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Identification of miRNAs with impact on melanoma cell invasion

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"Hold fast to dreams,

For if dreams die Life is a broken-winged bird,

That cannot fly."

— Langston Hughes

Declarations according to § 8 (3) b) and c) of the doctoral degree regulations:

a) I hereby declare that I have written the submitted dissertation myself and in this process have used no other sources or materials than those expressly indicated,

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Heidelberg, 25.11.2014 Claudia Weber

Contents

Part 1

Zusammenfassung

Das maligne Melanom ist ein aggressiver Tumor, der schon im frühen Stadium Metastasen bilden kann. Es wurden bereits miRNA Profile für verschiedenste Tumorentitäten, wie auch für das maligne Melanom publiziert. Allerdings unterscheiden sich diese substantiell in ihren Ergebnissen. Daher ist es vorteilhaft, direkt miRNA Netzwerke zu entschlüsseln, die eine bedeutende Rolle in der Tumorprogression aufweisen und z.B. das Invasionsverhalten von malignen Melanomzellen regulieren. In der vorgelegten Arbeit wurde die Melanomzelllinie A375 mit einer Bibliothek bestehend aus 988 miRNAs transfiziert und deren Effekt auf das Invasionsverhalten mit Hilfe eines Hochdurchsatz Boyden-Chamber Verfahrens untersucht.

Durch dieses funktionelle Screeningverfahren konnten miRNAs identifiziert werden, die entweder die Invasionsfähigkeit von Melanomzellen verstärken und somit onkogen wirken, oder als Tumorsuppressoren die Invasionsfähigkeit vermindern. Im nächsten Schritt wurde die Expression ausgewählter miRNA Kandidaten in 20 Melanomzelllinien im Vergleich zu normalen Melanozyten von 7 gesunden Spendern validiert. Im Invasionsversuch zeigte miR-339-3p einen deutlichen Effekt als Tumorsuppressor, außerdem war es in Melanomzelllinien signifikant niedriger exprimiert im Verlgeich zu normalen Melanozyten. Desweiteren zeigten A375 Tumorzellen mit stabiler miR-339-3p Überexpression eine niedrigere Metastasenanzahl im Lungenkolonisationsversuch in NSG Mäusen im Vergleich zur Leervektorkontrolle.

In anschließenden miRNA-Zielgenanalysen konnte das "myeloid leukemia cell differentiation protein" (MCL1) als neues Zielgen von miR-339-3p bioinformatisch identifiziert werden und in mehreren unabhängig voneinander durchgeführten Experimenten konnte nachgewiesen werden, dass miR-339-3p tatsächlich MCL1 reguliert: Die Transfektion von miR-339-3p führte zu einer Herabregulation von (i) MCL1 Protein und (ii) MCL1 spezifischer mRNA; (iii) die Applikation von miR-339-3p AntagomiRs bewirkte eine verstärkte Invasion von Melanomzelllinien; (iv) mit einem 3'UTR Bindungsassay konnte die direkte Interaktion zwischen miR-339-3p und der 3'UTR von MCL1 nachgewiesen werden; (v) eine verminderte MCL1 Expression durch MCL1 siRNA Transfektion zeigte außerdem eine vergleichsweise reduzierte Invasionsfähigkeit der Melanomzellen wie sie nach Transfektion mit miR-339-3p beobachtet wurde.

Die hier präsentierten Ergebnisse lassen vermuten, dass miR-339-3p als Tumorsuppressor eine Rolle in der häufig beobachteten unterschiedlich starken Aggressivität verschiedener Melanome spielen könnte. Der eingesetzte funktionelle Ansatz bietet eine gute Möglichkeit,

miRNAs und deren Zielproteine zu identifizieren, die eine wichtige Rolle in der Invasivität des malignen Melanoms spielen und stellt somit eine Grundlage für die Entwicklung neuer Therapieansätzen dar.

Part 2

Summary

Melanoma is a fast progressing tumor which tends to metastasize at small size and early time point. miRNA profiling has been performed for a variety of cancer types, but data differ substantially between studies. Therefore, it is of main interest to unravel miRNA networks which are involved in main aspects of melanoma progression, such as cell invasion. In order to obtain candidate miRNAs relevant for the control of invasion, a high-throughput invasion assay in a 96-well Boyden chamber format was performed for functional screening of a human miRNA mimics library consisting of 988 miRNAs (miRBase Version 13.0).

The identified miRNAs could be categorized into miRNAs increasing the invasive capacity of A375 melanoma cells (oncogenic miRNAs) or miRNAs decreasing the invasiveness, which were predominantly downregulated in melanoma and could function as tumor suppressors. miRNA expression analysis was performed to further validate these candidate miRNAs for their physiological role in different melanoma cell lines. miR-339-3p was defined as one promising candidate inhibiting invasion when transfected into different melanoma cell lines. miR-339-3p was expressed to significantly lower extent in melanoma cell lines compared to normal human epidermal melanocytes. Furthermore, A375 cells stably overexpressing miR-339-3p showed decreased lung colonization in NSG mice in comparison to cells expressing the empty vector control.

The myeloid leukemia cell differentiation protein (MCL1) was identified as a potential target of miR-339-3p by target prediction analysis and could be confirmed experimentally: miR-339-3p transfection resulted in downregulation of MCL1 (i) protein and (ii) mRNA levels; (iii) miR-339-3p antagomiR treatment increased melanoma cell invasion; (iv) the direct interaction of miR-339-3p and MCL1 3'UTR was shown in a 3'UTR binding assay; (v) MCL1 downregulation by siRNA inhibited melanoma cell invasion to comparable extent as mediated by miR-339-3p transfection.

These findings indicate that miR-339-3p can act as a tumor suppressor in melanoma and the extent of its expression levels might contribute to the varying aggressiveness of different melanomas. This presented approach may help to unravel possible therapeutic checkpoints within the miRNA network of malignant melanoma cells to counteract tumor spread.

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Abbreviations

Table 1: Abbreviations

Abbreviaton	Name
ABC	ATP-binding cassette
ABC B5	ATP-binding cassette, sub-family B, member 5
ACC	adrenal cortical carcinoma
ac-pre-miRNA	AGO2-cleaved precursor miRNA
AGO	argonaut protein
AJCC	American Joint Comitee on Cancer
AKT	v-akt murine thymoma viral oncogene homolog
ALM	acral lentiginous melanoma
AMACR	alpha-methylacyl-CoA racemase
ANGPTL4	angiopoietin-like 4
AP-1	activator protein 1
ATP	adenosine triphosphate
BASP1	brain acid soluble protein 1
Bcl-2	B-cell lymphoma 2
BH	Bcl-2 homology domain
BRAF	v-raf murine sarcoma viral oncogene homolog B1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CASP8	caspase 8
CCND1	cell-cycle mediator cyclin D1
CDC42	cell division cycle 42
CDK	cyclin-dependent kinase
CDKN1B	cyclin-dependent kinase inhibitor 1B
cDNA	complementary DNA
CDS	coding sequence
CPA4	carboxypeptidase A4
cFLIP	CASP8 FADD-like apoptosis regulator (CFLAR)
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	cytomelgalovirus
COX2	cyclooxygenase-2
CREB1	cAMP response-element binding protein 1
CtBP1	c-terminal binding protein 1
CTG	CellTiterGlo
CTGF	connective tissue growth factor
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DCT	dopachrome tautomerase
DGCR8	DiGreorge syndrome critical region gene 8
DKK1	dickkopf 1
DMSO	dimethyl sulfoxide
DNA	deoxyribonuleic acid
DTIC	dacarbazine
E2F1	E2F transcription factor 1
E2F3	E2F transcription factor 3
ECL	enhanced chemiluminescence
EMT	epithelial-mesenchymal transition
enh	enhancer
ERK	extracellular-signal regulated kinase
EZH2	enhancer of zeste homolog 2
FABP7	fatty acid binding protein 7
FADD	Fas-associated death domain protein
FC	fold change
FCRLA	Fc receptor-like A
FCS	fetal calf serum
FDA	US Food and Drug Administration
FER1L3	myoferlin
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
FOXO3	forhead box O3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPR143	G protein-coupled receptor 143
GSK3B	glycogen synthase kinase 3 beta
HBEGF	heparin-binding EGF-like grwoth factor
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
hr	hour(s)
HRAS	v-Ha-ras Harvey rat sarcoma viral oncogene homolog
hRluc	synthetic *Renilla* luciferase gene
HRP	horseradish peroxidase
HSV-TK	herpes simplex virus thymidine kinase
IEP	immediate early promoter
IGFBP3	insulin-like growth factor binding protein 3
IGF2R	insulin-like growth factor 2 receptor
IgG	immunoglobulin
IL1B	interleukin 1 beta
IL8	interleukin 8
IL11	interleukin 11
i.v.	intravenously
KCNMA1	potassium large conductance calcium.activated channel, subfamily M, alpha member 1
kDa	kilo dalton
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
MET	mesenchymal to epidermal transition
MeV	Multiexperiment Viewer
Mib1	mindbomb homolog 1
MIF	macrophage migration inhibitory factor

min	minute(s)
miRISC	miRNA-induced silencing complex
miRNA	microRNA
MT1X	metallothionein 1
MITF	microphthalmia-associated transcription factor
MLANA	melan-A
MMP 7	matrix metalloproteinase 7
mo	month(s)
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
MYCN	v-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)
MYOF	myoferlin
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
NSG mice	Non-obese diabetic scid gamma mice
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
PDCD1/4	programmed cell death 1/4
PEST	domain rich in proline, gulamic acide, serine and threonine amino-acids
PI(3)K	phoyphatidylinositol 3 kinase
PIP2	phoyphatidylinositol (4,5)-bisphosphate
PIP3	phoyphatidylinositol (3,4,5)-trisphosphate
PLC	protein kinase C
PLZF	promyelocytic leukemia zinc finger
PMEPA1	prostate androgen-induced protein A1

pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homologe
qPCR	real-time quantitative PCR
r	Pearsons'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
SCG5	secretogranin V
scFv	single-chain antibody fragment
SD	standard deviation
SDS	sodium dodecyl sulfate
sec	second(s)
SEMA6/5A	semaphorin-6/5A
SERPINA3	serpin peptidase inhibitor, clade 3
SILV	silver homolog (mouse)
siRNA	small interfering RNA
SLIAC	stable isotope labelling by amino acids in cell culture
SLC2A3	solute carrier family 2, member 3
snRNA	small nuclear RNA
SPRR2D	small proline-rich protein 2D
SSM	superficial spreading melanoma
STC2	stanniocalcin 2
SV40	simian vacuolating virus 40
T-ALL	T-cell acute lymphoblastic leukemia
TBS	Tris-buffered saline
TBS-T	TBS with 0.1% Tween-20
TGFBR2	transforming growth factor, beta receptor II
Tm	melting temperature
TM	transmembrane domain

TNF	tumor necrosis factor
TPM1	tropomyosin 1
TRIM2	tripartite motif containing 2
TRPM1	transient receptor potential cation channel, subfamily M, member 1
TSP1	thrombospondin-1
TYR	tyrosinase
TYRP1	tyrosine-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
WST	water soluble tetrazolium
YB1	Y box binding protein 1
yr	year(s)
ZEB1	zincfinger E-box binding homeobox 1

Part 3
Introduction

3.1 Melanoma

Skin cancer represents the cancer type most commonly diagnosed in the United Stated of America with a prevalence of two million new cases annually [1]. The most common forms are nonmelanoma skin cancer types including basal cell carcinoma (BCC), angiosarcoma, cutaneous B-cell lymphoma, cutaneous T-cell lymphoma, dermatofibrosarcoma protuberans, merkel cell carcinoma, sebaceous gland carcinoma or squamous cell carcinoma (SCC) [1, 340]. Malignant melanoma is the least common type of skin cancer representing only 5% of all skin cancer cases reported. Despite this fact malignant melanoma is considered the most aggressive form of human skin cancer with a ten-year survival rate of about 24% for melanoma patients without distant metastases [340]. Melanoma is responsible for about 80% of skin cancer related deaths [340]. In europe malignant melanoma is the ninth most common cancer type and accounts for around 3% of all cancer incidences. The highest incidence rates of malignant melanoma are found in Australia and New Zealand due to high exposure to UV radiation, which is the main cause of skin cancer [340].

Melanoma is a disease of epidermal melanocytic cells, so-called melanocytes. These pigment-producing cells are widely distributed in the body e.g. in the gastrointestinal tract, the inner ear and most commonly in the basal layer of the skin epidermis. Melanocytes are responsible for the skin pigmentation by a process called melanogenesis. Melanin, a skin pigment which absorbs UV-B light and therefore protects skin layers such as the hypodermis, is produced during this process [4]. Low amounts of melanin production in rather fair-skinned people and a high exposure to UV-B radiation are the main factors for skin cancer development in countries such as Australia and New Zealand [340]. Skin cancer screenings have been established world-wide as they have been shown to be a crucial step in the early detection of melanoma incidences, that is to say before the disease progresses and metastasis formation has begun [75].

3.1.1 Melanoma progression and invasion

The transformation of melanocytes to melanoma requires a multistep accumulation of genetic and molecular alterations [238, 360]. Melanocytes originate from melanoblasts which migrate from the neural crest for example into the skin and subsequently localize in the epidermis or the hair follicles [81]. The normal healthy skin is composed of the epidermis on the outside, the dermis and the hypodermis as the skins' most inner layer [4, 81]. Melanocytes are smaller in size than keratinocytes and inhibit the melanosomes, melanin-producing, membran-bound organelles, which can be observed by bright-field microscopy [81]. At their final destination, for example in the epidermis, melanoblasts differentiate into mature melanocytes and synthesize melanin that can be transferred to their neighbouring keratinocytes [81]. Melanocytes can be found in different organs, such as the ears, the eyes, mucosal membranes, the central nervous system and in the gastrointestinal tract [81, 112]. In the following the focus will be on skin cancer types and therefore explicitly on cutaneous melanocytes.

The transformation of melanocytes to melanoma is called melanogenesis and depends on various genetic factors of the host and on environmental influences [238]. It has been shown that different subsets of genetic alterations, so-called driver mutations, are necessary and sufficient to promote melanoma progression [145, 231]. In the normal healthy skin, melanocytes are evenly distributed in the epidermis [74]. However, melanogenesis begins when an aberrant proliferation of melanocytes leads to benign or dysplastic nevi formation. There then follows the radial growth phase when additional genetic and epigenetic mutations accumulate and the melanoma exhibits the ability to grow intraepidermally as can be seen in Figure 1. During the vertical growth phase melanoma cells can invade the dermis and form metastases. In this last step of the progression model the melanoma cells are able to disseminate to other locations of the skin or other organs through the vascular and lymphatic system. Despite this commonly proposed model, clinical data suggest that melanoma progression does not necessarily start with nevi formation or strictly follows the proposed model [68].

3.1.2 Melanoma classification

Melanoma can be classified into different types based on important characteristic features of melanoma progression and staging such as tumor thickness, mitotic rate and ulceration.

The mitotic rate is currently considered to be a novel important prognostic parameter of melanoma staging [20, 21]. Melanoma can be categorized into four basic types: *superficial spreading* melanoma, *lentigo maligna*, *acral lentiginous* melanoma and *nodular* melanoma [111, 65, 21]. *Superficial spreading* melanoma accounts for about 70% of all melanoma incidences especially among the younger population. This kind of melanoma is named due to persistent growth along the top layer of the skin in the period before it starts penetrating more deeply into the skin [112]. Starting with a benign mole the first indications are irregular discoloured flat or slightly raised patches that vary in colour. It is not restricted to any specific body part but most commonly it appears on the legs or the upper back.

The *in-situ* melanoma, *lentigo maligna,* shows similar features, however it is mostly found among the elderly, and on chronically sun-exposed or damaged skin on the face, neck, forearms and ears [285]. *Lentigo maligna* is the least common and least aggressive type of cutaneous melanoma with the highest incidence rates reported in Hawaii. The benign form of *lentigo maligna* might grow slowly for three to fifteen years. If this type acquires the ability to spread, it is thereupon called *lentigo maligna* melanoma [65].

The third superficially growing type of melanoma is called *acral lentiginous* melanoma and it appears mainly under nails and on feet or on hands. The progression of *acral lentiginous* melanoma is faster in comparison to *lentigo maligna* or *superficially spreading* melanoma and it is more commonly found among African-Americans and Asians than among Caucasians [21].

The most aggressive form of melanoma is called *nodular* melanoma and is found in ten to fifteen percent of melanoma incidences. It predominantly has a vertical growth phase while it significantly lacks the radial growth phase. Mostly during the time of diagnosis it is already invasive and has formed metastases [112]. It can appear without the indication of a previous lesion in a variety of colours, ranging from grey to blue, and white, brown to non-pigmented, but mostly it is black [111, 65, 238].

3.1.3 Melanoma staging

The different progression states of melanoma are mainly characterized depending on the thickness of the tumor and whether the tumor has already spread to lymph nodes or other organs of the body. Staging indicates possible treatment options, the likelihood of melanoma recurrence or if the tumor has already spread to adjacent lymph nodes and tissues [20, 21].

The Breslow scale indicates the thickness of melanoma occurrence whereas the Clark scale

measures the local invasion [74]. Five different levels are described by the Clark scale, reaching from melanoma *in situ* (level 1) where the melanoma cells are present only in the epidermis, to level 5, where tumor cells have penetrated through the dermis into the subcutaneous fat (Figure 1).

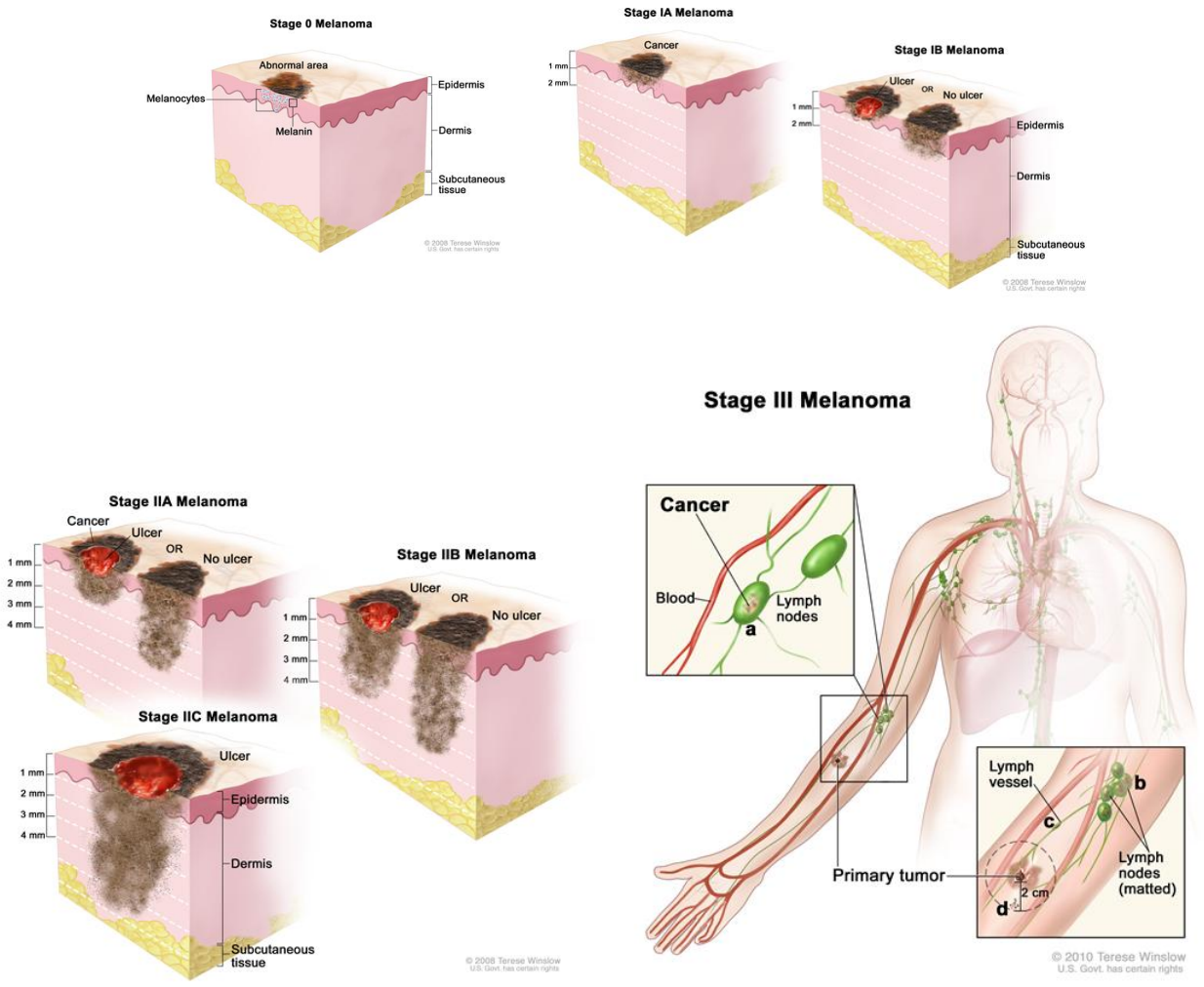

Figure 1: The Clark scale staging system for melanoma indicating its progression as it is categorized from stage 0 to stage III. The different stages are determined due to the penetration of melanoma cells into the skin. At stage 0 only the epidermis is affected, but with disease progression the cells invade the dermis, eventually the subcutaneous tissue and start to metastasize through the lymph or blood system. Adopted from the AJCC Cancer Staging Manual [74].

