF siRNA, but steady decrease at 48, 72 and 96 hr (**Figure 16**). Since MITF expression in Ma-Mel-86b cells is very high, the above observation may indicate that the moderate down-regulation of MITF protein level by miR-101 overexpression might slightly favor the proliferation of Ma-Mel-86b cells. In case of knockdown of MITF by siRNA, cell proliferation might be favored by the moderate reduction of MITF protein at early time, but might be inhibited due to a dramatic decrease of MITF protein at later time.

A few studies showed that decreased expression of MITF could increase the invasion of melanoma cells (179, 183), which disagrees with our results. Given that MITF is a transcriptional factor regulating many genes (175), it may well be that knockdown of MITF leads to a dysregulation of downstream genes involved in invasion. c-Met could be one of them as it promotes cell invasion and is up-regulated by MITF (176). Another possible mediator could be SNAI2, which can be transcriptionally activated by MITF and can induce EMT-like process in melanoma through activation of ZEB1 (184, 185). Further studies are warranted to clarify this question.

EZH2 is a member of the poly-comb group proteins which repress gene transcription through methylation of histone H3 lysine 27 (186). EZH2 was initially reported to interact with the Vav proto-oncogene involved in hematopoietic signal transduction (187) and recently, many studies demonstrated that this protein is oncogenic (188). EZH2 is involved in the regulation of cell cycle progression, proliferation, tumor cell invasion and metastasis (188).

Overexpression of EZH2 was found in different types of cancer, including prostate(189), breast (190), bladder(191), and gastric cancer (192). Overexpression of EZH2 is linked to melanoma by immunohistochemical analysis conducted in two studies which showed that EZH2 was up-regulated in melanoma compared to benign nevi (193) and was associated with cell proliferation markers and aggressiveness of melanoma (155). However, detailed functional studies of EZH2 in melanoma are still lacking. Recently, several groups reported that miR-101 could target EZH2 in a variety of cancers, leading to decrease of cell proliferation, invasion and tumor growth (87-91), but this targeting interaction was not tested in melanoma. In the present study, we confirmed that miR-101 down-regulates EZH2 protein expression in melanoma cells leading to suppression of cell proliferation, invasion and migration.

4.4.2 miR-137 and its target genes

In the progression of melanoma, invasion is a prerequisite for tumor metastasis (4). Notably, several miR-137 targets tested in our study are associated with the invasion of tumor cells. c-Met is overexpressed or mutationally activated in a variety of solid tumors (194). The majority of melanoma cells, but not melanocytes, can produce HGF that induces continuous activation of c-Met, leading to down-regulation of E-cadherin and invasive growth (150). YB1 acts as a transcriptional and translational factor (195), which is up-regulated in melanoma and involved in proliferation, survival, invasion and chemosensitivity of melanoma cells (151). EZH2 as mentioned above, is involved in tumor invasion and its overexpression has been associated with the aggressiveness of melanoma (155, 188). Our study shows that overexpression of miR-137 in melanoma cells suppressed their invasive activity which could be phenocopied by knockdown of these targets.

miR-137 was able to inhibit the migration and proliferation of both cell lines. This raises the concern that a decrease of proliferation may affect the result of a migration assay, leading to misinterpretation. However, three facts argue against this concern in our experiments: (i) the reduction of cell migration caused by miR-137 overexpression was more pronounced than that of cell proliferation; (ii) Ma-Mel-86b cells almost stopped proliferating 96 hr post transfection of miR-137, whereas they could still migrate in wound healing assay; (iii)

knockdown of YB1 in Ma-Mel-79b or EZH2 in Ma-Mel-86b significantly suppressed the migration of the respective cell line, but did not affect the proliferation.

Malignant melanoma is notorious for its high resistance to current therapeutic drugs. One of the underlying mechanisms is the impairment of the apoptosis pathway (196). Melanoma cells are malignantly transformed melanocytes, the natural UV-protectors of the skin, which shield neighboring keratinocytes from radiation by transferring melanin in melanosomes to these cells (196). The enhanced survival feature of melanoma cells may derive from their paternal melanocytes and can be fostered by additional alterations acquired during tumor progression (196). Interestingly, several miR-137 target genes (MITF, c-Met, YB1) tested in the present study are associated with melanoma cell survival. MITF, the first identified target of miR-137, acts as a master regulator of melanocyte development and melanoma oncogene. Continued MITF expression is essential for melanoma cell survival (17). BCL2, a key anti-apoptotic factor, has been found to be a direct MITF target (180). Apoptosis induced by MITF disruption in melanocytes and melanoma could be partially rescued by BCL2 overexpression (180). Both MITF and BCL2 were found to be associated with resistance to chemotherapies against melanoma (180, 196, 197). Puri *et al.* reported that tyrosine kinase inhibitor of c-Met induced apoptosis in melanoma cells (198). Schitteck *et al.* showed that increased expression of YB1 in melanoma stimulated proliferation and inhibited apoptosis (151). In this study, we found that overexpression of miR-137 in melanoma cells induced apoptosis, and down-regulated MITF, BCL2, c-Met and YB1, which might sensitize melanoma cells to chemotherapeutic agents.