The Breslow scale is calculated based on the depth of tumor growth and divides the tumor occurence in five stages. In the Tis stage melanoma cells are only present in the uppermost layer of the skin surface and less than one millimetre thick. With increasing thickness the tumor is classified into T1 stage (> 1mm), T2 stage (1mm-2mm), T3 stage (>2mm) and finally the T4 stage, when the tumor reaches a thickness of more than four millimetre. This staging system is also the basis for the third type of classification for malignant tumors, the TNM staging [21].

Pierre Denoix introduced the first staging system, so-called TNM classification of malignant tumors, which is still the basis of current staging criterias [317]. Denoix' system assesses the size of the primary tumor (T), if regional lymph nodes are affected (N) and the occurrence of distant metastasis (M) [317, 74, 328]. The TNM classification does not only consider tumor thickness but also its spreading to lymphnodes and distant organs. The TNM classification is the most common system for cancer prognosis published by the American Joint Comitee on Cancer/Union for International Cancer Control (AJCC/UICC) [265, 74, 20]. As mentioned above, this staging system describes the tumor thickness according to the Breslow scale. Additionally, ulceration of tumors is indicated with small letters: un-ulcerated tumors are indicated with "a" and ulcerated tumors with "b". The risk of tumors to metastasize is reported to be increased for ulcerated tumors [21]. Four stages describe the spreading of melanoma cells to nearby lymph nodes or lymphatic ducts. If the tumor has not spread to other parts of the body it is indicated by the M0 stage. In the M1 stage metastases in different organs can already be detected. Lower case letters from a to c define the sizes of metastases from (a) small which can only be seen under the microscope to (b) macrometastases and (c) small satellite metastases close to the primary tumor or in the lymphatic system of the skin [265, 20].

In the last ten years studies have emerged, which show that ulceration might not be the most sensitive marker [21, 294]. Therefore, the mitotic rate was considered to assess the presence or absence of mitoses during the vertical growth phase of the tumor in addition to the amount of overall mitotic events [294].

Until today, all melanoma staging systems are solely focussing on the tumor itself and do not consider the role of the tumor microenvironment in provoking or restricting tumor progression [328]. Several parameters have to be included into the common staging criteria, such as the presence and characterization of T cell subsets in the tumor, potential adaptive immune resistance mechanisms of the tumor in combination with the local immune milieu [329, 332]. Various immune cell subsets, e.g. regulatory T cells, have been reported to have ambivalent prognostic importance in different tumor entities [98]. Furthermore, not only the

host adaptive immune system has to be taken into account, also acquired tumor immune resistance mechanisms as PD-L1 expression playing a role in tumor progression or rejection [328, 17, 164, 329].

The availability of accurate staging criteria is crucial for patients in order to receive the best available treatment in regard to long or short term cytotoxicities, the possibility of tumor relapse and estimated life expectancy [294].

3.1.4 Important signaling pathways in melanoma

Only the combination of staging criteria with a fundamental understanding of melanoma cell biology, malignant initiation and progression will enable new therapeutic approaches. Therefore, crucial intracellular signaling pathways involved in melanoma development are highly investigated at the moment.

3.1.4.1 The Ras/Raf/MEK/ERK pathway

The Ras/Raf/MEK/ERK signaling pathway with its respective receptor tyrosine kinases, cytokines and heterotrimeric G-protein-coupled receptors is able to induce and translate cell fate decisions after outside stimuli.

The activation of growth factor receptors lead to Ras activation and further downstream signaling via Raf activation that in turn activates the MAPK/ERK kinase (MEK). MEK activates mitogen-activated protein kinase (MAPK, also known as extracellular-signal regulated kinase (ERK)) which phosphorylates various targets intracellularly e.g. in the cytoplasm and interacts with other pathways involved in cell death and survival. MAPK also translocates into to the nucleus and regulates gene expression that promotes proliferation and cell survival [305, 232].

This signaling cascade can be activated by growth factors, such as stem-cell factor (SCF) or fibroblast growth factor (FGF) leading to a transient ERK activation and an intermediate mitogenic effect on the cell (Figure 2) [314, 58, 64]. Thus, until now it is thought that only the combination of several growth factors would increase donwnstream signaling and lead to a sustained ERK activation. In around 90% of melanomas ERK is hyperactivated leading to sustained melanoma cell proliferation [58]. The most common mutated component of this

signaling pathway found in around 70% of melanomas is BRaf, for which over 30 different mutations are currently known. The most common mutation, a substitution of glutamic acid for valine at position 600 (V600E), is widely investigated for targeted therapy [46, 135]. BRaf is one of the three human Raf genes, the others being ARaf and CRaf. The V600E mutation leads to constitutive active ERK signaling and stimulation of proliferation and sustained survival of tumor cells. Several other genes were recently identified to function downstream of V600E BRaf inducing several tumor growth and maintenance functions such as neoangiogenesis by vascular endothelial growth factor (VEGF) secretion [321] and the expression of transcription factors as MITF (microphthalmia-associated transcription factor), cell cycle regulators as cyclin D1 and the matrix metalloproteinase-1 (MMP-1), enzymes important for tumor maintenance and progression [151, 32].

Another mode of action for sustained ERK activation can be induced by autocrine stimuli or gain-of function mutations in NRas. NRas represents one of the three Ras genes present in humans and commonly mutated in up to 30% of melanomas. Oncogenic Ras are key regulators of tumor maintenance and tumor initiation in p16 INK4a-deficient mice [2, 59]. The Ras/Raf/MEK/ERK signaling cascade inhibits key players and checkpoint molecules of cell survival and proliferation indicating its importance in tumor progression.

3.1.4.2 The PTEN/PI(3)K/AKT pathway

Another important pathway in melanoma is the PTEN/PI(3)K/AKT pathway that regulates cell proliferation, survival and motility (Figure 2). Phosphatidylinositol 3 kinases (PI(3)Ks) mediates phosphorylation of membrane lipids such as phosphoinositides, which then function intracellularly as second messengers to induce various downstream effector pathways [308]. Typically, growth factors bind to their receptor tyrosine kinases which subsequently activate PI(3)K, leading to the conversion of plasma membrane lipid phosphatidylinositol (4, 5)-bisphosphate (PIP2) to phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3). In turn, PIP3 functions as second messenger by phosphorylating v-akt murine thymoma viral oncogene homolog (AKT), that induces numerous mitogenic processes including survival gene transcription, inhibition of apoptosis, cell cycle progression, protein translation and proliferation. Dephosphorylation of PIP3 by phosphatase and tensin homologue (PTEN) terminates this signaling cascade [366, 64]. In 3% of metastatic melanomas PI(3)K mutations can be detected, up to 20% of late-stage melanomas were found to lack PTEN function and overexpression of PI(3)K effector protein kinase B (PKB or AKT) is present in around 60%

of melanomas [366, 263, 320].

To inhibit melanoma cell proliferation and growth, inhibition of both signaling cascades Ras/Raf/MAPK/ERK and PTEN/PI(3)K/AKT is required [314]. BRaf and PI(3)K are acting downstream of Ras. Therefore BRaf and PTEN mutations often occur simultaneously whereas NRas and BRaf mutations as well as NRas and PTEN mutations could be shown to be mutually exclusive [356, 357, 366]. Overall Ras mutations are less common in melanoma than BRaf mutations, reflecting a genetic or biological benefit for malignant progression of melanomas with mutant BRaf over those with NRas mutations [357].

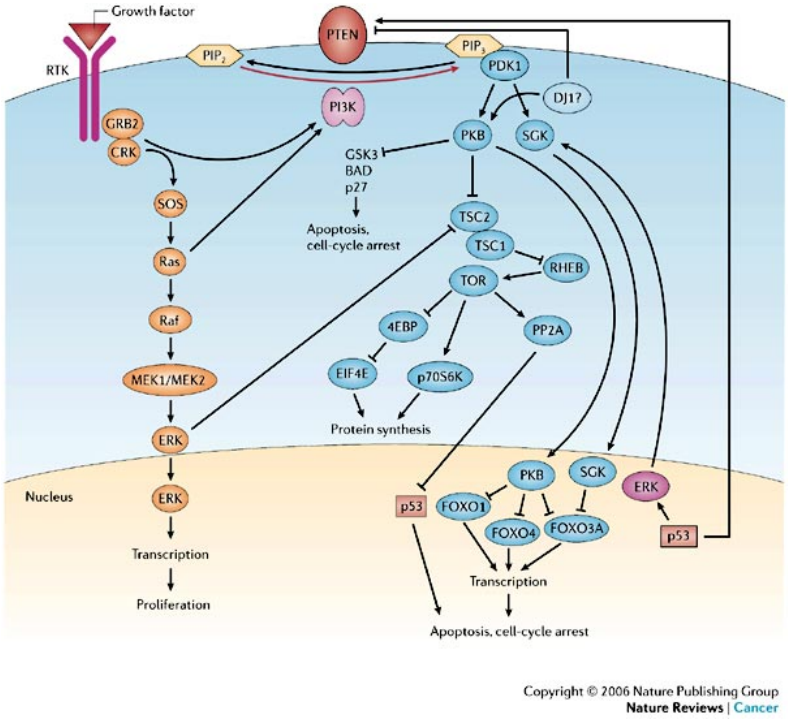

Figure 2: Important signaling pathways in melanoma. This scheme highlights the interaction and physiological consequences of aberrant Ras/Raf/MEK/ERK and PTEN/PI(3)KAKT signaling in disease progression. Both pathways are important regulators of cell proliferation and apoptosis. Adopted from Cully et al. 2006 [64].

3.1.4.3 MITF signaling pathway

MITF is called the "master regulator" of melanocyte development and differentiation and plays an important role in melanoma initiation and progression [238, 200]. It is a dimeric transcription factor with a basic helix-loop-helix leucine zipper tertiary structure. Physiologically, MITF regulates melanoblast survival and melanocyte lineage commitment. The oncogenic properties of MITF is mediated due to its transcriptional regulation of melanogenic proteins such as, the glycoprotein 100 (gp100) and melanoma-associated antigen recognized by T cells-1 (MART-1) [105, 200]. MITF itself is transcriptionally regulated through several pathways interacting with its promoter such as, the melanocortin and the β-catenin pathways [238, 305].

MITF transcription can be mediated via cAMP responsive-element binding protein 1 (CREB1) activation after interaction of adenylate cyclase and alpha melanocyte stimulating hormone (α-MSH) with the G protein-coupled melanocortin receptor 1 (MC1R). The transcription factor CREB1 in turn activates MITF transcription by binding to its promoter. Additionally, growth factors such as the tyrosine-protein kinase c-KIT and the hepatocyte grwoth factor (HGF) can induce MAPK pathway activation resulting in MITF phosphorylation. MITF downstream targets are genes involved in cell survival (Bcl-2, MCL1), melanin synthesis (TYR, TYRP1, DCT) or are markers for melanoma therapies (MLANA, SILV, TRPM1). In the β-catenin pathway, Wingless-related integration site (WNT) proteins bind to receptors of the frizzled family to inactivate the glycogen synthase kinase 3 beta (GSK3B) which phosphorylates β-catenin to be degraded by the proteasome. Thus, WNT signaling results in increased β-catenin levels, which may translocate into the nucleus to activate the transcription of target genes including MITF, G1/S-specific cyclin-D1 (CCND1) and matrix metalloproteinase 7 (MMP7)[161, 238].

MITF expression needs to be tightly controlled as different levels of expression regulate distinct functions in melanocytic cells. Only intermediate levels of MITF seem to favour cell proliferation. Cell cycle arrest and differentiation is induced by excessively high MITF levels whereas extremely low levels predispose to cell cycle arrest and apoptosis [200]. The complex regulation level of MITF seems to be controlled by ERK phosphorylation and subsequent MITF degradation to counteract constitutive active MITF. Oncogenic BRaf fails to induce melanoma cell proliferation in the presence of increased MITF expression, explaining its significantly lower expression level of BRaf in melanoma cells compared to melanocytes [356]. Additionally, it was shown that MITF regulates anti-apoptotic protein Bcl-2 expression synergistically [228]. Cells lacking MITF were rescued from programmed cell death after Bcl-2

overexpression [228]. This complex MITF regulation system highlights its importance in cell physiology and its potency to drive malignant progression.

3.1.4.4 Senescence mechanisms

As described above, mutant BRaf influences melanoma proliferation, survival and metastasis. Strategies inhibiting oncogenic BRaf resulted in remarkable clinical responses,however also to rapid acquisition of resistance to BRaf inhibitors due to the activation of alternative downstream signaling cascades. In addition, melanoma cell resistance to cytotoxicity is mainly attributed to unbalanced levels of anti-apoptotic proteins, e.g. Bcl-2 and MCL1. This suggests that MCL1 overexpression may contribute to the resistance of melanoma cells to various therapies targeting BRaf and MEK [388, 362].

Therefore strategies are currently being investigated to specifically target cell survival pathways. The Bcl-2 protein family is mainly involved in mitochondrial mediated cell death and survival [121]. It consists of pro-apoptotic family members, Bax, Bak and Noxa, and anti-apoptotic members, Bcl-2, Bcl-xL, Bcl-w and MCL1. Bax and Bak are known to be key mediators of apoptosis induction as cells fail to undergo induced programmed cell death after their loss [355]. The ratio of pro- to anti-apoptotic members of this family mediates the decision for cell death or survival [262, 355]. Activated death receptors or DNA damage triggers the cell-intrinsic pathway of apoptosis. Several pro-apoptotic members of the Bcl-2 family are activated, for example Bax and Bak to induce mitochondrial cytochrome C release and consequently caspase 3 dependent apoptosis. MCL1, Bcl-2 and Bcl-xL are able to bind and dimerize with Bax or Bak to counteract this signaling cascade and sustain cell survival [224, 121]. Thus, targeting therapies to counter-act acquired cell death resistance, as one of the hallmarks of cancer [133], might lead to new possibilities in cancer therapy [133].

3.1.5 Melanoma treatment and therapy

Before melanoma progression reaches the metastatic state most melanomas can be cured by surgical resection. Metastatic melanoma, however, needs to be treated with individualized combination of surgery, chemotherapy, radiotherapy and targeted immunotherapy.

In chemotherapy several monotherapies are currently available, such as Dacarbazin (DTIC),

alkalyting agents, hormonal agents, Tamoxifen, Paclitaxel and other plant derived agents [19]. Only Dacarbazin (DTIC) is an FDA-approved chemotherapeutic agent for treatment of advanced melanoma with a maximum response rate of 20% and a median response duration of four to six month. Recently, combination chemotherapies have been investigated to increase therapeutic effectiveness.

All different combinations, such as Cisplatin and DTIC alone, additionally with Vindesine, Vinblastine and Carmustine (BCNU) or Vinblastine, Bleomycin, Cisplatin together with DTIC, Dibromodulcitol (DBD) are currently applied to investigate the best individual combination for each melanoma patient [19].

At the moment, intensive studies are being carried out to elucidate checkpoint molecules for melanoma progression and crucial signaling pathways to identify new possibilities for targeted therapy. As indicated in Figure 3 a variety of molecules are already being targeted by melanoma agents [258]. The main players of melanoma progression are being explored and can be targeted by various therapeutics in the clinics, such as the receptor tyrosin kinases (RTKs) by Imatinib, Ras by Lonafamib and BRaf targeted by Vemurafenib.

One of the first targeted therapies aimed at targeting receptor tyrosine activation of MAPK, PI(3)K or Jak/STAT pathways (Figure 3). Imatinib, the first drug in clinical phase II studies targeting c-KIT, BCR-ABL and the platelet derived growth factor receptor (PDGFR)-α and -β, did not show any beneficial effect [339, 369]. This result can be explained due to missing patient preselection. Interestingly, c-KIT is mainly expressed in mature melanocytes and diminished or absent in metastatic melanoma [251]. In case of c-KIT positive tumors, a mutation or amplification rate of 39% for mucosal, 36% for acral and 28% for chronic melanoma was reported [66, 67]. In recent clinical studies, the effect of c-KIT suppression was validated by Imatinib treatment in c-KIT mutant metastatic melanoma[44, 128]. Additional c-KIT inhibitors are currently under investigation including Dasatinib targeting the c-KIT L576P mutation [363] and the second generation tyrosine kinase inhibitor, Nilotinib (NCT01028222). These studies support the potency of c-KIT inhibition after careful evaluation of its expression and mutation status in the respective patient.

As already mentioned before, Ras plays a distinct role in the activation of the Ras/Raf/MEK/ERK and PTEN/PI(3)K/AKT pathways promoting melanoma survival and proliferation and is therefore an optimal target for cancer treatment [144]. Several studies tried to inhibit Ras activation for example with farnesyltransferase inhibitors (Tipifarnib, Ionafarnib) but no clinical response was observed for Tipifarnib [281, 102] (Figure 3). Despite the fact that Ras inhibition alone did not negatively influence melanoma progression, ionafarnib in combination with chemotherapy induced melanoma cell apoptosis or increased the effects of second agents, such as apoptosis induction via receptor tyrosin kinase inhibitors

[315, 242, 256]. Conclusively, Ras inhibition alone might not be a promising melanoma therapeutic, nevertheless its combination with secondary agents might enhance the therapeutic effect.

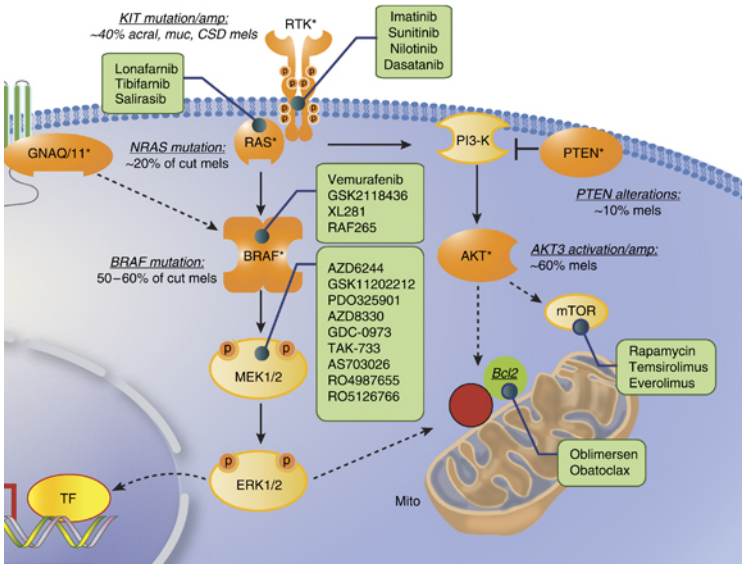

Figure 3: Therapeutic strategies in melanoma targeting several members of the the MAPK pathway (RTK, Ras, BRAF, MEK1/2, ERK), the PI(3)K pathway (PI(3)K, AKT, PTEN) inducing apoptosis (Bcl-2) or inhibiting aberrant DNA methylation (DNMT, HDAC). Adopted from Nikolaou et al. 2012 [258].

As more than half of all melanomas patients harbour mutation in BRaf, this protein is an interesting targeting candidate. Two BRaf inhibitors will be mentioned in the next paragraph. Sorafenib, which is an unspecific receptor tyrosine kinase inhibitor affecting not only BRaf, but also PDGF receptor and c-KIT activity [168]. Monotherapies as well as combination therapies of Sorafenib with chemotherapy (DCIT, Temozolamide) failed to show clinical benefits for metastatic melanoma patients [46]. Currently Vemurafenib and Dabrafenib are highly investigated BRaf inhibitors which selectively target V600E mutated BRaf [94]. A first phase I clinical study indicated a positive clinical response rate for Vemurafenib in metastatic melanoma patients [94]. Also Dabrafenib indicated promising response rates with only mild side effects in patients with V600E mutant BRaf. Additionally, studies have shown that Dabrafenib impacts on distinct cancer types [166, 236, 135]. Several Baf inhibitors are currently under investigation in clinical trials [170, 93]. Despite the promising data on metastatic melanoma patients responding upon BRaf inhibitor treatment alternative pathways were ac-

tivated in most patients to circumvent BRaf inactivation, resulting in secondary resistance and disease relapse [252].

Other important signaling molecules in the Ras/Raf/MEK/ERK signaling cascade is MEK. Interestingly, the inhibiting effect of MEK inhibitors, such as the MEK1 MEK2 inhibitor AZD6244, was dependend on MCL1 activity [3, 134]. Therefore, further studies are elucidating the cross talk between mechanisms for apoptosis induction and the signaling pathways downstream of MEK [60, 57]. Moreover, it was shown that combinatory treatments such as of BRAF and MEK inhibitors could counteract secondary resistance [155].

Furthermore blocking anti-apoptotic molecules, e.g. Bcl-2, Bcl-xl and MCL1 with small molecule inhibitors (e.g. Obatoclax) is reported to sensitize melanoma cells to Bcl-2 homology domain 3 mimetic drug (ABT-737) and proteasome inhibitor Bortezomib treatment [258]. The combination of chemotherapy and targeting of anti-apoptotic proteins was investigated by inhibiting Bcl-2 or Bcl-xL or the combination of both by Obatoclax [254]. Obatoclax was shown to target MCL1 BAK interaction counteracting resistance development after Bcl-2, proteasome inhibitor or chemotherapies [254, 383]. Reported in Oncogene this year [25], constitutive expression of MCL1 in melanocytes and melanoma can be promoted by oncogenic BRaf V600E. BRaf V600E mediated MCL1 promoter activation is dependent on STAT3 activity [25]. Furthemore, STAT3 is phosphorylated by BRaf V600E and therefore required for MCL1 expression. Thus, MCL1 dependent chemoresistance and melanoma survival can be disrupted by missing STAT3 activity.

Another therapeutic approach uses adjuvant therapies to treat melanoma patients with for example interferon-α [239] and interleukin-2 [11]. Both cytokines are among previously approved therapeutics that are widely used in adjuvant immunotherapy for metastatic melanoma. Thus, patients treated with one of these cytokines, are reported to experience side effects such as flu-like syndrome or vitiligo and the response rates are low [33, 240, 327].

Additionally, the specific targeting of melanoma cells by the immune system is currently under investigation and clinical studies indicate promising future therapeutic options. Different strategies try to stimulate tumor-specific T cell response of the patient. Such stimulation can be achieved by vaccination with tumor specific antigens (e.g. TYR, gp100 and MART-1) or adoptive T cell transfer. Another approach aims to inhibit T cell checkpoint molecules, such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) [122, 268], or programmed cell death 1 (PD-1) or its ligand PD-L1 [29, 164, 337].

An increasing number of therapeutic options arise every year targeting for example members of the PI(3)K pathway, tumor angiogenesis, modulating the immune system or trying to counteract tumor suppressive pathways which are investigated in basic research and clinical studies until now. In conclusion, therapeutic options for melanoma patients must be carefully

selected and individualized for every melanoma case itself.

3.2 microRNAs

microRNAs (miRNAs) are small endogenous ribonucleic acid (RNA) molecules with a length of around 22 nucleotides (nts). These small single stranded RNA molecules are able to pair with messenger RNAs (mRNAs) of protein-coding genes and induce mRNA cleavage or translational repression. Today, the major view is that this class of regulatory molecules comprises between 0.5 to 1% of predicted genes in humans and controls various cellular processes in development, differentiation and cell maintenance. The first miRNAs to be discovered were lin-4 and let-7 and their role in larva development [359, 194]. At the discovery it was surprising to realize that the lin-4 gene is not protein-coding but encodes for a small RNA molecule. These small RNAs could interact with mRNAs due to their antisense complementarity and negatively regulate mRNA expression levels, such as lin-4 negatively regulates LIN-14 protein expression [194, 359, 18]. Various roles of these short RNA molecules, termed miRNAs, are currently reported not only in human cellular processes, but in leaf and flower development in plants [12] and also in fat metabolism, cell proliferation and cell death in flies [12, 8, 38]. Despite the fact that miRNA targets are mostly unknown, it is estimated that there might exist hundreds of targets for a single miRNA, in total targeting around 30% of all protein coding genes within the human genome [204, 203]. Thus, a strongly increasing number of studies focus on miRNAs, demonstrating their capabilities to regulate complex signaling networks, cellular processes and malignant progression [24, 203].

3.2.1 miRNA biogenesis

miRNAs are encoded either in intronic regions of protein-coding genes or located solely as single genes. RNA polymerase II transcribes the first miRNA structure called the primary miRNA (pri-miRNA) in the nucleus as depicted in Figure 4 [9]. Subsequently, this pri-miRNA is processed into a approximately 70-nucleotide hairpin precursor (pre-miRNA) by a complex consisting of a member of ribonuclease (RNase) III family, Drosha, and a double-stranded

RNA binding protein, DGCR8. The pre-miRNA is then exported into the cytoplasm by Exportin 5 in a Ran-GTP dependent manner and captured by the RNA binding protein TRBP together with the RNase II enzyme Dicer. The so formed approximately 70 bp guide miRNA (miRNA)/ passenger miRNA (miRNA*) duplex indcuces Argonaute 2 (AGO2) cleavage of the 3' arm of some pre-miRNAs before Dicer-mediated cleavage occurs. This intermediate nicked hairpin precursor, called AGO2-cleaved precursor miRNA (ac-pre-miRNA), is only found in mammals and requires a high degree of complementarity along the hairpin stem for its functionality. As mostly only one strand (guide strand) of the so formed miRNA/miRNA* duplex is preferentially loaded into the miRNA-induced silencing complex (RISC) to form the miRISC complex, the other strand, the so-called passenger strand (miRNA*) is degraded.

In some cases both strands are functional and play a role in target regulation [222, 127]. The functional miRNA guides the miRISC complex to the complementary target mRNA, leading to translational repression, mRNA deadenylation or degradation. Several miRNA biogenesis pathways are known, which do not require this specific Drosha-DGCR8, or the Dicer processing. As shown in Figure 4 some pre-miRNAs mainly processed from short introns (mirtrons) take the alternative pathway due to splicing, debranching and further resection by nucleases, therefore bypassing the Drosha-DGCR8 step. Also shRNAs, which are produced by cleavage from endo- or exonucleases or by RNA polymerase III transcription, may not have a stem to allow Drosha-DGCR8 processing and are therefore directly incorporated into AGO proteins. Pri-miR-451 is another special case, as it does not require Dicer-mediated cleavage. After Drosha-DCGR8 cleavage this pre-miRNA can directly form a complex with AGO2. In turn AGO2 cleaves its 3'-arm but further processing occurs by an yet unknown mechanism to generate mature miR-451 [9, 379].

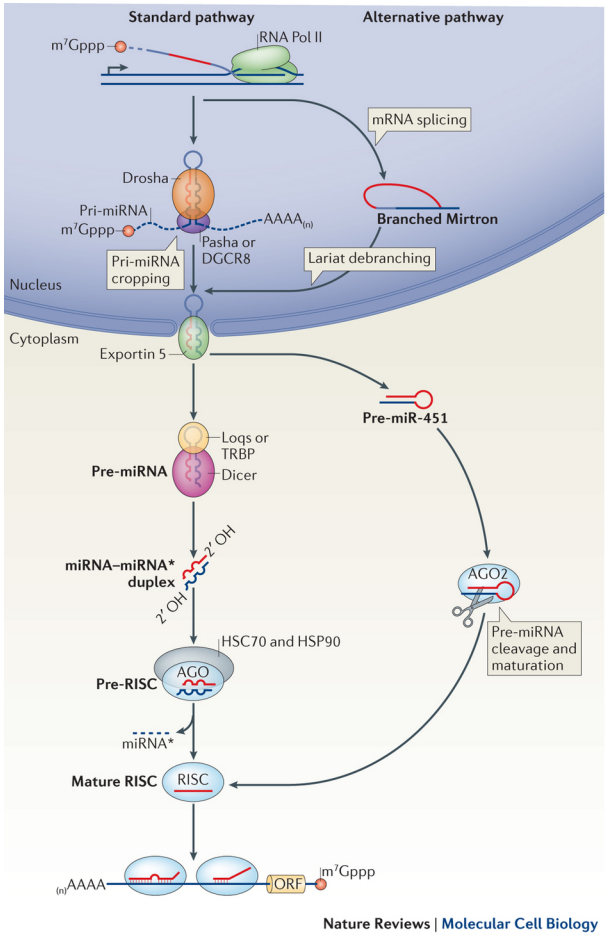

Figure 4: miRNA biogenesis: A comparison of the standard miRNA biogenesis pathway on the left and the proposed alternative pathway on the right side. m7Gpp, 7-methylguanosine cap; RNA Pol II, RNA polymerase II; AAAA, poly(A) tail; Loqs, Loquacious; TRBP, human immunodeviciency virus (HIV-1) transactivating (TAR) response RNA-binding protein; 2'OH, hydroxyl group; HSC70, heat shock protein 70 kDa protein 8; HSP90, heat shock protein 90; AGO, argonautprotein; RISC; RNA-induced silencing complex; ORF, open reading frame. Adpoted from Ameres et. al. 2013 [9].

3.2.2 miRNA target identification

The identification of miRNA targets is essential to understand the function of miRNAs. miRNA target prediction has its limitations as most studies focus on only a small number of targets ignoring the global impact on gene expression that one miRNA can have [304]. This complex regulatory capacity is most likely controlled by the interaction of approximately six nucleotides of the miRNA called the nucleotide seed sequence defined as nucleotides 2-7

in the miRNA sequence with a complementary sequence at the 3'UTR of its target genes [204, 120, 104, 101]. As already mentioned, every miRNA is able to regulate several hundreds of target genes hence making *in vitro* analysis and *in silico* target predictions challenging. Experimental investigation of miRNA target gene interaction is mainly based on reporter assays. The 3'UTR of putative target genes can be integrated into a reporter plasmid containing a luciferase or GFP reporter gene. After combined transfection of the reporter construct and the miRNA *in vitro* the interaction of both is translated into a decreased reporter signal [176]. Additionally, quantitative real-time polymerase chain reaction (qRT-PCR) experiments can indicate mRNA changes for genes under investigation due to miRNA overexpression. A more global approach comprises microarray expression analysis to either identify deregulated miRNA profiles associated with different malignancies or detect changes in gene expression after miRNA deregulation by introducing chemically modified complementary oligonucleotides or gene knockdown [41, 137, 193, 185]. Another rather indirect method for miRNA target identification is called stable isotype labeling with amino acids in cell culture (SILAC). With this approach differences in protein expression levels on a proteome-wide scale can be assessed [306, 17]. More recently a novel experimental approach has been reported for the identification of miRNA targets in a more direct fashion. Immunoprecitpitation of RISC components sequester mRNAs targeted by miRNAs within this complex. Subsequently, high-throughput sequencing of the obtained mRNAs allows the identification of specific miRNA targets and their putative miRNA binding sites [27, 72, 139]. High-throughput sequencing of RNA isolated by crosslinking and immunoprecipitation (HITS-CLIP) [69, 206, 55] of AGO2 and two more recent variants of this method, photoactivatable-ribunucleoside-enhanced crosslinking and immunoprecipitation (PAR-CLIP) [130] of miRNA containing ribonucleoprotein complexes (miRNPs) and the individual nucleotide resolution (iCLIP) [152] approach, are currently used to elucidate larger miRNA networks. These techniques enable the investigation of miRNA-mRNA interactions and the identification of new binding events [56]. Despite these promising *in vitro* tools for miRNA research the complexity of miRNA interactions is still not fully understood.

Therefore, *in silico* prediction tools are of growing interest and several computational target prediction algorithms are currently available. Correlating mRNA and miRNA expression and sequence data might highlight the global genome wide regulation mediated by miRNAs. miRBase is one of the current available prediction platforms, which mediates the crosslinking between sequencing databases provided by miRNA registry and target identification by gene name [115, 116]. This online tool aims to integrate all online available sequencing information to directly indicate the possibility of an individual miRNA to interact with the 3'UTR of genes from all species available in the Ensembl database [95]. Target gene prediction with

miRBase is based on the 5' seed region complementarity, the thermodynamic stability and the conservation between species is determined precisely. miRBase does not only provide miRNA target identification but also integrates published miRNA sequence information and annotations known so far. Recently the updated version miRBase21 was launched online [115, 118, 117, 182, 181]. In addition another group at the Memorial Sloan-Kettering Cancer Center (NewYork City, USA) established a target prediction algorithm, called miRanda that is available online (www.miRNA.org) [80, 162, 163]. This miRNA target gene prediction tool also scans currently available RNA sequences of miRNAs and DNA or RNA sequencing information, available for the species of interest, to determine minimum free energy and rank possible interactions accordingly [30]. The mode of interactions is based on up-to date knowledge of target miRNA interaction rules [31]. Both target prediction databases provide direct links to additional resources on miRNA target prediction available online such as PicTar (http://pictar.mdc-berlin.de/ [183]), MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), TarBase [307, 345] and TargetScan. TargetScan was developed from the Whitehead Institute for Biomedical Research (Camebridge, USA). At the moment, TargetScan release 6.2 is available online. Their algorithm is able to predict interaction of the seed region of individual miRNAs with a conserved 7mer or 8mer region within biological targets [204, 100, 120]. Up to now TargetScan indicates miRNA target interactions at the 3'UTRs, their orthologs and just recently started to indicate possible interaction within open reading frames [120, 104].

Despite the wide range of available tools for *in silico* miRNA target prediction, most targets remain unidentified. Prediction algorithms do need to improve in the future due to increasing amounts of high-throughput data available to train computation-based systems [7]. Novel prediction rules have to be established as miRNA seed interaction cannot account for all miRNA dependent regulations observed so far. More recently several groups identified possible miRNA interaction sites within the coding regions, open reading frames or the 5'UTR of protein coding genes [204, 220]. Nevertheless, binding within the 3'UTR is still considered as the most prominent mode of interaction between miRNAs and their target genes [17]. Identification of alternative binding sites will be critical to unravel the complete interaction network of individual miRNAs. It is important to overcome the difficulties in miRNA target prediction to elucidate miRNAs in their whole complexity for future prognostic markers and therapeutic options for example in cancer.

3.3 miRNA and cancer

The first report that connects miRNA and cancer implied frequent downregulation or deletion of miR-15a and miR-16, shown in chronic lymphocytic leukemia (CLL) patients [41]. In melanoma the first direct link between dysregulation of miRNAs and disease progression was shown by Bemis et al. [28], where they demonstrated that miR-137 expression correlated with MITF expression [28]. Since then, increasing numbers of miRNA expression profiling and functional studies on miRNAs in cancer are reported, demonstrating the importance of miRNAs as key players in cancer development and progression.

3.3.1 miRNA as an oncogene

Deregulated miRNA expression profiles are key features of cancer cells in comparison to healthy cells under physiological conditions. The classification of genes into oncogenes and tumor suppressor genes also can be applied for miRNA networks. A previous study has shown that miRNAs can act either as oncogenes or tumor suppressors depending on their targets and downstream signaling pathways [82]. The term "oncogene addiction" was created to characterize the potential of an individual oncogene to promote malignant progression regardless of the complex process of cancer development [133]. Individual miRNAs are currently known to have oncogenic properties in specific malignancies. To determine possible relations between miRNA expression and malignant phenotype, expression profiles of several miRNAs were investigated by distinct groups. Differences were observed for miR-155 and miR-21 expression between benign melanoytes, melanocytic lesions and metastatic melanoma. Overexpression of both miRNAs correlated with increased malignancy in melanoma [276, 303, 119, 199]. Contradictory, Levati et al. reported [199] that miR-155 expression *in vitro* correlates with reduced cell proliferation and enhanced apoptosis induction due to gene silencing of SKI, a transcriptional co-regulator over expressed in melanoma [199]. The oncogenic potential of miR-155 was further supported by the finding, that miR-155 transgenic mice develop lymphoblastic leukemia after blockage of the pre-B-cell to B-cell differentiation step [91]. Until now the multifunctional role of miR-155 is intensively discussed as its differential expression profiles seem to indicate a complex role in different maligancies. Despite cancer, it plays a role during viral infections of DNA viruses, in hematopoietic lineage differentiation, inflammation, immunity and cardiovascular diseases [85]. Levati et al. [199] also reported a

differential expression of miR-21 which was supported by another recent report showing that decreased miR-21 levels induce apoptotic cell death in melanoma cells. This finding correlated with a highly increased amount of miR-21 in primary melanoma compared to benign nevi [299, 199].

One of the early identified oncogenic miRNAs, miR-182 located at chromosomal region 7q31-34, is predominantly amplified in melanoma cells [302]. Analysis of tissue microarrays of benign melanocytic nevi, primary and metastatic melanomas showed a correlation between increased miR-182 expression and melanoma progression and malignancy. miR-182 was shown to influence melanoma cell invasion and metastasis formation in several recent studies [211, 282, 302]. While Segura at al. showed that loss of miR-182 expression led to apoptosis induction, Huynh et al. investigated the importance of miR-182 in facilitation of anchorage-independent growth and increased lung metastasis formation in the murine B16F10 melanoma mouse model [302, 154]. Due to its distinct role in invasion and metastasis formation, several groups are currently investigating the mode of action of miR-182 in more detail. A wide range of putative targets of miR-182 are currently under investigation, e.g. FOXO3 (forkhead-box-protein O 3, FKHRL1), FOXO1 (FKHR), MITF, CDKN2C (p181INK4C), CASP2 and FAS, but only MITF and FOXO3 could be identified as direct targets so far [302]. In combination with miR-203, miR-182 was able to induce mesenchymal to epithelial transition (MET) probably due to snail family zinc finger 2 (SNAI2) repression in prostate epithelial cells (EPT1 cells) [282, 211]. Recently, miR-182 expression in two different melanoma cell lines was investigated due to epigenetic modulation after treatment with demethylating agents by Liu et al. [211]. This group could identify a CpG island in close proximity to the miR-182 locus as being selectively methylated in melanoma cells [211]. This epigenetic regulation indicates an auxiliary level of miR-182 regulation that has to be considered in future therapeutic options.

miR-221 and miR-222 are overexpressed in a variety of different cancer types, as for example pancreatic cancer [193] and glioblastoma [192]. In papillary thyroid carcinoma miR-221/222 were reported to target c-KIT [137]. Furthermore, expression of c-KIT was found to decrease with melanoma progression [241, 309]. In addition, Felicetti et al. [86] showed that miR-221/222 expression reversely correlates with c-KIT expression in primary vertical growth phase and metastatic melanomas. Based on these observations, the same group reported that miR-221/222 inhibition by antagomir treatment interfered with tumor growth and miR-221/222 overexpression in turn promoted tumor growth *in vivo* [86].

Another miRNA cluster whose members are reported to have oncogenic properties, is the miR-17-92 cluster. Members of this cluster promote cell proliferation, apoptosis resistance, the induction of tumor angiogenesis and are known to be overexpressed in hematopoietic

malignancies and solid tumors [234, 136, 267, 346].

One of the so-called "melano-miRs", miR-214 was identified due to its oncogenic phenotype after overexpression in melanoma cells [274]. The metastatic phenotype resulting from miR-214 overexpression is regulated in a double manner [274, 275]. miR-214 controls several members of an important pathway for melanoma progression and migration, namely the AP-2 transcription factors (TFAP2) and the activated leukocyte cell adhesion molecule (ALCAM). On the one hand, miR-214 mediates downregulation of TFAP2 and on the other hand controls ALCAM expression promoting melanoma progression [274, 275]. miR-214 regulates ALCAM expression on two individual levels. On the post-transcriptional level miR-214 mediates ALCAM upregulation via targeting of miR-148b whereas transcriptionally ALCAM is upregulated via TFAP2 downregulation. This signaling cascade is reported to influence several prometastatic properties such as cell migration, invasion and extravasation [275, 186].

Recently, miR-9 was reported to influence tumor cell motility and metastasis formation. It is overexpressed in breast cancer and differentially expressed in many other tumor entities [141, 196, 210, 221, 354, 387]. Several requirements need to be full filled for successful metastasis formation, such as sufficient angiogenesis induction, which is tightly controlled by the JAK-STAT pathway. miR-9 overexpression was observed in tumor-associated endothelial cells leading to enhanced cell migration and angiogenesis induction [387]. Furthermore, these cells showed reduced levels of the putative miR-9 target, suppressor of cytokine signaling 5 (SOCS5). miR-9 downregulation could mimic JAK inhibitor treatment and reduced the migrative potential in endothelial cells [387].

miR-9 overexpression in melanoma cells is reported to result in a decreased migrative and proliferative phenotype *in vitro* and *in vivo* [380, 210]. In addition melanoma progression and metastasis seems to correlate with miR-9 downregulation. This observed increased motility is induced on the one hand by miR-9 mediated cytoskeleton remodelling processes as well as increased E-cadherin expression and on the other hand, by NF-$\varkappa$B downregulation [210]. Further findings indicate miR-9 in combination with miR-9* and miR-92b as a marker for primary brain tumor formation [250]. The combination of all three miRNAs allows one to distinguish brain metastasis from primary brain tumors as they are found to be overexpressed solely in primary brain tumors [250]. To conclude, the oncogenic role of miR-9 is controversially discussed until today as its function seems to be context and tumor type specific.

3.3.2 miRNA as a tumor suppressor

Until now numerous miRNAs are reported for their tumor suppressive role in different cancer types. The let-7 miRNA family was one of the first miRNAs to be identified and therefore heavily investigated in the last decades [194, 359]. Therefore it was not surprising that this miRNA family was found to be one of 72 differentially expressed miRNAs in primary melanomas, benign or melanocytic nevi [301]. During this miRNA expression screening they identified members of the let-7 family to impact cell cycle regulation. This could then be validated *in vitro* as for example let-7b was able to downregulate cyclin A, D1 and D3. In addition, further studies directly linked let-7b and let-7a downregulation to malignant transformation form nevi to primary melanoma as they were found to be significantly reduced in melanoma. Until now the let-7 family has been reported to act as tumor suppressor by targeting oncogenes such as Ras and c-Myc in different cancer types [344]. Therefore, loss of this miRNA family favours tumor progression and malignant transformation [165, 246].

Focussing on changes in the miRNA expression pattern during melanoma progression unravels the complexity of the miRNA network. miR-137 targeting MITF was the first functional study of an individual miRNA with a key role in melanoma [28]. Soon miR-137 was identified as a potent tumor suppressor in a variety of cancers including brain tumors, colorectal cancer, head and neck cancer, gastric cancer and melanoma [70, 190, 209, 313, 49]. Another study indicated shorter survival times for stage IV melanoma patients with reduced miR-137 expression indicated by a correlation of miR-137 expression with melanoma patients' clinical outcome [218]. The at present identified targets of miR-137, c-Met, Y-box protein 1 (YB1) [218], enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) and MITF highlight the crucial role of miR-137 in melanoma development and progression [28]. Furthermore, miR-137 was demonstrated to inhibit migration, invasion and proliferation of melanoma cells through multiple targets [218], emphasizing the tumor suppressive function of miR-137 in melanoma. Yet, preclinical studies are still missing to clearly define miR-137 susceptibility as therapeutic agent in the treatment of melanoma.