Another mechanism involved in chemo-resistance is the decreased intracellular drug accumulation partly due to the enhanced drug efflux mediated by ABC transporters (199). More than 40 ABC transporter proteins have been identified so far and some of them were found to be expressed in melanomas, including ABCA9, ABCB1, ABCB5, ABCB8, ABCC2, and ABCD1 (200). ABCB1 was detected at low frequencies in cutaneous melanoma, but at high frequencies in non-cutaneous melanomas. Overexpression of ABCB1 in human cutaneous melanoma cell line induced resistance to different drugs (201). Notably, ABCB1 is transcriptionally up-regulated by YB1 (202). ABCB5 is frequently expressed in melanoma and mediates doxorubicin resistance via its function as an efflux transporter (199, 200). Therefore,

it is worthwhile to test whether miR-137 is able to reduce the chemoresistance of melanoma by targeting YB1 and ABCB5 in future.

4.5 Therapeutic potential of miRNAs

The identification of molecular pathways involved in melanoma provides opportunities to develop targeted therapies for melanoma patients (2). A number of important molecules (e.g. RAF, RAS, BCL2, PI3K and integrins) have been selected as targets in preclinical research or clinical trials which showed encouraging results (203-207). As increasing evidence shows that miRNAs are regulators of key signaling pathways in cancer, attention has been attracted to the development of miRNA based therapy for cancer (208). Since miRNAs can act as either a tumor suppressor or an oncogene, two strategies could basically be taken in the therapeutic application: reintroduction of tumor suppressive miRNAs or inhibition of oncogenic miRNAs using antagomirs that have complementary sequences (208, 209).

Several preclinical studies have been carried out to explore the feasibility of using miRNAs as therapeutic targets. Trang *et al.* reported that delivery of let-7 mimic through intranasal passage to xenograft NSCLC tumors in mouse model significantly reduces the tumor burden (210). In another study, intravenous and intratumoral delivery of liquid-formulated miR-34a mimic into xenograft NSCLC tumors in mice led to accumulation of miR-34a in the tumor tissue, down-regulation of the target genes (c-Met, BCL2, and CDK4), and inhibition of tumor growth (211). Chen *et al.* developed a liposome-polycation-hyaluronic acid (LPH) nanoparticle decorated with tumor-targeting single-chain antibody fragment (scFv) for specific delivery of siRNA and miRNAs (212). Using this system, they delivered a cocktail of siRNAs (against c-Myc, MDM2 and VEGF), miR-34a, or a combination of siRNAs and miR-34a into the lung metastasis of B16F10 melanoma mouse model. Their results demonstrated that targeted nanoparticles enhanced the uptake of siRNA and miRNA in B16F10 lung metastasis and inhibited tumor growth.

Cancer is a disease associated with many signaling pathway dysregulations (213). Since a single miRNA can regulate expression of multiple genes and thereby affect multiple pathways, miRNAs could be promising therapeutic targets or agents in cancer treatment. Our studies

show that miR-101 and miR-137 act as tumor suppressors by affecting crucial cellular processes (proliferation, invasion and migration) in melanoma through targeting multiple genes that regulate different pathways. It would be interesting to test these two miRNAs in preclinical models and finally in therapeutic applications.

4.6 Outlook

Based on our results, the Illumina miRNA microarray platform is not reliable and other methods like deep-sequencing may be more suitable for high-throughput expression analysis of miRNAs.

Our studies on individual miRNAs demonstrated that low expression of miR-101 or miR-137 was associated with poor survival of stage IV melanoma patients. We have not only identified and validated novel targets for miR-101 (MITF) and miR-137 (c-Met, YB1 and ABCB5), but also confirmed previously reported targets for miR-101 (EZH2) and miR-137 (MITF and EZH2) in melanoma cells. We showed that overexpression of miR-101 or miR-137 could inhibit melanoma cell invasion, migration and proliferation, which suggests a tumor suppressive role of miR-101 and miR-137 in melanoma. Thus, down-regulation of miR-101 and miR-137 in stage IV melanoma patients may increase the aggressiveness of melanoma cells and thereby worsen the patient outcomes.

Further preclinical studies are needed to test whether the *in vivo* application of miR-101 and/or miR-137 may be therapeutically effective by lowering the aggressiveness melanoma or may improve treatment for melanoma when combined with conventional therapies.

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