The decreased copy number of miR-101 detectable during prostate cancer progression suggested its potential tumor suppressive role [342]. Furthermore, miR-101 directly targets EZH2 and therefore inhibit cell proliferation, invasion and tumor growth in glioblastoma [316], non-small cell lung cancer (NSCLC) [385], gastric cancer [350] and melanoma [217]. Notably, Luo et al. [217] showed that miR-101 inhibits melanoma cell invasion and proliferation and could additionally identify MITF as a direct target of miR-101 in melanoma cells. Thus, miR-101 and miR-137 share two common target genes namely, MITF and EZH2 [217].

Another miRNA family with a potent role in melanoma progression was discovered later,

namely the miR-200 family. Its members - miR-200a, miR-200b, miR-200c and miR-141 - are reported to be potent tumor suppressors in several types of cancer [78, 90, 311, 348, 373]. During melanomagenesis they are found to be significantly downregulated together with several miRNAs not belonging to this family, miR-205, miR-203 and miR-211 [373]. Decreased expression of miR-141 was detected in melanoma metastases in comparison to melanoma nevi. Additionally, cells that underwent transforming growth factor (TGF) induced epidermal to mesenchymal transition (EMT) had strongly decreased amounts of miR-200a, miR-200b, miR-200c, miR-141 and miR-429 [113]. As EMT favours the acquisition of a migratory phenotype, cancer progression benefits from downregulation of the miR-200 family. These studies underline the tumor suppressive role of this miRNA family, but it was shown that members of this miRNA family can have controversial effects on tumor progression [78].

Particularly, miR-200c inhibited cell proliferation, migration and drug resistance due to downregulation of BIM-1, a known regulator of stem cell self-renewal in breast cancer stem cells [311]. On the contrary miR-200c induced EMT and targeted the zinc finger E-box binding homeobox 1 (ZEB1) in various types of cancer [39, 113]. Overall, miR-200 family members differentially regulate melanoma cell plasticity and morphology in a context dependent manner [78]. Several tumor suppressive miRNAs are reported to target similar pathways or even the same genes, as for example ZEB1 and ZEB2 are targeted by miR-200 family members and miR-101 in ovarian cancer [113, 114, 126]. The ZEB proteins are interesting targets to investigate tumor suppressive miRNAs as they play a pivotal role in EMT transition and cell migration.

Two different methods are frequently applied to identify miRNAs with distinct function in malignant transformation. Most studies intensively focus on miRNA expression analysis, identify respective target genes and subsequently use functional assays to confirm predicted roles. Another approach is the identification of specific miRNAs based on their functional effect on cancer cells. Therefore a number of functional screenings were performed using sense and antisense miRNA libraries to investigate distinct miRNA induced effects on lung [52], breast [269], colorectal [188] and pancreas cancer [157]. In this respect, Levy et al. [201] performed a miRNA library screening to investigate the effect of miRNAs on melanoma cell invasion. miR-211 was found to be able to inhibit melanoma cell invasion significantly [201]. miR-211 is located intronically within the TRPM1 gene. Both, miR-211 as well as its host gene, transient receptor potential cation channel subfamily M member 1 (TRPM1) were found to be downregulated in metastatic melanoma, but only miR-211 overexpression could reduce the migrative potential of highly invasive melanoma cells. Surprisingly, increased TRPM1 expression did not exhibit the same effect on melanoma cells with low levels of miR-211 and TRPM1. Epigenetic blockade of the TRPM1 locus during melanomagenesis is

thought to favour tumor progression by additionally inhbiting the tumor supressor miR-211 [201, 226].

The miR-29 family was downregulated in leukemia [106] and several solid tumors including breast cancer [156], cutaneous melanoma [255] and hepatocellular carcinoma [371]. miR-29 family members are potent inducers of apoptosis in hepatocellular cell lines as they are downregulating MCL1 and Bcl-2 protein expression [371]. In melanoma miR-29c downregulation correlates with the methylation status during melanoma progression and favours abberant hypermethylation [255]. This was confirmed by the identification of two miR-29c target genes, the DNA (cytosine-5)-methyltransferases (DNMT) DNMT3A and DNMT3B which are important mediators to epigenetically regulate gene expression. It could be shown that miR-29c is significantly downregulated in advanced stage IV melanomas in comparison to primary melanoma which correlated with increased DNMT3B expression [255].

Rarely both miRNA duplex strands can be loaded into the RISC complex, leading to translational repression, mRNA deadenylation or degradation of target genes. This was shown for miR-126 and its complement miR-126*. In all vertebrates both miRNAs are encoded by the intron of the EGF-like domain-containing protein 7 (EGFL7) gene, a regulator of blood vessel formation [92, 352]. Comparing malignant tumor to non-malignant tissue, miR-126 was significantly downregulated or lost in lung [323], gastric [89], cervix carcinoma [352] and melanoma [88, 245]. In accordance, loss of its passenger strand miRNA, miR-126*, was observed in various cancer cell lines of the colon [125] and prostate [248]. Loss of miR-126/126* could be shown to induce melanoma progression, whereas restored expression of miR-126/miR-126* decreased neoplastic behaviour *in vivo* and *in vitro* [88]. miR-126/126* are potent tumor suppressor miRNAs as they inhibit cancer progression via various signaling pathways that control tumor progression, migration, invasion and survival, including inflammatory processes and angiogenesis [233, 92, 87]. However, detailed mechanisms and possible additional functions of miR-126/126* still have to be further investigated.

A highly investigated tumor suppressor in cancer research is p53. Interestingly, the expression of miR-34 family, miR-34a, miR-34b, miR-34c, was induced by p53 [326, 284]. Therefore, it does not seem surprising, that decreased levels of miR-34 are reported in lung [169], prostate [177, 53], pancreatic[160], gastric cancer [159] and melanoma[227]. Several target genes of the miR-34 family are known e.g. two anti-apoptotic genes, the silent information regulator 1 (SITR1) and Bcl-2 [376, 177]. Furthermore, important cell proliferation and differentation regulators, Notch and the stem cell marker CD44 [160, 159]. In p53 wild type melanoma the miR-34 family not only inhibits cell survival but also seems to inhibit melanoma cell invasion *in vitro* [378]. Overall, recent studies not only associate miR-34 expression with various non-

malignant processes, namely ageing, neuronal development, stem cell differentiation but also with decreased tumor growth and invasion *in vivo* [293, 261, 213, 140, 173, 174]. Members of the miR-34 family are one of the first miRNAs reaching the clinics as replacement therapeutic [14, 335].

3.3.3 miRNAs as biomarker

Prognostic and clinical biomarkers are widely investigated to clinically estimate tumor burden, tumor type, therapy response of patients or patients survival time. Markers assessed in melanoma patients are mainly important genes for tumor growth, enzymes for extracellular matrix degradation, angiogenic factors, molecules for cell Signaling , cell-cell interaction and several immunomodulatory molecules [296, 279, 146, 341, 50]. Necessary properties for molecules to be used as biomarkers are for example lineage specificity, tissue and fluid stability, which can be fulfilled by miRNAs as well [304]. Their stability would allow retrospective as well as longitudinal studies in patients to evaluate disease progression or even the onset of melanoma. For different cancer types including melanoma, miRNA profiles in the blood are already used to determine tumor burden [86, 96]. Furthermore, it was shown that histological differences of melanoma spreading, nodular or superficial spreading, can be assessed by changes in the miRNA expression profile [278, 112]. One study assessed the serum levels of miR-221 in melanoma *in situ*, stage I-IV patients, which were significantly increased in comparison to the control group [86, 167]. Additionally, Kanemaru et al. linked changes in miR-221 expression to disease burden, by analysing recurrent patients before and after primary excision [167]. Despite the fact that histological differences between melanoma cases were not considered, these results cannot be generalized but they hold promises for the prognostic value of miRNAs. The detection of early stage melanoma by changes in the miRNA expression profiles would be favorable to counteract tumor progression. In this respect, Leidinger et al. [197] reported a set of 16 deregulated miRNAs in the blood of melanoma patients in comparison to the healthy control group [197]. Nevertheless, the source of the measured miRNA profile has to be carefully evaluated, as it can often not be clarified whether the measured miRNA profiles reflect the situation of circulating tumor cells or the host's immunresponse [280, 298].

miRNAs could be shown to be stable in tissue samples irrespective if they are formalin-fixed paraffin embedded (FFPE) tissues or frozen [370, 207]. Therefore, retrospective studies have the potential to assess miRNA expression profiles of frozen material or FFPE tissue sam-

ples of primary melanomas [304]. Upon investigation of lymphnode samples of melanoma patients or tumor tissues from stage I-IV patients, deregulated miRNA expression profiles of either single miRNAs such as miR-29c [255], miR-193b [42] as well as of distinct sets of miRNAs (e.g. miR-150, miR-342-3p, miR-455-3p, miR-145, miR-155 and miR-497) correlated with clinical outcome [303, 304]. Overall miRNAs can predict patients' recurrence and clinical outcome however the detection of dysregulated miRNA profiles has to be optimized to assess accurately and reproducible miRNA expression levels as trustworthy risk biomarkers.

Recently a number of miRNAs were linked to suppressive effects on melanoma cell proliferation *in vitro.* Poell et al. performed a cell viability screening on a genome-scale with a lentiviral human miRNA expression library [277]. They showed that several miRNAs, including miR-16, miR-497, miR-141, miR-184, miR- 96 and miR-203, showed long-term (> 1 month) suppressive effects on melanoma cell proliferation *in vitro* [277].

miRNA expression profiles in patients with malignancies cannot only indicate oncogenic or tumor inhibitory miRNA candidates, they can also highlight treatment success or failure. Additionally, changes in the miRNA expression profile of cancer patients during treatment might indicate new therapeutic options. Recently, a study showed that targeted therapies as for example with Temsirolimus and Bevacizumab induce changes in the miRNA expression profile in melanoma tissues [349]. The combination treatment induced a significant upregulation of 12 miRNAs with reported tumor suppressive properties, therefore suggesting the inhibition of oncogenic pathways by this combinatorial treatment and miRNAs as potential indicators for successful therapy and patients' prognosis [349].

3.3.4 miRNA therapy

The discovery of molecular pathways involved in melanoma development and progression opened new opportunities for the development of targeted anti-melanoma therapies [111]. The number of clinical trials investigating miRNAs targeting a variety of regulatory proteins including kinases (Ras, Raf, PI(3)K), anti-apoptotic proteins (Bcl2- MCL1) or integrins is increasing constantly with partly encouraging results [26, 76, 79, 336, 375]. As increasing evidence shows that miRNAs can have potent functions as oncomirs or tumor suppressors, attention has been drawn to the development of miRNA based cancer therapies [15]. Unfortunately, most of the mentioned studies have been conducted only *in vitro.* Therefore, for most miRNAs with a potential oncogenic or tumor suppressive effect, their specific role in human malignancies *in vivo* is still lacking [304]. Recently, two strategies (Figure 5) for miRNA-

based anti-tumor therapy approaches are investigated. On the one hand the reintroduction of tumor suppressive miRNAs into the cells, the so-called miRNA replacement therapy is applied, or on the other hand the inhibition of oncogenic miRNAs using antagomirs [15, 367]. Several delivery systems are used to specifically transport miRNAs to the cells of interest. For systemic *in vivo* delivery these delivery systems need to be neutrally charged, of nanosize (10-100nm) and target specific [300]. Additional obstacles are the potential immunogenicity of naked, unmodified intravenously delivered miRNAs inducing cleavage by endonucelases and innate immunity activation [253, 191, 158]. Therefore, different materials and formulation methods are tested to shield miRNAs, for example lipid-based systems, nanoparticels, PEG-conjugated copolymers or modified cyclodextrins in combination with monoclonal antibodies as novel ligands [179, 300, 6]. Thus, possible interactions between these delivery particles and various serum components have to be taken into account and thoroughly investigated [361, 6]. In view of the possible induction of immune and inflammatory responses or oncogenic integration, viral delivery would be an alternative approach.

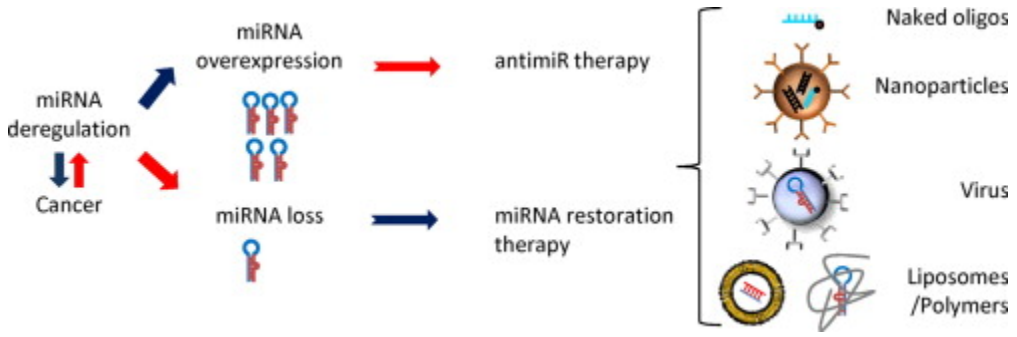

Figure 5: Two options for miRNA based therapeutics by either antimiR therapy or miRNA restoration therapy. Both are dependent on successful miRNA delivery strategies as for example naked oligos, nanoparticles viruses or within liposomes or polymers. Adopted from Segura et al. 2014 [304]

During several preclinical studies the feasibility of these approaches in the treatment of cancer has been explored. In the first preliminary *in vivo* study, antagomirs against miR-211/222 were tested and growth inhibition of xenografted melanomas in athymic nude mice were observed [86]. A more recent study investigated a spleen-to-liver melanoma metastasis mouse model after intraperitoneal injection of anti-miR-182 with modified oligonucleotides and a phosphorothioate backbone. In this model Huynh et al. observed a decreased number of liver metastasis in the treated compared to the control group [154]. They showed that

miR-182 levels were decreased and previously known targets of miR-182 were upregulated in the metastatic lesion of the anti-miR-182 treated mice. Furthermore, the feasibility of anti-miRNA treatment *in vivo* as a new therapeutic tool was proven [154].

Another approach was developed by Chen et al. [51], where liposome-polycation-hyaluronic acid nanoparticles tagged with a tumor-targeting single-chain antibody fragment (scFv) were designed that specifically delivered miRNAs or siRNAs to the site of interest [51]. With this system they could enhance the uptake of delivered small oligonucleotides into the tumor. These nanoparticles used in the study were delivering a potent cocktail of siRNAs (against c-Myc, MDM2 and VEGF) and miR-34a, resulting in inhibition of B16F10 lung metastasis [51].

The first clinical trials to use small oligonucleotides mostly focused on single therapies. More recently, combinations of miRNAs and siRNAs with already approved anticancer agents such as Bcl-2 inhibitors (e.g. ABT-263) are investigated. Lam et al. [188] described a synthetic lethal screening for 810 synthetic miRNAs in combination with ABT-263 in a colorectal cancer cell line. They identified 19 miRNAs to overcome ABT-263 resistance in this cell line by directly downregulating MCL1 [188]. This year another phase I clinical study will finish with primary liver cancer patients to assess the safety of MRX34, a synthetic mimic of miR-34, delivered in so far unpublished nanoparticles by liposomal injection [NCT01829971]. Previously, in pre-clinical studies the anti-tumor activity of miR-34a has been already shown [138, 335]. Also the miR-29 family targets members of the same signaling pathway, mainly MCL1 and Bcl-2 of the mitochondrial apoptosis pathway [371]. It has already been shown that intratumoral injection of miR-29b inhibited myeloid leukemia development of tumor xenografts [106].

Despite the promising results of miRNA replacement therapies or antagomir introduction, potential off target effects of miRNAs always have to be tested by bioinformatical prediction tools. Additionally, the development of miRNA-based therapies is still limited due to the high amount of putative, not identified targets of a single miRNA, complicating precise predictions for systemic miRNA delivery *in vivo*.

3.4 Aim of this study

In melanoma research it is of outermost interest to unravel mechanisms of cancer progression and metastasis. Metastatic melanoma has been shown to advance extremely fast often resulting in a poor patient prognosis. Melanoma cell dissemination, migration and invasion into distant organs of the body are important steps during disease progression. Some important checkpoints for melanoma progression are already known and clinically targeted, such as members of Ras/Raf/MEK/ERK or apoptosis signaling cascades. Only recently the role of miRNAs in malignant progression came into focus.

The aim of this study was to:

1. Identify miRNAs that influence melanoma cell invasion in a functional screening approach;
2. Validate these candidate miRNAs and investigate their role in melanoma cell invasion;
3. Identify and validate novel miRNA candidate target genes;
4. Access miRNA candidate effects on lung colonization *in vivo*.

Part 4

Materials and Methods

4.1 Materials

4.1.1 General instrumentation

Table 2: General instrumentation

Equipment	Manufacturer
ABI 7300 Real-time PCR System	Applied Biosystems, Foster City, CA
Biofuge Fresco Centrifuge	Heraeus, Hanau, Germany
Biological Safety Cabint	Heraeus, Hanau, Germany
BioPhotometer	Eppendorf, Hamburg, Germany
BioRad Mini-gel apparatus	Bio-Rad, Richmond, CA
CO_2 incubator	Binder, Tuttlingen, Germany
CASY1 Cell counter	Schaerfe System, Reutlingen, Germany
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
Pipetboy	Brand, Wertheim, Germany
Pipette (P2, P10, P100, P200, P1000)	Gilson, Bad Camberg, Germany
Power PAC 300 power supplier	Bio-Rad, Richmond, Germany
Refrigerator	Liebherr, Ochsenhausen, Germany
Sorvall RT7 Centrifuge	Sorvall, Newton, CT
Thermomixer	Eppendorf, Hamburg, Germany
Verti 96-Well Thermal Cycler	Applied Biosystems, Froster City, CA
Fluoroskan Ascent Microplate Fluorometer	Thermo Scientific, Dreieich, Germany
FACS Calibur Flow Cytometer	Becton Dickinson, Heidelberg, Germany
FACS Canto	Becton Dickinson, Heidelberg, Germany

4.1.2 General consumables

Table 3: General consumables

Material	Manufacturer
Falcon tubes 15ml, 50ml	Greiner, Frickenhausen, Germany
Pipette filter tips (10, 20, 100, 200, 1000µl)	Starlab, Milton Keynes, United Kingdom
Pipette tips (10, 20, 100, 200, 1000µl)	Greiner, Frickenhausen, Germany
Combitips (2.5, 5ml)	Eppendorf, Hamburg, Germany
Sterile serological pipettes (5, 10, 25ml)	Greiner, Frickenhausen, Germany
Safe-Lock tubes (0.5, 1.5, 2ml)	Eppendorf, Hamburg, Germany
Tissue culture flasks (25, 75, 150cm^2)	TPP, Trasadingen, Switzerland
Cell culture test plates, flat bottom (6, 12, 24 wells)	TPP, Trasadingen, Switzerland
Round bottom 96-well plates	TPP, Trasadingen, Switzerland
96-well Transwell plate	Corning Incorporated, Lowell, MA
96-well Reciever plate	Corning Incorporated, Lowell, MA
PCR strip tube	Greiner, Frickenhausen, Germany
MicroAmp Optical 96-well pate	Applied Biosytems, Foster City, CA
MicroAmp Optical adhesive film	Applied Biosytems, Foster City, CA
Petri Dishes	Greiner, Frickenhausen, Germany
Cryotubes	Greiner, Frickenhausen, Germany
Nitrocellulose membrane	Whatmann, Dassel, Germany
Needles (18G, 27G)	Becton Dickinson, Heidelberg, Germany
Inject-F, Syringes	Braun, Melsungen, Germany
Liquid reservoirs	Carl Roth GmbH, Karlsruhe, Germany
FACS tubes	Becton Dickinson, Heidelberg, Germany

4.1.3 General chemicals and reagents

Table 4: General chemicals

Equipment	Manufacturer
Trypsin/EDTA 10x	PAA Laboratories GmbH, Pasching, Austria
Phosphate Buffered Saline (PBS)	Biochrom AG, Berlin, Germany
Dimethyl sulfoxide (DMSO)	Applichem, Darmstadt, Germany
Agarose	Sigma, Saint Louis, MO

Tris Base	Sigma, Saint Louis, MO
Ammonium Persulfate (APS)	Sigma, Saint Louis, MO
Tetramethylethylendiamine (TMED)	Bio-Rad, Saint Louis, MO
Sodium dodecyl sulfate (SDS)	Sigma, Saint Louis, MO
Glycine	GERBU Biotechnik, Gaiberg, Germany
Tween20	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
Ethidium Bromide	Carl Roth GmbH, Karlsruhe, Germany
β-Mercaptoethanol	Sigma, Saint Louis, MO
Paraformaldehyde	Sigma, Saint Louis, MO
Tryptone	Sigma, Saint Louis, MO
Yest extract	GERBU Biotechnik, Gaiberg, Germany
Sodium Chloride (NaCl)	Sigma, Saint Louis, MO
Agar	Sigma, Saint Louis, MO
Crystal violet	Sigma, Saint Louis, MO
Leukosept	Greiner, Frickenhausen, Germany

Table 5: General reagents

Material	**Manufacturer**
Gene Ruler 100bp DNA Ladder	Fermantas, St. Leon-Rot, Germany
O´Gene Ruler 1kb DNA Ladder	Fermantas, St. Leon-Rot, Germany
6x Orange Loading Dye	Fermantas, St. Leon-Rot, Germany
Precision Plus Protein Standard	Bio-Rad, Richmond, CA
Cell Lysis Buffer	Cell Signaling Technology, Beverly, MA
Bio-Rad Protein Assay Reagent	Bio-Rad, Richmond, CA
Restriction enzymes (DpnI,PvuI, KpnI, NotI, XbaI)	Fermantas, St. Leon-Rot, Germany
FastAP Thermosensitive Alkaline Phosphatase	ThermoFisher Scientific, Schwerte, Germany
Trypsin/EDTA 10x	PAA Laboratoires GmbH, Pasching, Austria
DharmaFect 1 Reagent	Dharmacon, Lafayette, CO
BD Matrigel Basement Membrane Matrix	BD Biosciences, Bedford, MA

Cell Proliferation Reagent WST-1	Roche Applied Science, Mannheim, Germany
CellTiter Glo Reagent	Promega, Mannheim, Germany
BCIP/NBP Liquid Substrate System	Sigma, Saint Louis, MO

4.1.4 Plasmids

Table 6: Plasmids

Plasmid	**Manufacturer**
pLight Switch 3'UTR MCL1	Active Motif, La Hulpe, Belgium
pEZX-MR04	GeneCopoeia, Rockville, MD 20850 USA

4.1.5 Oligonucleotides

Table 7: Oligonucleotides

Name	**Manufacturer**
DNA primers	Eurofins, MWG GmbH, Ebersberg, Germany
miRNA mimics library	Sigma Aldrich, St. Louis, USA
siRNAs	Sigma Aldrich, St. Louis, USA
miRCURY LNA inhibitor	Exiqon, Vedbaek, Denmark
AllStars Hs cell death control siRNA	Qiagen, Hilden, Germany

Table 8: DNA primers for sequencing

Name	**Sequence 5'-3'**
pLS MCL1 3'UTR for	GGGAAGTACATCAAGAGCTTCGT
pLS MCL1 3'UTR rev	CCCCCTGAACCTGAAACATAAA
pLS mut #1 for	TCCCTGAGAGAAGCGTAAGAC
pLS mut #1 rev	CCTGGGATTGAGAGGTTGATG
pLS mut #2 for	CTGAGAGAAGCGTAAGACAAA
pLS mut #2 rev	TGGGATTGAGAGGTTGATGAATGG

4.1.6 Kits

Table 9: Kits

Material	Manufacturer
miRNeasy Mini kit	Qiagen, Hilden, Germany
TaqMan miRNA Reverse Transcription	Applied Biosystems, Foster City, CA
TaqMan miRNA 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
Rapid DNA ligation	Roche, Applied Science, Mannheim, Germany
Phusion High-Fidelity PCR	Finnzymes, Espoo Finnland
QuickChange Lightning Site-Directed Mutagenesis	Stratagene, La Jolla, CA
Luciferase Reporter Assay Systems	Promega, Madison, WI
CellTiter Glo Luminescencent Cell Viability Assay	Promega, Mannheim, Germany
Vybrant Apoptosis Assay Kit #4	Life technologies, CA, USA
QIAquick Gel extraction Kit	Qiagen, Hilden, Germany
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden, Germany
DNA isolation kit	Qiagen, Hilden, Germany
Effectene Transfection Reagent Kit	Qiagen, Hilden, Germany
ECL Plus Western blotting Detection System	GE Healthcare, Buckinghamshire

4.1.7 Antibodies

Table 10: Antibodies used for Western Blot analysis

Material	Manufacturer
anti-MCL1 monoclonal (sc819)	Santa Cruz Biotechnology, Heidelberg, Germany
mouse anti-β-actin monoclonal (#691001)	MP Biomedicals, Solon, OH

4.1.8 Cell culture

Table 11: Cell culture medium and supplements

Material	Manufacturer
RPMI 1640	PAA Laboratories, Pasching, Austria
αMinimum Essential Medium Eagle (MEM)	Sigma, Saint Louis, MO
Fetal Calf Serum (FCS)	PAA Laboratories, Pasching, Austria
Penicillin/Streptomycin (Pen/Strep)	PAA Laboratories, Pasching, Austria
DMEM	PAA Laboratories, Pasching, Austria
Lipopolysacharides (LPS)	Sigma, Saint Louis, MO
Glutamine	Gibco-Invitrogen, Karlsruhe, Germany

Table 12: Human cells

Cells	Sources
NHEMs from adult skin	PromoCell, Heidelberg, Germany
NHEMs from juvenile foreskin	Department of Surgery, University Hospital Heidelberg, Germany

Table 13: Melanoma cell lines

Cell line	Source	Cell line type
A375	ATCC	human metastatic melanoma cell [107]
MaMel-12	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-13	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-19	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-20	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-21	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-36	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells

MaMel-37b	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-38	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-5	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-51	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-53	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-53a	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-57	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-61e	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-68	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-73a	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-79b	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
MaMel-86b	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
UKRV Mel-17	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
UKRV Mel-21	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
WM 115	Division of Preventive Oncology, DKFZ, Heidelberg	human primary melanoma cells
WM 266.4	Division of Preventive Oncology, DKFZ, Heidelberg	human primary melanoma cells
WM 13.41	Skin Cancer Unit, DKFZ, Heidelberg	human melanoma cells
WM 98.1	Division of Preventive Oncology, DKFZ, Heidelberg	human melanoma cells

Table 14: Bacterial cells

Material	Source
TOP10 E.coli competent cells	Invitrogen, Carlsbad, CA

4.1.9 Software

Table 15: Software

Software	Source
Microsoft Office 2010	Microsoft, Redmont, USA
GraphPad Prism 5	GraphPad Software, Inc., San Diego, USA
Cell Quest	Becton Dickinson, Heidelberg, Germany
Leica Application Suite	Leica, Wetzlar, Germany
Ascent Software	Thermo Scientific, Dreieich, Germany
Adobe Illustrator	Adobe, San José, CA, USA
FlowJo	Becton Dickinson, Heidelberg, Germany

4.2 Methods

4.2.1 Preparation of buffers and medium

Table 16: TBS-T

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

Table 17: 50 x Tris-acetate-EDTA (TAE) buffer, pH 8.0, 1L

Component	Amount
Tris base	242 g
Acetic acid	57.1 ml
500mM EDTA solution	100 ml
H_2O	Adjust final volume to 1L

Table 18: 1 x PBS, pH 7.4, 1L

Component	Amount
PBS Dulbecco w/o CA^{2+}, Mg^{2+}	9.55 g
H_2O	Adjust final volume to 1L

Table 19: 10 x SDS-PAGE running buffer, IL

Component	Amount
Tris base	30 g
10% SDS solution	100 ml
Glycin	144 g
H_2O	Adjust final volume to 1L

Table 20: 1x Tris-buffered saline (TBS), pH 7.6, 1L

Component	Amount
Tris base	2.24 g
Acetic acid	57.1 ml
500mM EDTA solution	100 ml
H_2O	Adjust final volume to 1L

Table 21: Transfer buffer, pH 8.5, 1L

Component	Amount
Tris base	3 g
Glycin	17.5 g
Methanol	200 ml
H_2O	Adjust final volume to 1L

Table 22: Stripping buffer, pH 6.8, 100 ml

Component	Amount
0.5M Tris-HCL (pH 6.8)	6.25 ml
10% (v/v) SDS solution	20 ml
β-Mercaptoethanol	700 µl
H_2O	Adjust final volume to 1L

Table 23: Cell freezing medium

Component	Amount
FCS	90% (v/v)
DMSO	10% (v/v)

Table 24: LB medium, pH 7.5, 1L

Component	Amount
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
H_2O	Adjust final volume to 1L

Table 25: FACS buffer

Component	Amount
FCS	3% (v/v)
2% NaN_3	5 ml
PBS	Adjust final volume to 500 ml

Table 26: MACS buffer

Component	Amount
0.5M EDTA	4 ml
BSA	5 g
PBS	Adjust final volume to 1L

Table 27: Cell culture medium

Component	supplements
culture medium (RPMI 1640)	10% FCS superior

4.2.2 Cell culture

All malignant melanoma cell lines in this study were established from metastasis of patients with stage III or IV melanoma (Table 13) Biopsies were obtained from either a solid metastatic lesions or a malignant effusion after a patient's informed consent. A375 cell line was purchased from ATCC. WM-266-4 and WM-155 primary melanoma cells were kindly provided by Dr. Eva Frei, Division of Preventive Oncology, DKFZ German Cancer Research Center. Melanoma cell lines 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% CO_2 incubator, except the melanoma cell line WM-155, which was cultivated at 35°C in a humidified 5% CO_2 incubator.

4.2.3 Generation of a stable cell line overexpressing miR-339-3p

The melanoma cell line A375 was seeded in the appropriate amount in a 6-well plate. After 24h a confluency of 70% was reached and the cells were transfected with the clone pEZX-MR04 encoding for miR-339 and the green fluorescence protein (GFP) which allows to further select for GFP positive cells (Figure 6). In addition that vector encodes a puromycin resistance encoded, therefore successfully transfected cells would survive puromycin treatment. After 24h transfected cells were selected by adding 0.2ng/ml puromycin. Cells which were successfully transfected would show a GFP positive signal and would survive the applied selection pressure by puromycin. After 5 days only positively transfected cells remain to be expanded.

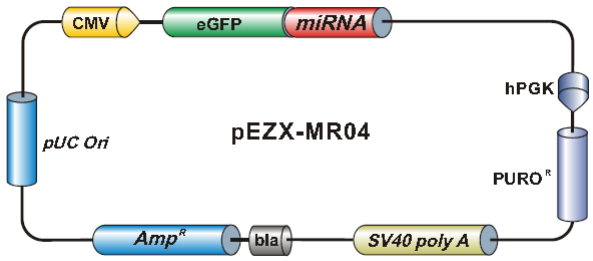

Figure 6: miR-339 expression vector: pEZX-MR04

4.2.4 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. The final RNA concentration was determined by NanoDrop, according to the manufacturer´s protocol.

4.2.5 Reverse transcription and qPCR

For the quantification of a specific miRNA, reverse transcription and qPCR was performed using TaqMan MicroRNA Reverse Transcription Kit and TaqMan miRNA assay (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

In a first step 40ng of total RNA was reverse transcribed in a 15µl reaction using specific stem-loop primers for mature miRNAs. In a second step 2µl of cDNA was used for the PCR amplification in 20µl reaction using the TaqMan Universal PCR Master Mixture kit. For quantitation of mRNA expression 500ng of total RNA was reverse transcribed in a 20µl reaction using oligo (dT)18 as primer and subsequently reverse transcription and PCR were carried out using Transcriptor First Strand cDNA Synthesis Kit and Taqman Gene Expression Assays. For the Taqman Gene Expression Assay, the cDNA was diluted 1:5, of what 2µl was used for a total volume of 20µl PCR reaction using the TaqMan Universal PCR Master Mixture kit. qPCR was performed in duplicates or triplicates as indicated. The thermal conditions are summarized in Table 28. 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 ΔCt-method according to the manufacturer's recommendation.

Table 28: 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	

4.2.6 Transfection of miRNA mimics, miRNA inhibitor 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 was performed the next day. 50nM of miRNA mimics, miRNA inhbitor or siRNAs were transfected into melanoma cells using DharmaFect 1 reagent according to the manufacturer's protocol. Medium was changed 6h or 24hr post transfection.

4.2.7 Site-directed mutagenesis

To introduce site-specific mutations into the pLS-plasmid containing the 3'UTR of MCL1 primers were designed with the program provided by Agilent Technologies (QuickChange

Primer Design). Site-directed mutagenesis was then performed according to the manufacturers protocol using the Quick Change Site-Directed mutagenesis kit. After successful PCR amplification of the mutated construct, it was transformed and amplified by Top10 bacteria and subsequently sequenced.

4.2.8 Transformation and amplification of cloned plasmid

Amplification of the construct was achieved by transformation of Top10 bacteria and subsequent growing of colonies on LB agar with ampicillin. Colonies were picked and used to inoculated 3 ml of LB-medium containing 100 µg/ml ampicillin. Positive clones were further expanded by using the Maxi-Prep Kit (Qiagen) accordingly to the manufacturers instructions. DNA concentration was determined by a BioPhotometer.

4.2.9 Luciferase reporter assay

Appropriate amount of cells were seeded into 96-well plates to achieve 40% - 60% confluency before transfection the next day. 50nM miR-339-3p and 50ng/µl pLS-MCL1-3'UTR were transfected into Melanoma cell lines using Effectene Transfection Reagent (Figure 7). 48hr after transfection, medium was removed and 20µl/well of Passive Lysis Buffer was added to lyse the cells followed by 15min incubation at room temperature. 100µl/well of Luciferase Assay Reagent II was added to quantify Renilla lucifease activity. Renilla luciferase ratio was calculated and further normalized to the negative control. Significance was tested by Student's t-test.

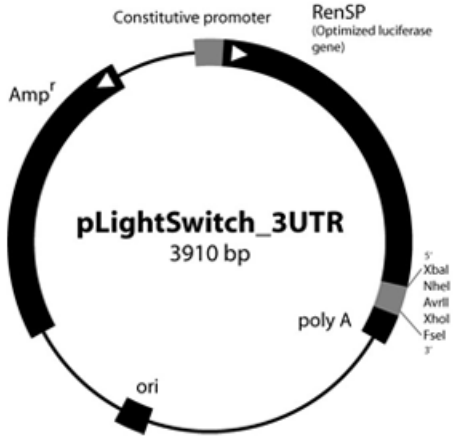

Figure 7: pLS-MCL1 3'UTR vector map

4.2.10 Protein detection by Western Blot analysis

Cell lysates were generated using appropriated amounts of the Cell Lysis Buffer (Cell Signaling) and incubated for 15min on ice followed by 25min centrifugation at 13,000rpm, 4°C. Protein concentration was determined by Bio-Rad Protein Assay reagent on a BioPhotometer. Cell lysates were stored at -20°C.

Heat denatured whole cell protein samples (15µg- 50µg) were mixed with 5 x loading dye and separated on a 10% polyacrylamid gel, and electro-transferred onto nitrocellulose membranes. Successful protein transfer was confirmed by Ponceau S staining of the nitrocellulose membrane. Before blocking of the membrane with 5% of non-fat milk in TBS-T, Ponceau S was washed away with TBS-T buffer. After blocking, the membranes were incubated with the respective primary antibody diluted in 0.5% non-fat milk in TBS-T buffer and left rotating at 4°C over night. Next, membranes were washed in intervals of 1 x 10min and 2 x 5min with TBS-T before incubated with the respective horseradish peroxidase conjugated secondary antibody diluted in 0.5% non-fat milk in TBS-T for 1h at RT. Following another interval of washing with TBS-T buffer, protein signals were detected using the enhanced chemiluminescence (ECL) system by a charged-coupled device (CCD)-camera. Densitometric quantification of specific bands was performed using ImageJ software.

4.2.11 Automated scratch assay

Melanoma cells were seeded into clear-bottom 96-well plates and transfected after 24h with the respective miRNAs or siRNAs indicated. After 48h the melanoma cells formed a dense monolayer, they were fluorescently stained with a live cell dye and the scratch was performed. The media was removed and the cells were washed once with PBS. Then the scratch was performed homogeneously and simultaneously in all 96-wells by a robot. Afterward the melanoma cells were washed again, media was added and the 0h time point of migration was imaged by fluorescence microscopy. After 24h a second fluorescence image was attained and the average migration width was determined. Significance was tested by Student's t-test.

4.2.12 96-well Boyden chamber assay

Invasion assay was performed using a Matrigel (50ng matrigel / well) coated 96-well transwell plate. Briefly, 48hr post transfection cells were seeded into the upper insert with serum-free medium and the lower chamber was filled with RPMI 1640 medium containing 10% FCS as chemo-attractant. Cells were kept at 37°C in a humidified 5% CO2 incubator. After 24h or 6h for the whole library screen, the invaded cells were washed twice with PBS, detached from the membrane b cell dissociation solution (CDS), fluorescently stained with calcein and measured with the Fluoroskan Ascent™ Microplate Fluorometer according to the manufacturer's protocol.

4.2.13 96-well Boyden chamber assay: Screening

During the screening of miRNA effects on melanoma cell invasion the matrigel invasion assay was performed in exactly the same fashion. $1x10^5$ A375 cells per well were seeded into a 96-well cell culture plate and transfected with the respective miRNAs after 24h. 48h posttransfection the cells were detached and three times one third was transferred into three inserts of a 96-well transwell plate resuspended in serum free media. The whole assay was therefore performed in technical triplicates for the invasion assay. After 6h the invaded cells were stained with calcein and the amount of invaded cells was determined with the Fluoroskan Ascent™ Microplate Fluorometer according to the manufacturer's protocol. Significance was tested by Dr. Tim Holland-Letz with a one way anova in the first step and a Dunnetts test in the second step.

4.2.14 Cell Viability Assay: CellTiter Glo

Cell viability was assessed using a luminescence CellTiter Glo (CTG) assay. 100µl CellTiter Glo reagent was added to 100µl RPMI media with 10% FCS per well in a 96well plate. The solutions were mixed for 2min on an orbital shaker and incubated for 10min in room temperature (RT) to stabilize the luminescence signal. Due to the occurring cell lysis a luminescence signal can be detected proportional to the ATP content present in each well. Therefore, the ATP amount measured is directly proportional to the amount of lysed cells in the well. The luminescence signal was measured with the Fluoroskan Ascent™ Microplate Fluorometer according to the manufacturer´s recommendation. Significance was tested with Student's t-test.

4.2.15 Apotosis assay

The apoptosis staining uses the features of YO-Pro. Apoptotic cells become permeable to it but remain impermeable to propidium iodide (PI), a dead cell stain. Live cells are not stained with YO-Pro, allowing them to be used in subsequent experiments. Melanoma cells were seeded in the appropriate amount in 24-well plates. After 24h they reached around 70% confluency and were transfected with the specific miRNAs and AllStars Hs Cell Death Control siRNA. 48h post transfection the cells were washed with PBS, detached and stained for apoptotic and necrotic cells. One µl of YO-Pro and/or PI stock solution was added to 1ml of cell suspension and incubated for 30min. The YO-Pro and PI signals were detected by FACS analysis.

4.2.16 Illumina gene expression profiling

For Illumina gene expression profiling two different melanoma cell lines, A375, MaMel-86b were transfected in biological triplicates with miR-339-3p. The cells were harvested 48h post transfection and RNA concentration was determined. In total six samples per cell line were subjected to gene expression analysis. Three independent biological triplicates either transfected with miR-339-3p or untreated were subjected to Illumina gene expression analysis. The assay itself and differential gene expression analysis was performed by the gene expression Core Facility of the DKFZ. In brief, they could group the data accordingly to the provided triplicates and differentially analyzed gene expression by comparing untreated melanoma cells with miR-339-3p transfected melanoma cells by Chipster analysis. Additionally, follow up data analysis and heatmap generation was also performed with the Chip-

ster software. To determine processes and signaling pathways differentially regulated after miR-339-3p overexpression a DAVID (Database for annotation, visualization and integrated discovery) enrichment analysis and subsequent MetaCore enrichment analysis in cooperation with Dr. Agens Hotz-Wagenblatt was performed for genes two fold differentially regulated both melanoma cell lines, A375, MaMel-86b. MetaCore enables the analysis of complex microarray genexpression data as it is based on databases containing transcription factors, receptors, ligands, kinases, drugs, and endogenous metabolites as well as other molecular classes to directly analyze species-specific directional interactions between protein-protein, protein-DNA and protein-RNA, drug targeting, and bioactive molecules and their effects on signaling and metabolic pathways represented on maps and networks or ontologies for diseases and processes with hierarchical or graphic output (MetaCore MetaCore Bioinformatics software from Thomson Reuters,https://portal.genego.com/).

4.2.17 Lung metastasis assay *in vivo*

Three different A375 melanoma cell lines were investigated for their impact on lung colonization in non-obese diabetic scid gamma (NSG) mice. NSG mice were injected *i.v.* with $1x10^6$ or $5x10^5$ A375 melanoma cells stably transfected with either the vector encoding for pre-miR-339, the empty vector or the parental A375 cells. The cells were harvested, washed three times in cold PBS and injected intravenously (*i.v.)* into ten mice per group. 14 days later the mice were sacrificed and metastasis formation in the lungs was investigated. Despite lung metastasis, hepatic colonization was observed. Therefore, metastasis in lungs and livers of all mice were counted. Significance was tested by Student's t-test.

4.2.18 Bioinformatic analysis

4.2.18.1 Statistical analysis of screening result

After the functional screening approach the effect of every single miRNA candidate displayed by its mean fluorescence intensity normalized to the mock control was logarithmized and analyzed for its significant effect on A375 cell invasion with the Dunnett's test displayed in Table S1. This statistical analysis was performed by Dr Tim Holland-Letz.

Furthermore, z-scores of all miRNA candidates were calculated to standardize the screening result. The z-score represents the number of standard deviations a individual sample value is different from the mean of the complete data set according to the following equation:

$$\mathbf{z = (x\text{-}\mu)/\sigma}$$

x represents the sample value (mean MFI)

μ represents the mean of the population

σ represents the standard deviation of the population

MFI values of every individual miRNA candidate were used to calculate the z-scores of all miRNA candidates and are represented in Table S2.

4.2.18.2 miRNA target prediction analysis

Most promising miRNA candidates were subjected to target identification databases, TargetScan and miRBase. Putative miRNA targets were subsequently subjected to the Database for Annotation, Visualization and Integrated Discovery (DAVID) for enrichment analysis [148, 149] and subsequent MetaCore (MetaCore by Thomson Reuters) analysis performed by Dr. Agnes Hotz-Wagenblatt for targets of miR-339-3p and miR-576-5p. These effects were classified into different signaling pathways mainly influenced by miRNA overexpression, respectively.

Part 5

Results

The aim of this thesis was to investigate the role of miRNAs in melanoma cell migration and invasion. In this respect a functional screening approach had to be established which can detect changes in melanoma cell migration or invasion as read-out.

5.1 The model system

To set up a functional screening approach a model cell line has to be selected, the most suitable assay and test its screening feasibility with robust positive and negative controls. Therefore, the first step was to investigate different properties of a panel of melanoma cell lines. They were tested for their proliferative capacity and cell viability in a 96-well format (Figure 8). Different cell lines with different cell numbers were seeded and the amount of viable cells was measured 48h post seeding Figure 8A. The amount of viable cells positively correlates with luminescence intensity. Most cell lines could be titrated, such as melanoma cell line A375, MaMel-38, MaMel-12 and MaMel-17 with the strongest proliferative potential. Several melanoma cell lines showed intermediate proliferative capacity, e.g. MaMel-20, MaMel-51, MaMel-57, MaMel-61 and MaMel-103b. Whereas, other cell lines, such as MaMel-21, MaMel-73a, WM 98.1 and WM 13.41 showed a low proliferative potential. Overall, differences in the amount and cell viability for all melanoma cell lines tested could be determined.

The melanoma cell line A375 is one of the most common melanoma cell lines used in melanoma research and performed reliably in all assays. Thus, its properties are well-known and widely investigated, which would be in favor for its usage in a functional screening approach. The ability to form metastasis *in vivo* might be of further interest for follow-up studies after the functional invasion assay. Therefore, an experimental tail vein assay for lung metastasis formation in non-obese diabetic scid gamma (NSG) mice mice was performed. $1x10^6$ cells were injected *i.v.* and metastasis in the lung could be observed 14 days later. 21 days post injection increasing numbers of metastases could be detected in the lungs as well as in the liver of these mice (Figure 8C, a, b, d, e). Additionally, the lymph nodes of these mice

were increased at day 21 post injection (Figure 8C, c). The lung metastasis formation was in accordance with already published results by Carreno et al. [43]. This proves the metastatic potential of the A375 cells that is important for a functional migration or invasion assay screening and the cell line A375 was chosen as model cell line for the functional screening approach.

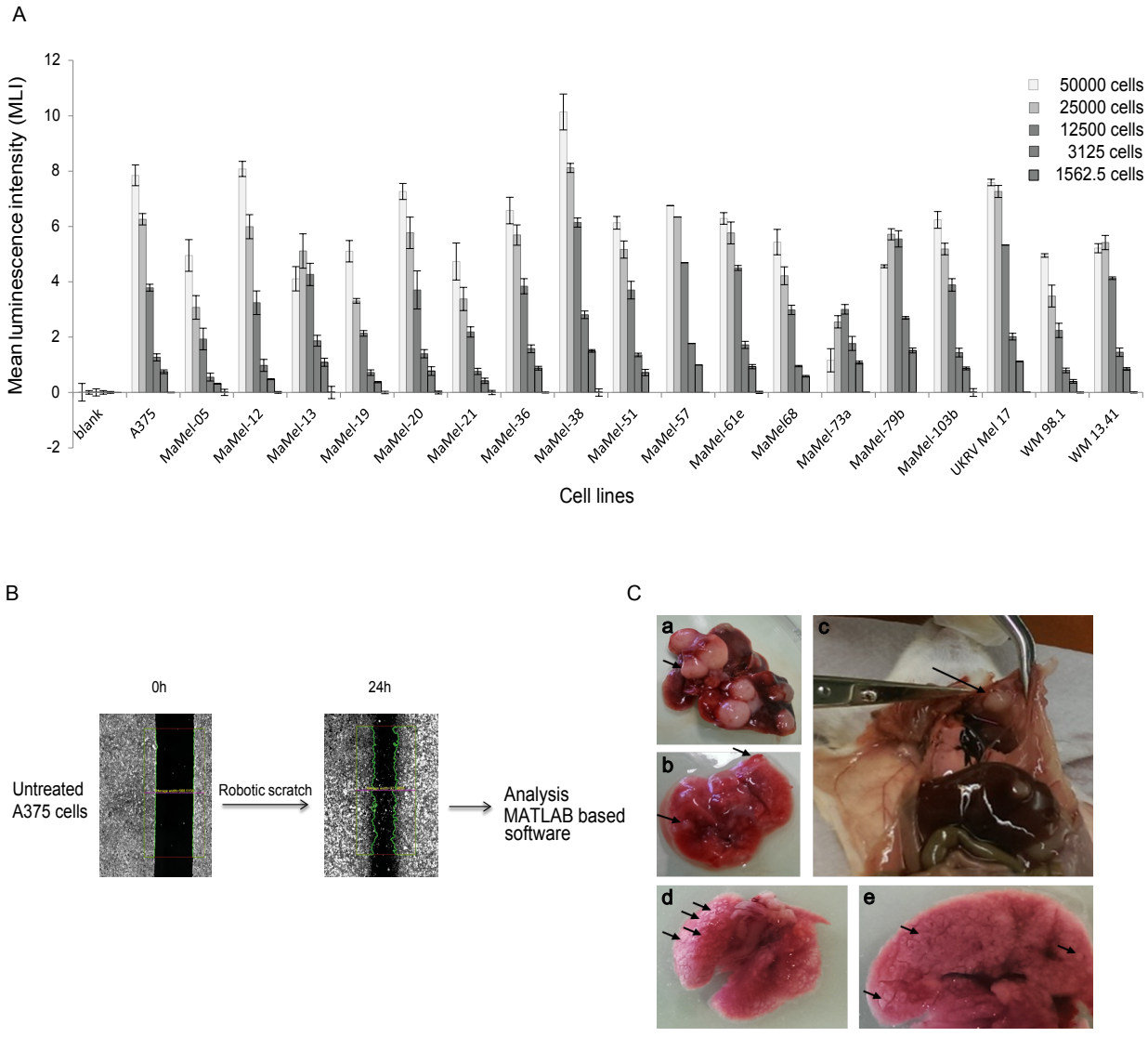

Figure 8: Characteristics of the melanoma cell line A375. **A**: Different melanoma cell lines were seeded with different cell density and cell viability was determined 48h post seeding. **B**: The Automated cell migration assay. The average scratch width (ASW) was determined after fluorescence staining of the living cells with a fluorescence microscope at the starting point (0h time point) and after 24h to attain the migration distance. **C:** The metastatic potential of A375 cells *in vivo* was tested in NSG mice. $1x10^6$ cells were injected *i.v.* and metastasis formation in lung and liver was investigated (a, b, d, e). 21 days post *i.v.* injection of the tumor cells; additionally, elevated lymph nodes could be detected (c).

In a next step we had to select a suitable migration assay. The assay of choice, was a high-throughput automated wound-healing scratch assay, was previously established in the lab of Prof. Dr. Stefan Wiemann [178, 382]. This assay allows one to determine changes in the migrative potential of melanoma cells after transient miRNA overexpression via fluorescence imaging.

For this reason, the model cell line A375 was seeded into a 96-well glass bottom plate. After formation of a dense cell monolayer the cells were fluorescently stained and a robot performed the scratch as displayed in Figure 8B (0h time point). Fluorescence images were taken at 0h and 24h post scratching to determine the average migration width of A375 cells. The scratch width was determined by an automated program [178,382] which identifies and marks the boarder of fluorescently stained cell monolayers.

Overall, the cell line A375 showed all important characteristics to be suitable for a scratch assay screening. It forms a homogenous cell monolayer, metastasizes *in vivo* and does migrate *in vitro*. In addition, A375 cells showed an intermediate migration potential after 24h which can still be increased or decreased during the screening. Consequently, the cell line A375 was chosen as model cell line for the functional screening approach.

5.2 miRNA library screening in A375 melanoma cells

5.2.1 Assay establishment

A screening approach requires the establishment of stable positive and negative controls to monitor and compare independent experiments. To control the whole screening procedure positive controls for inhibiting or accelerating melanoma cell migration have to be established. Different miRNAs and siRNAs were transfected 24h post seeding and 24h before the automated scratch was performed. The 0h and 24h time points were recorded by fluorescence microscopy and the average migration width was determined.

The siRNA against CSNK1G2 and all miRNAs, miR-182, miR-221 and miR-211 were influencing A375 cell migration. It was possible to accelerate A375 cell migration via miR-182 overexpression during 24h of migration. miR-182 and miR-206 transfected cells showed the strongest migrative potential compared to untransfected cells or cells transfected with miRNA control 1 (ctrl1) (Figure 9A, B). miR-221 and miR-211 as well as siRNA against EGFR,

CSNK1G2 and EZH2 did not show a significant difference to mock treated cells (Figure 9B). All possible positive controls tested in this assay could not alter the migration of A375 cells significantly during these 24h of migration. Therefore, no reliable assay control could be established and consequently another assay was considered which allows to investigate not only the migration but the more physiological invasion of melanoma cells.

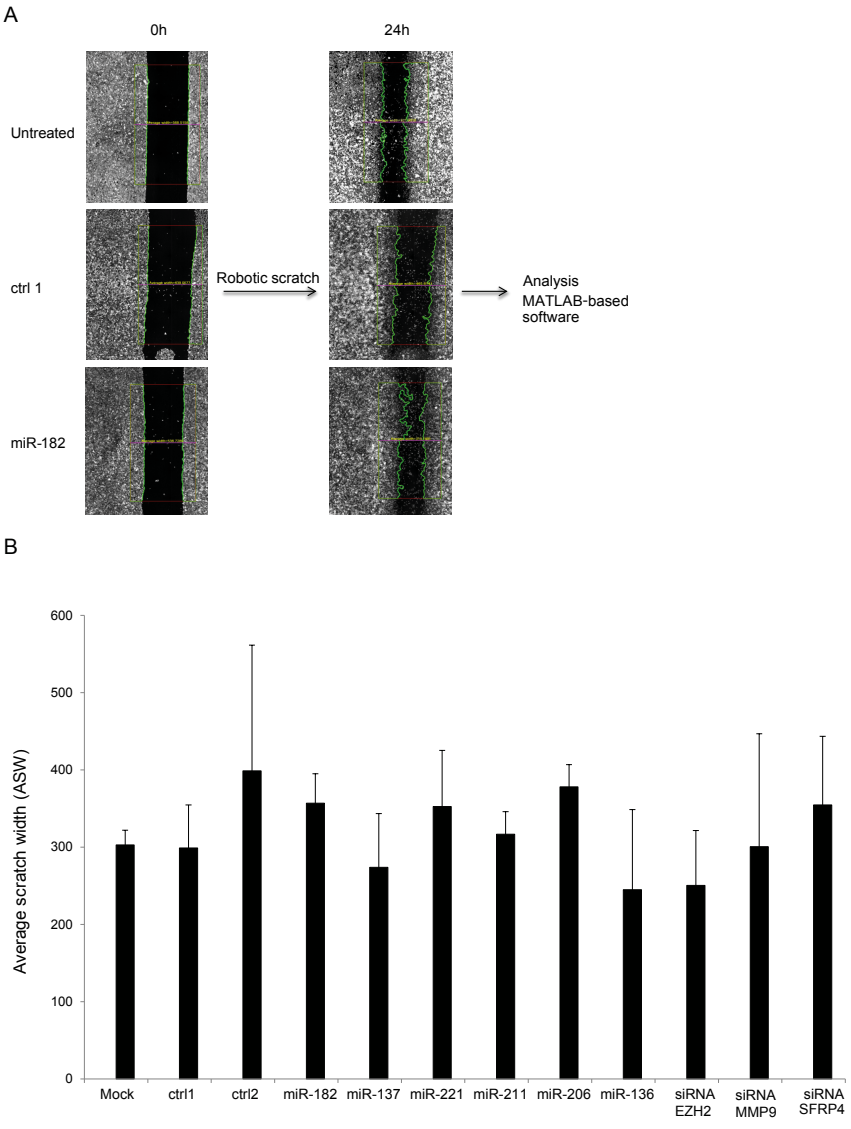

Figure 9: Migratory capacity of the cell line A375. **A**: The automated cell migration assay. The average scratch width (ASW) was determined after fluorescence staining of the living cells at the starting point (0h time point) and after 24h to attain the migration distance. **B**: The cell line A375 was transfected with different siRNAs or miRNAs which are thought to reduce migration, siRNAs against EGFR, MMP9 and EZH2 or miR-137, miR-136 and miR-206 or accelerate migration, siRNA against CDKN1G2 and miR-182 and miR-211. The mean ASWs of triplicates are represented in the bar graph after 24h of migration to compare changes in the migration distance for every siRNA or miRNA treatment.

The assay of choice was the 96-well Boyden chamber assay. In this assay viable melanoma cells are seeded into the upper Boyden chamber inserts in serum free media, whereas media with 10% in the lower chamber serves as a chemoattractant. After a certain amount of invasion time the invaded cells are detached and fluorescently stained with calcein which allows a direct fluorescence measurement of the invaded cells. The strength of the fluorescence signal thus directly indicates the amount of invaded cells.

To establish the assay, in a first step different cell numbers (Figure 10A) of the A375 cells were seeded into each of the 96-well Boyden chamber inserts and different invasion times (Figure 10B) were used to determine the best combination. It was important that increased as well as decreased cell number can still be detected later during the screening procedure. $5x10^4$ cells per insert and an invasion time of 6h could be determined as the best combination as indicated by the highlighted white bars (Figure 10A, B). As positive controls a panel of six miRNAs and siRNAs was tested: miR-182, miR-101, miR-221, miR-211, siRNA against MMP9 and siRNA against EZH2. A375 cells transfected with miR-182 showed a significant increased invasive potential compared to mock or ctrl1 transfected cells (Figure 10C, purple). Therefore, miR-182 was selected as positive control for increased invasive potential for the functional screening assay. miR-101 could be validated as positive control for decreasing invasive potential of A375 cells (Figure 10C, green). Both positive controls are highlighted in color in Figure 10C. With a stable assay and a suitable cell line the next step was to standardize the screening procedure to minimize variances between different experiments. The standardized work-flow used during the whole screening procedure is presented in Figure 10D.

For the functional invasion screening A375 cells were transfected with the two validated positive controls, miR-182, miR-101, two irrelevant controls ctrl1 and ctrl2 (miRNA control 2) as well as mock 24h post seeding. After additional 24h the medium was exchanged and 48h post transfection the cells were detached and transferred into the matrigel coated 96-well Boyden chamber inserts. Six hours later the invaded cells were detached from the membrane of the insert, fluorescently stained and the fluorescence intensity was measured. This protocol was stringently followed during the whole screening procedure. Every miRNA mimic of the library was tested in functional triplicates and all five controls (miR-182, miR-101, ctrl1 miRNA, ctrl2 miRNA and mock) were present in biological replicates and functional triplicates on every screening assay plate.

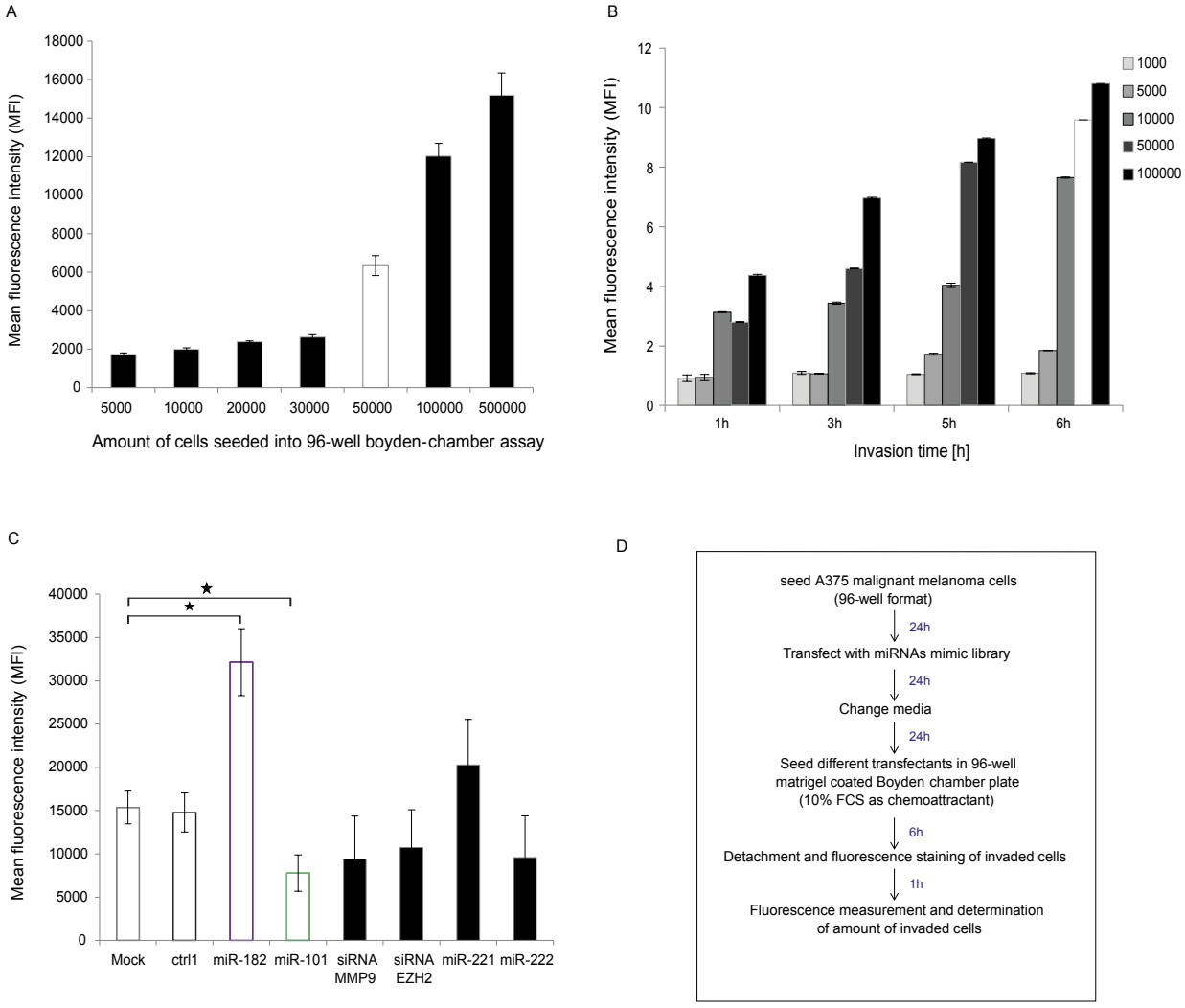

Figure 10: Setting up a functional screening approach for melanoma cell invasion. A375 cells were investigated for their invasive capacity. At different cell amounts after 6h of invasion **(A)** were seeded into the 96-well Boyden chamber wells and the amount of invaded cells was determined by fluorescence staining of living cells with calcein AM at different time points **(B)**. **C**: Different miRNAs or siRNAs were investigated for their effect on A375 cell invasion. Cells were seeded in a 96-well plate, transfected 24h post seeding and transferred into the 96-well Boyden chamber assay plate 48h post transfection. After 6h of invasion the invaded cells were fluorescently stained and measured. **D**: Workflow of the 96-well Boyden chamber assay screening procedure. The selected cell number and time of invasion is represented in white bars **(A, B)**, the selected controls are represented in color (**C**: purple accelerating control, green inhibitory control). Comparisons significant at the 0.05 level are indicated by asterisks (*).

5.2.2 microRNA library screening using the functional invasion assay

The miRNA mimics library consists of 988 miRNA mimics which were directly used to transfect A375 cells in a 96-well format. $5x10^4$ A375 cells were seeded into each well of the 96-well plate and transfected with 50nM miRNA mimic of the library or with one of the respective positive or negative controls. 24h later the medium was exchanged and after additional 24h the cells were detached and transferred into the 96-well Boyden chamber inserts. Every transfection was split into three 96-well Boyden chamber inserts to attain functional triplicates. After 6h of invasion through a matrigel coated membrane directed towards FCS as chemoattractant the invaded cells were simultaneously detached, fluorescently stained and the fluorescence intensity was measured to determine the amount of invaded cells (Figure 10D).

In Figure 11A the whole screening result is presented. Every bar represents the effect of one miRNA mimic on A375 cell invasion in functional triplicates normalized to mock control transfected cells on every individual assay plate. The higher the measured fluorescence intensity the more cells invaded through the matrigel coated membrane. On the left hand side of the waterfall plot miRNA mimics are represented which induced a high fluorescence signal, therefore had an accelerating effect on A375 melanoma cell invasion. miRNA mimics located on the right showed the opposing effect, an inhibiting effect on A375 cell invasion (Figure 11A, Supplement Table S2). The effect of every single miRNA candidate displayed by its mean fluorescence intensity normalized to the mock control was logarithmized and analyzed for its significant effect on A375 cell invasion with the Dunnett's test by Dr. Tim Holland-Letz. The result is displayed in the supplement, Table S1, and summarized in Figure 11B. 50% of miRNA candidates did not show a significant effect on A375 cell invasion in this functional screening approach. 37% increased A375 cell invasion significantly and 13% significantly decreased the invasive capacity of this melanoma cell line. Subsequently, only miRNA candidates which changed melanoma cell invasion significantly compared to the irrelevant control miRNAs transfected melanoma cell invasion after statistical analysis were further considered for investigation according to Table S1.

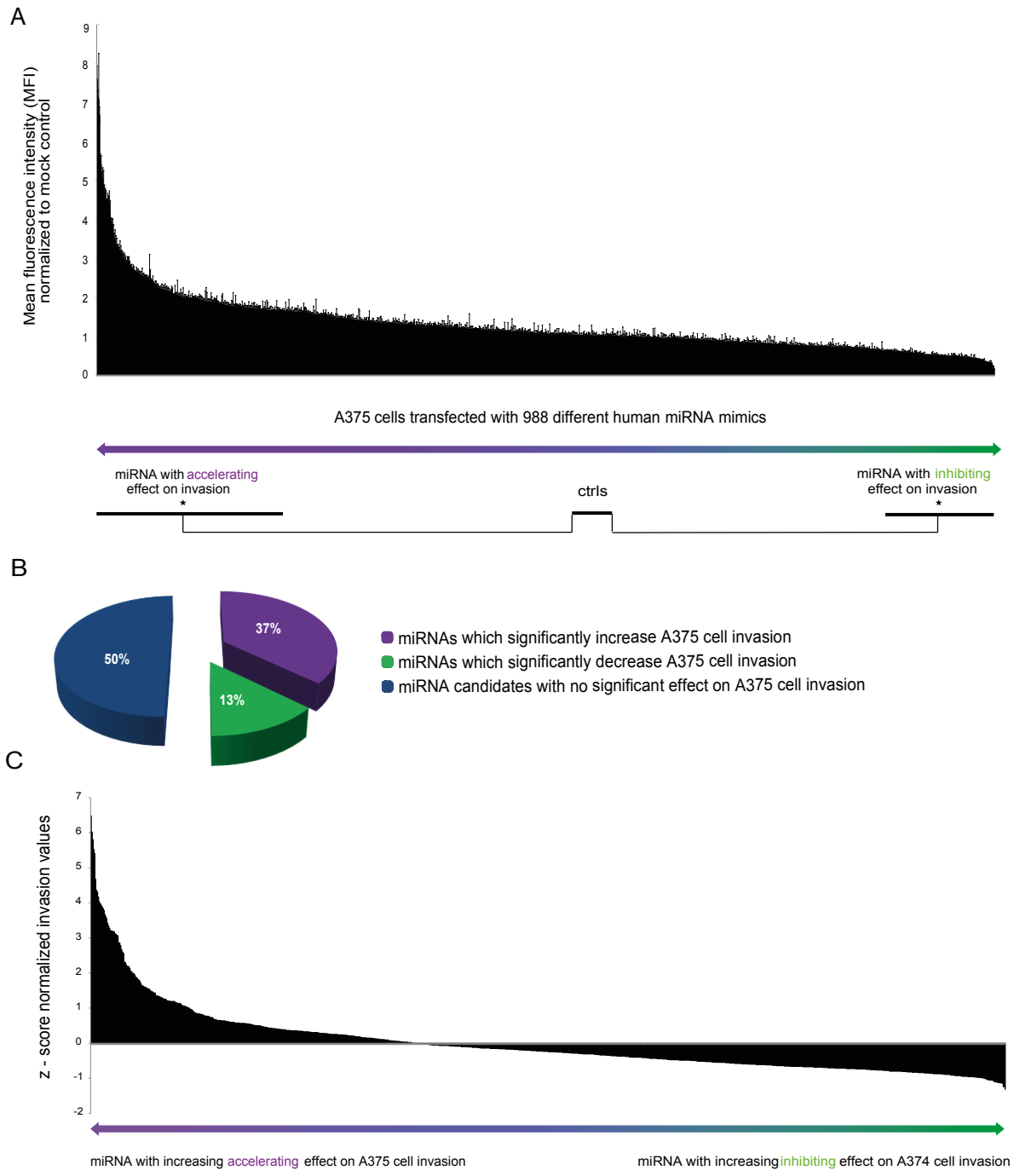

Figure 11: Result of the functional invasion assay screening with the melanoma cell line A375. **A**: 988 miRNA mimics were investigated for their effect on A375 cell invasion in technical triplicates. The invaded cells for every miRNA treatment were fluorescently stained and are given as the mean fluorescence intensity +/- STD. All individual plates were normalized to the mock control present on every 96-well Boyden chamber plate. **B**: The miRNAs which showed an either significant accelerating effect on invasion (37%) or a significant inhibiting effect on invasion (13%) were determined by the Dunetts test. **C**: The z-scores for all 988 miRNA mimics were calculated to display the number of standard deviations a data point is from the mean. Comparisons significant at the 0.05 level are indicated by asterisks (*).

The represented z-score was calculated for every miRNA triplicate individually (Figure 11C). The z-score represents the distance in standard deviations of a sample from the mean. In this case it represents the difference of the mean of one miRNA candidate to the mean +/- standard deviation of the whole data set. Therefore, the stronger the effect of the miRNA candidates the higher, for miRNAs with positive effect on A375 cell invasion, or lower, for miRNA candidates with negative effect on A375 cell invasion, is the z-score (Figure 11C).

Several miRNAs included in our miRNA mimics library are known in literature to play a role in melanoma or cancer cell invasion. Some miRNAs could be verified in our functional screening and showed significant effects on A375 cell invasion according to their published function (Figure 12A).

The z-score for 15 different miRNAs are displayed in Figure 12A. Among these 15 miRNAs nine miRNAs are shown with reported function on cancer cell invasion. The two negative control miRNAs, ath-miR-416 and cel-miR-243 are displayed in the center. Above miRNAs with negative z-score and therefore, an inhibiting effect on A375 cell invasion are shown and below the miRNAs with accelerating effect on A374 cell invasion during the functional screening. The two miRNA candidates with the strongest effect during the screening assay are displayed on both edges of the graph, miR-576-5p and miR-325. For representative reasons also the two positive controls of the invasion screening approach are displayed (Figure 12A, filled bars).

All miRNAs shown in Figure 12A are significant for their effect on A375 cell invasion in comparison to the negative controls, supporting the aim of this project to identify new miRNAs with potential effect on melanoma cell invasion. The 50 miRNA candidates with the strongest effect to accelerate or inhibit A375 cell invasion are shown in Figure 12B and C. All these miRNAs show a significant effect on A375 cell invasion and would be therefore interesting candidates for further analysis.

The complete list of 988 miRNAs and their effect on A375 cell invasion can be found in Table S2. In addition, all miRNA candidates with their significant effect on A375 cell invasion analyzed by Dr. Tim Holland-Letz are listed in the Table S1. Overall, this result shows that the 96-well Boyden chamber assay can be used in a functional screening approach to investigate differences in the invasive potential of a distinct cell population. The assay could be shown to be a reliable method to investigate the invasive behavior of different melanoma cell lines, however the possible effects of specific miRNA candidates on cell viability have to be taken into account.

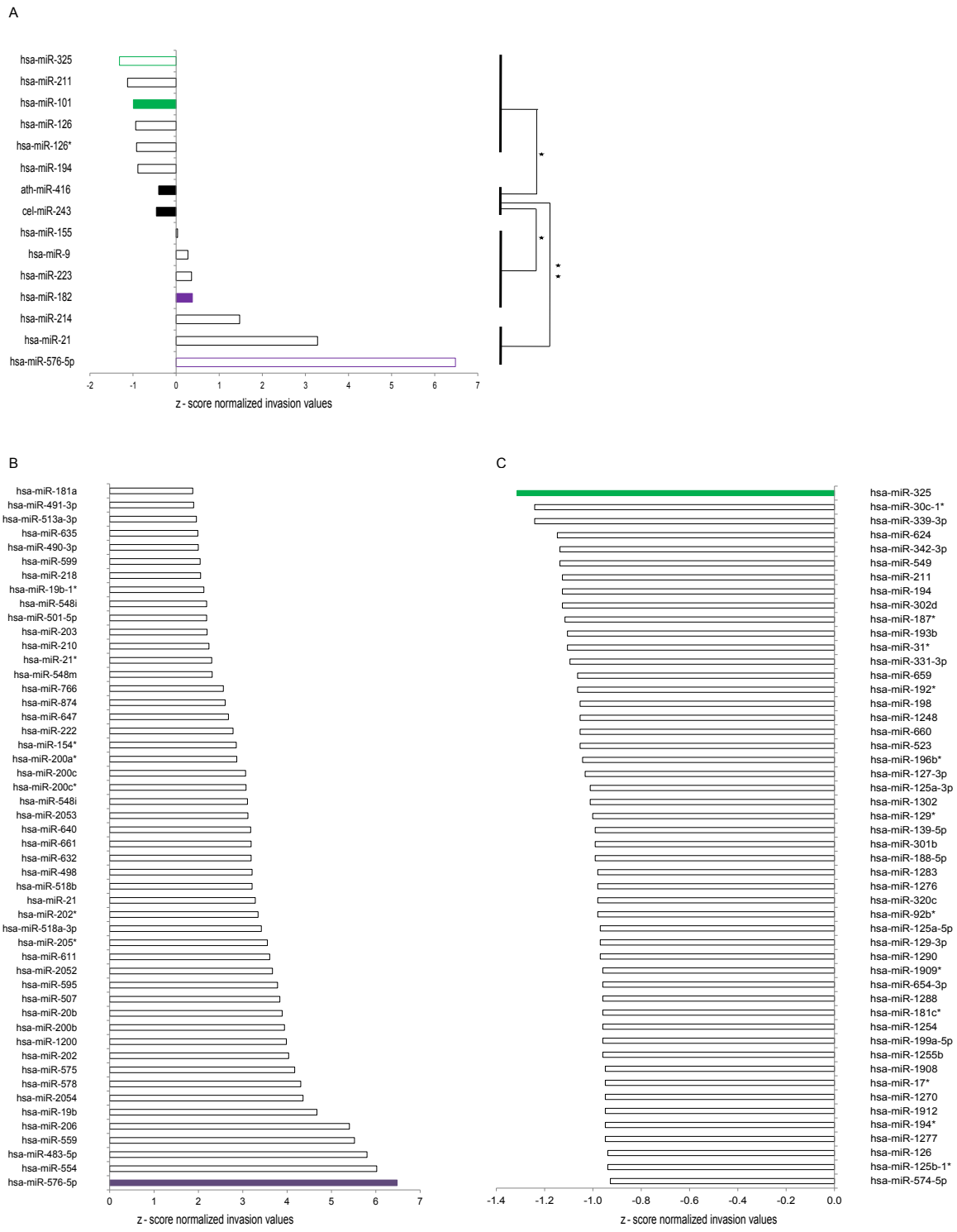

Figure 12: miRNA candidates with significant increase or decrease of A375 cell invasion. **A**: miRNAs already published for their role in cancer cell invasion which could be confirmed in this functional screening assay. **B**: The 50 strongest miRNA candidates which showed a significant accelerating effect on A375 cell invasion **C**: The 50 strongest miRNA candidates which showed a significant inhibiting effect on A375 cell invasion. The control miRNAs are displayed with filled bars in the respective colors, cel-miR-324 (ctrl1), ath-miR-416 (ctrl2) in black, miR-101 in green and miR-182 in purple. The miRNA candidates considered for further investigation follow the same color scheme. Comparisons significant at the 0.05 level are indicated by asterisks (* $p<0.05$, ** $p<0.01$).

The validation and further candidate selection following the screening is presented in Figure 13. The strongest miRNA candidates were investigated for their putative impact on melanoma cell viability. Additionally, miRNA candidates were further investigated with an independent invasion assay using several melanoma cell lines. In these assays, cell numbers were determined before starting a 24h invasion phase. Subsequently, miRNA candidates with a validated effect on melanoma cell invasion and no effect on melanoma cell viability were bioinformatically characterized for their putative target genes. Furthermore, these miRNA candidates were further characterized for their expression profile in normal human melanocytes in comparison to several melanoma cell lines (Figure 16). This approach identifies the most promising miRNA candidates for additional functional assays.

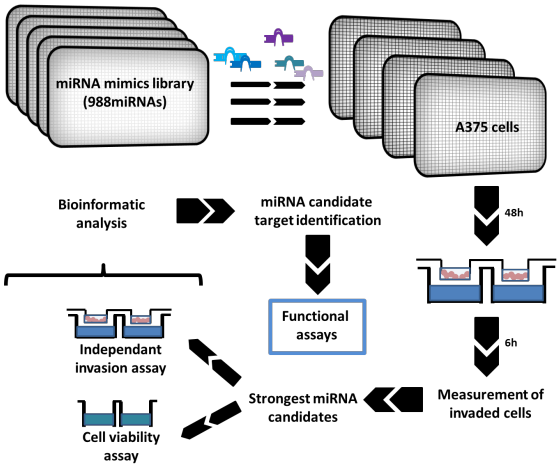

Figure 13: Functional invasion assay screening work flow: The outline of the screening, the validation phase with independent invasion assay and cell viability assays and the identification and follow-up of specific miRNA candidates in functional assays.

5.3 Cell viability effects of specific miRNA candidates

While miRNA effects on cell viability may affect the outcome of the invasion assay, it was not possible to test these simultaneously during the screening. Therefore, the 97 strongest miRNA candidates were further investigated for their effects on A375 melanoma cell viability. CellTiter Glo (CTG) assay was performed in a 96-well format for 48 miRNA candidates with inhibiting effect on melanoma cell invasion and 49 miRNA candidates which were capable of accelerating A375 cell invasion. To investigate any cell viability effects that could possibly

influence the screening result the same time intervals were used. A375 cells were transfected 24h post seeding and consequently 48h post transfection the effect on cell viability was measured (Figure 14).

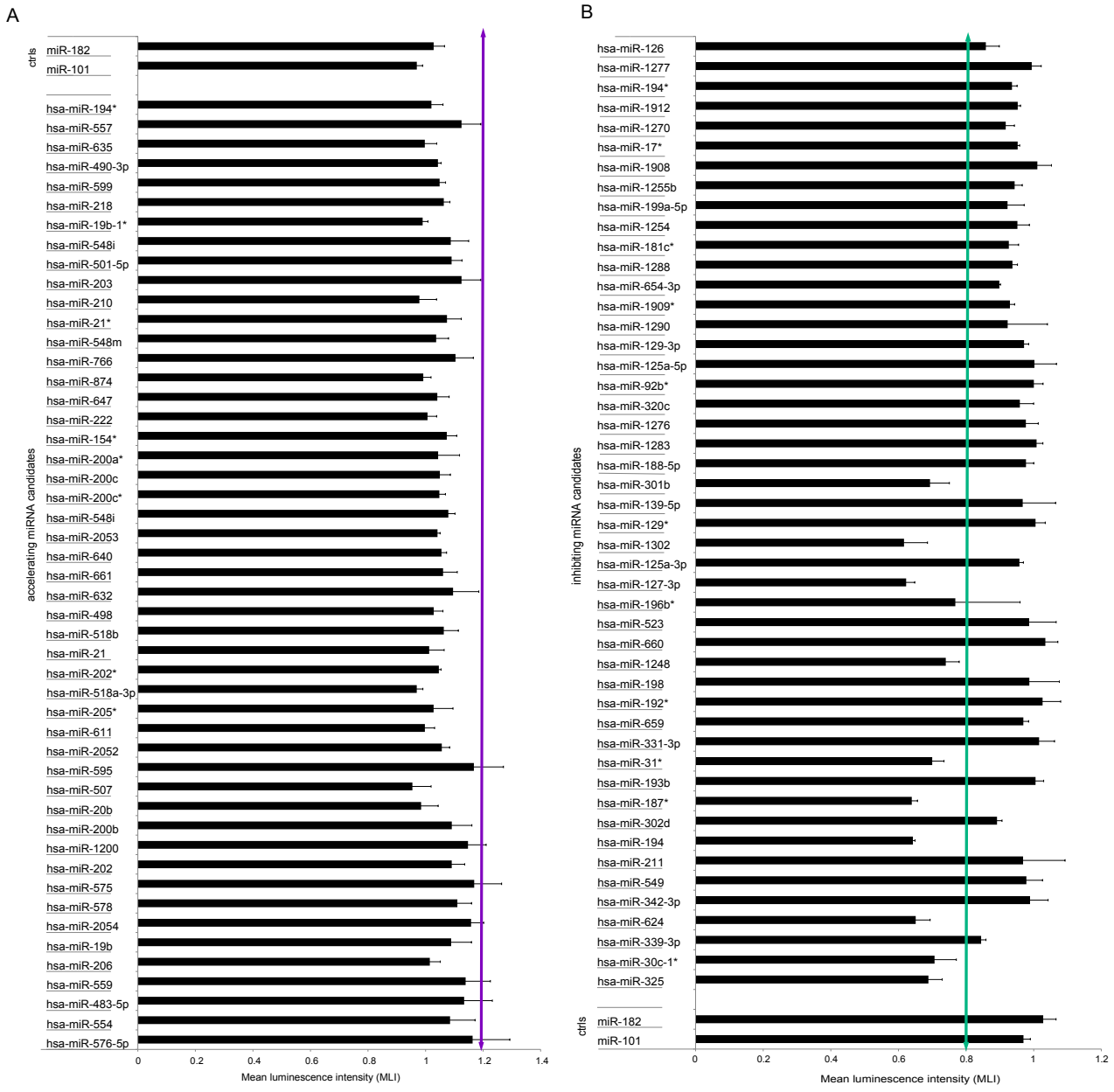

Figure 14: Cell viability assay for the 98 most effective miRNA candidates accelerating **(A)** or inhibiting **(B)** A375 invasion. A375 cells were seeded into 96-well plates and transfected with the respective miRNA mimics after 24h. 48h post transfection the number of viable cells in culture was determined based on the quantification of the ATP present, as an indicator of metabolically active cells resulting in a luminescence signal. The green and purple lines indicate a more than 20% decrease or increase on A375 cell viability, respectively. Data are shown as mean +/- STD of biological triplicates.

As it can be seen in Figure 14 some miRNA candidates affected A375 cell viability. For example miR-1302, miR-127-3p, miR-187*, miR-194, miR-624, miR-30c-1* and miR-325 showed a more than 20% decreased A375 cell viability (green line). Within the miRNA candidates accelerating the A375 invasive potential some slightly enhanced cell viability, as for example miR-576-5p, miR-575 and miR-595-5p. This observed influence on cell viability has to be considered for further investigation of the respective miRNA candidates. All these candidates are within the strongest candidates to decrease or increase A375 cell invasion during the functional screening. This indicates, that for some miRNA mimics, the observed decrease or increase of A375 invaded cells might not be solely due to changes in the number of invaded cells, but might also be influenced by effects on cell viability.

To further clarify miRNA effects on A375 cell viability altering the screening result, the invasive potential observed during the screening procedure was normalized to detected changes in cell viability for 98 miRNA candidates. miRNA candidates with inhibiting or accelerating effect on A375 cell invasion which could only be explained due to changes in cell viability would reach values around one, as it can be seen for ctrl1 transfected cells in Figure 15. If the effects on A375 cell viability were stronger than the effects on cell invasion the values would range below one for miRNA candidates accelerating A375 cell invasion and above one for miRNA candidate inhibiting A375 cell invasion (Figure 15).

As it can be seen in Figure 15, miRNA candidates inhibiting melanoma cell invasion reach normalized values between 0.2 and 0.7, whereas in the case of miRNA candidates accelerating A375 cell invasion the values lie above 1.6. The overexpression of several miRNA candidates with a potential inhibiting effect on A375 cell invasion show cell viability effects after overexpression of the respective miRNA candidate, e.g. miR-301b, miR-1302 and miR-127-3p affected A375 cell viability in this setting. However, this effect alone can not explain the strong decrease of invasion that could be observed during the invasion assay screening (Figure 15). Also the positive control miR-101 and additional candidate miRNAs, miR-1248, miR-31*, miR-187* and miR-194 slightly affected A375 cell viability.

None of the miRNA candidates accelerating A375 cell invasion showed indications for a strong effect on melanoma cell viability, that could solely explain the invasion assay screening result (Figure 15B).

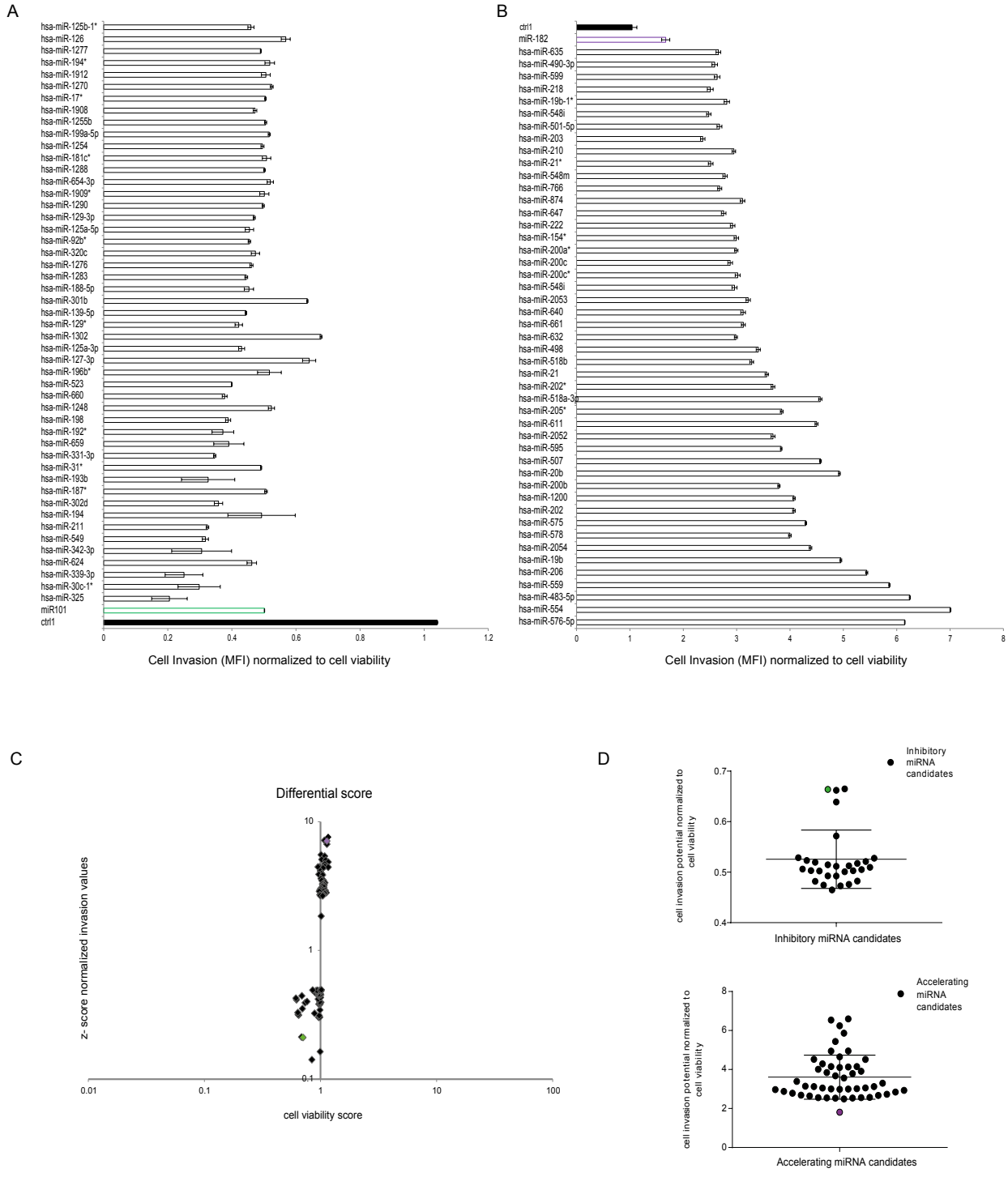

Figure 15: Cell viability effects influence the observed cell invasion but do not explain the observed effects on cell invasion. **A, B**: Cell invasion effects of 98 most promising miRNA candidates in relation to their influence on A375 cell viability. **A:** miRNA candidates inhibiting or **(B)** accelerating melanoma cell invasion. Data represent the mean of triplicates +/- STD. **C**: The of z-score of the invasion screen in relation to the cell viability score is represented by the differential score for each miRNA candidate. **D**: Distribution of effects for every single miRNA candidate. The assay positive controls, miR-101 and miR-182 are shown in the respective color.

In addition, the differential score was determined for all 98 miRNA candidates. The differential score represents the z-score of every single miRNA candidate to its cell viability score (Figure 15C) and the accumulated results are represented in two dot plots (Figure 15D). The lower the effect of the individual miRNA on A375 cell invasiveness, the closer the values allocate around one. Two populations can be observed for miRNA candidates inhibiting A375 cell invasion. One population of 24 miRNA candidates around the value 0.5 representing miRNA candidates which showed a strong inhibition effect on melanoma cell invasion and only a slight effect on cell viability. The second population represents three miRNA candidates, miR-301b, miR-1302 and miR-127-3p, which reach closer to one due to slight effects on cell viability and their lesser, but still significant effect on A375 cell invasion (Figure 15A). miRNA candidates accelerating melanoma cell invasion are represented by one homogeneously distributed population (Figure 15B, D) distributed according to their strength during the functional invasion assay screening.

For all miRNA candidates the effect on invasion observed during the screening could not exclusively be explained by their influence on cell viability. Therefore, all 98 miRNA candidates were considered for further investigation.

5.4 miRNA candidate expression profiling

5.4.1 Expression of miRNA candidates in melanoma cell lines compared to NHEMs

The possibility of introducing artificial effects always has to be considered in miRNA overexpression experiments. Therefore, the endogenous expression of seven selected miRNA candidates was validated in 16 different melanoma cell lines (MM) and seven normal human melanocytes (NHEMs). These miRNA candidates represent candidates to either accelerate, miR-576-5p, miR-559-5p and miR-483-5p or inhibit A375 cell invasion. As shown in Figure 16, significant different expression levels were observed between melanoma cell lines and NHEM in case of miRNAs, miR-339-3p. miR-30c-1* and miR-193b. However, only a very low expression level of miR-193b could be detected. miR-339-3p and miR-30c-1* displayed significant higher expression in NHEMs compared to melanoma cell lines and showed a higher overall expression in comparison to miR-193b.

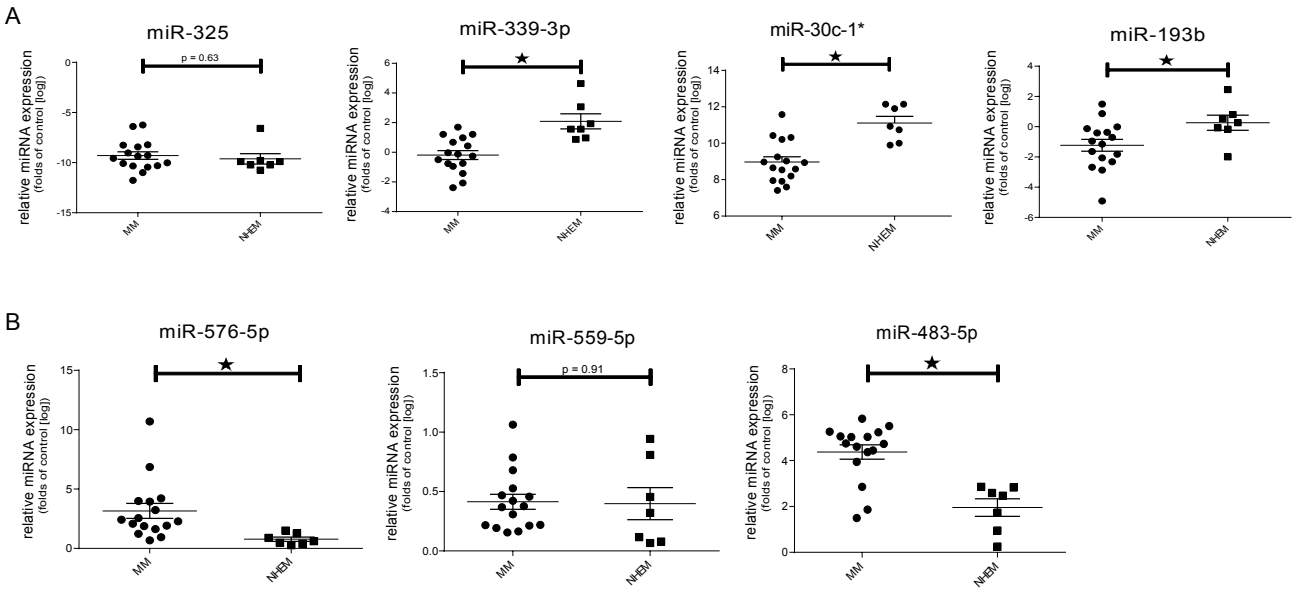

Figure 16: Profiling of seven miRNAs in 16 different human melanoma cell lines (MM) and 6 normal human melanocytes (NHEM). **A:** Four miRNA candidates inhibiting A375 cell invasion **B:** Three miRNA candidates to accelerate miRNA invasion during the functional invasion assay screening. Expression was normalized to the internal control RNU6B. The horizontal line shows the average expression level in each group. Comparisons significant at the 0.05 level are indicated by an asterisk (*).

For the miRNA candidates with accelerating effect on A375 cell invasion (Figure 16B) a significant difference between melanoma cell lines and NHEMs could be observed for miR-576-5p and miR-483-5p, but not for miR-559-5p. Both miRNA candidates are among the five strongest accelerators of A375 cell invasion according to the screening result (Figure 12B).

5.4.2 Correlation of miRNA expression and invasive capacity of different cell lines

In order to correlate the expression levels of candidate miRNAs and the invasive potential of melanoma cell lines a set of experiments was performed. Initially, the invasive capacity of 22 cell lines was analyzed using the Boyden chamber assay. Cell lines differed substantially in their invasive behavior in this assay ranging from very low, MaMel-79b, to extremely high, MaMel-19, invasion levels (Figure 17).

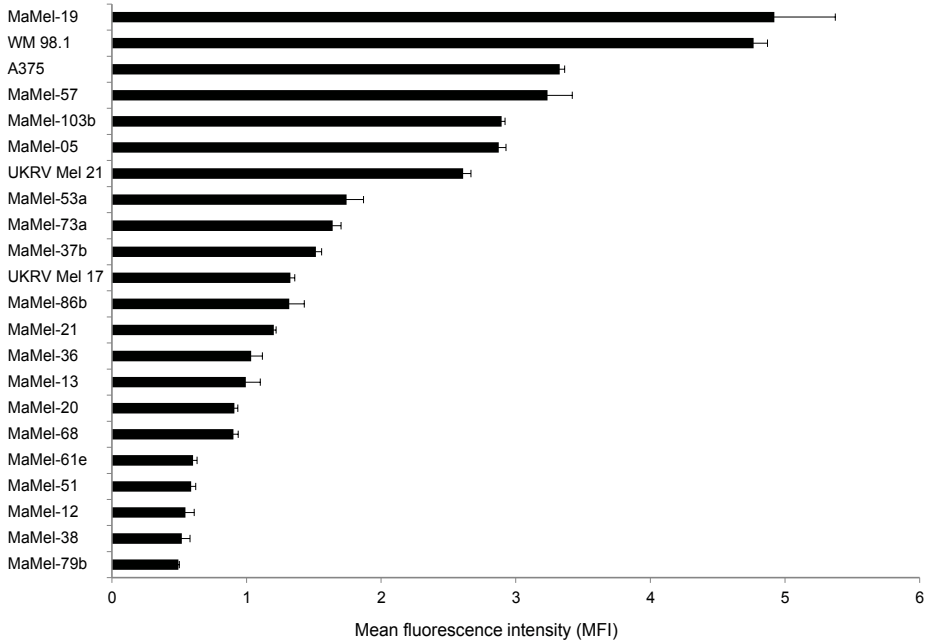

Figure 17: Analysis of the invasive capacity of 22 different melanoma cell lines. The Boyden chamber assay was performed with all 22 cell lines in triplicates and the invaded cells were fluorescently stained and measured after 24h invasion time. This panel of melanoma cell lines shows a wide range of different invasive potentials. Data represents biological triplicates in mean +/- STD.

Subsequently, fourteen cell lines with determined miRNA expression and known invasive behavior were analyzed for the correlation of their invasive potential with endogenous miRNA expression, respectively (Figure 18). A perfect correlation between both parameters would indicate R square (R^2) values close to one. When analyzing miRNA candidates which inhibited invasion in the screening assay, both miR-339-3p and miR-30c-1* expression levels showed a significant negative correlation to the invasive potential with R^2 values of 0.60 and 0.47, respectively (Figure 18A). In contrast, miR-325 and miR-139b did not show a significant correlation, though the trend was also negative.

Additionally, the same correlation was performed for three candidate miRNAs accelerating A375 cells invasion, miR-576-5p, miR-559-5p, miR-438-3p (Figure 18B) and a positive correlation tendency for all miRNA candidates, but only miR-576-5p and miR-483-3p were found to correlate significantly.

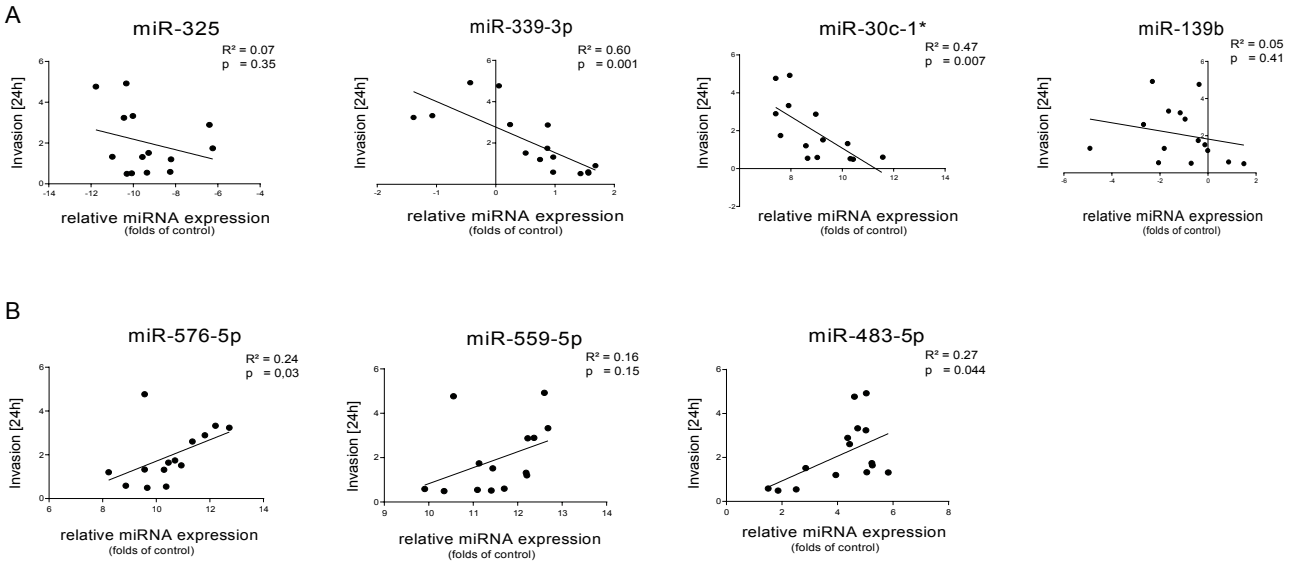

Figure 18: Correlation of miRNA candidate expression to the invasive capacity of 14 different melanoma cell lines. **A:** miRNA candidates with inhibiting effect on melanoma cell invasion; miR-325, miR-339-3p, miR-30c-1* and miR-193b. **B**: miRNA candidates with accelerating effect on melanoma cell invasion; miR-576-5p, miR-559-5p and miR-483-5p. R^2 as coefficient of determination of how well the regression line approximates the real data points. R^2 equals one represents the perfect fit.

After this first validation step two miRNAs were selected for further analysis, namely miR-339-3p, inhibiting A375 cell invasion and miR-576-5p, accelerating A375 cell invasion. Both were among the miRNA candidates with the strongest effects in the functional invasion assay and showed only minor effects on cell viability. Additionally, they are significantly differentially expressed in melanoma cells in comparison to NHEMs.

5.5 *In silico* miRNA target prediction and candidate selection

miRNAs are known to interact, inhibit or degrade specific target molecules and thus are regulating protein expression and influencing cell signaling pathways. To investigate the specific regulation both selected miRNA candidates were subjected to miRNA target identification databases which analyze putative miRNA binding sites within the 3'UTR of mRNAs and indicate possible targets by Dr. Agens Hotz-Wagenblatt. A combination of miRBase and TargetScan was chosen to identify targets of interest (Table 29).

The following gene enrichment analysis using the DAVID computational software categorized putative targets for both miRNA candidates into three groups, proteins influencing melanoma cell apoptosis, proliferation and melanoma cell motility via the cytoskeleton. Some targets are present in two categories indicating a combinatorial effect on cell motility and cell proliferation or apoptosis. These target prediction programs only indicate putative targets for specific miRNAs, therefore the actual interaction and exact binding sites of miR-339-3p or miR-576-5p and their predicted targets had to be further validated *in vitro*.

Physiological effect	Predicted targets for miR-339-3p (miRBase/TargetScan)
Apoptosis	AKT1 substrate 1, CASP8 and FADD-like apoptosis regulator, DnaJ homolog, GLI family zinc finger 3, TNF receptor-associated factor 3, alpha 1A voltage dependent calcium channel, Cardiotrophin-like cytokine factor 1, Glutamate receptor , Homeodomain interacting protein kinase 2, Inositol hexakisphosphate kinase 2, Insulin-like growth factor 1 receptor, Lymphotoxin beta, Myeloid cell leukemia sequence 1 (MCL1), Nucelar rezeptor subfamily 3, Nucleolar protein 3, Pim-2 oncogene, Protein kinase C, Retinoid X receptor alpha, Telomerase reverse transcriptase, Tubulin beta
Proliferation	SRY (sex determining region Y), Aristaless related homeobox, Epithelial membrane protein 2, Low density lipoprotein receptor-related protein associated protein 1, Neurofibromin 2 (Merlin), Pim-2 oncogene, Retinoid X receptor alpha, Scavenger receptor class B, Similar to mitogen-activated protein kinase phosphatase x, Solute carrier family 11, Taxilin alpha
Motility (Cytoskeleton)	CDC42 binding protein kinase alpha, DnaJ homolog, NCK adaptor protein 2, Rootletin, Cytohesin 2, Growth arrest-specific 7, Microtubule-associated protein tau, Neurofibromin 2 (Merlin), NudE nuclear distribution gene E homolog 1, Tubulin polymerization promoting protein, Tubulin beta
Physiological effect	**Predicted targets for miR-576-5p (miRBase/TargetScan)**
Apoptosis	CASP8 and FADD-like apoptosis regulator, CD38 molecule Kruppel-like factor 10, SH3-domain GRB2-like endophilin B1, TNF receptor associated factor 6, Cullin 3 cyclin-dependent kinase 5 (p35), Cyclin dependent kinase inhibitor 1A (p21,Cip1), Cystein-serine-rich nuclear protein 3, Forkhead box O3, Glutamate receptor, Interleukin 2 receptor, Junction mediating and regulatory protein p53 cofactor, Neurofibromin 1, Pleckstrin homology-like domain family A member 1
Proliferation	CD38 Kruppel-like factor 10, CNCK adaptor protein 1, Rho GTPase activating protein 5, Bone morphogenetic protein receptor type 1A, Bradykinin receptor B2, Caveolin 1, Cullin 3, Cyclin-dependent kinase inhibitor 1A, Epiregulin fatty acid binding protein 7, Fibroblast growth factor 2, Gap junction protein alpha 1, Insulin-like growth factor 1, Interleukin 2, Receptor interleukin 6, Neurofibromin 1, cKit
Motility (Cytoskeleton)	NCK adaptor protein 1, TAR DNA binding protein VAMP (vesicle-associated membrane protein)-associated protein B, Actin alpha, Caldesmon 1, Coronin, Formin 1, Formin-like 3, Kinesin family member 1B, Moesin, Myosin VA, Parvin, Phosphatase and actin regulator 2, Potassium large conductance calcium-activated channel, Protein phosphatase 1 regulatory (inhibitor) subunit 9A, Protein tyrosine phosphatase, Tropomodulin 2, Tropomyosin 3

Table 29: Target predictions for miR-339-3p and miR-576-5p based on two different prediction websites, miRBase and TargetScan. This table only displays targets identified simultaneously by both algorithms. Putative targets were grouped into three distinct physiological functions by DAVID analysis: apoptosis, proliferation and effects on cell motility (cytoskeleton).

5.6 Validation of the effects of miR-339-3p and miR-576-5p on melanoma cell invasion

5.6.1 Independent melanoma cell invasion assay

An independent functional invasion assay was performed according to the work flow presented in Figure 10D with five different melanoma cell lines to validate the effect of miR-339-3p and miR-576-5p on melanoma cell invasion. In comparison to the screening work-flow the time of invasion was extended to 24h and exactly $5x10^4$ cells were seeded per transwell insert. For all five cell lines MaMel-61e, WM 266.4, MaMel-103b, MaMel-86b and A375 under investigation miR-339-3p showed an inhibiting effect on invasion (Figure 19). This effect was more pronounced in cell lines with a rather strong invasive potential, such as WM 266.4, A375 and MaMel-103b, than in less invasive cell lines, e.g. MaMel-86b and MaMel-61e (Figure 19). Thus, miR-339-3p could be confirmed for its inhibitory impact on melanoma cell invasion.

miR-576-5p showed the strongest accelerating effect on A375 cell invasion during the functional screening assay. This effect could be reproduced in three additional cell lines, MaMel-103b, MaMel-86b and MaMel-61e (Figure 19). A more pronounced effect on invasion acceleration was observed for the less invading cell lines, MaMe-61e and MaMel-86b in comparison to WM 266.4, A375 and MaMel-103b (Figure 19) rather fast invading cell lines. Consequently, both miRNA candidates could be confirmed for their effect on melanoma cell invasion in an independent assay.

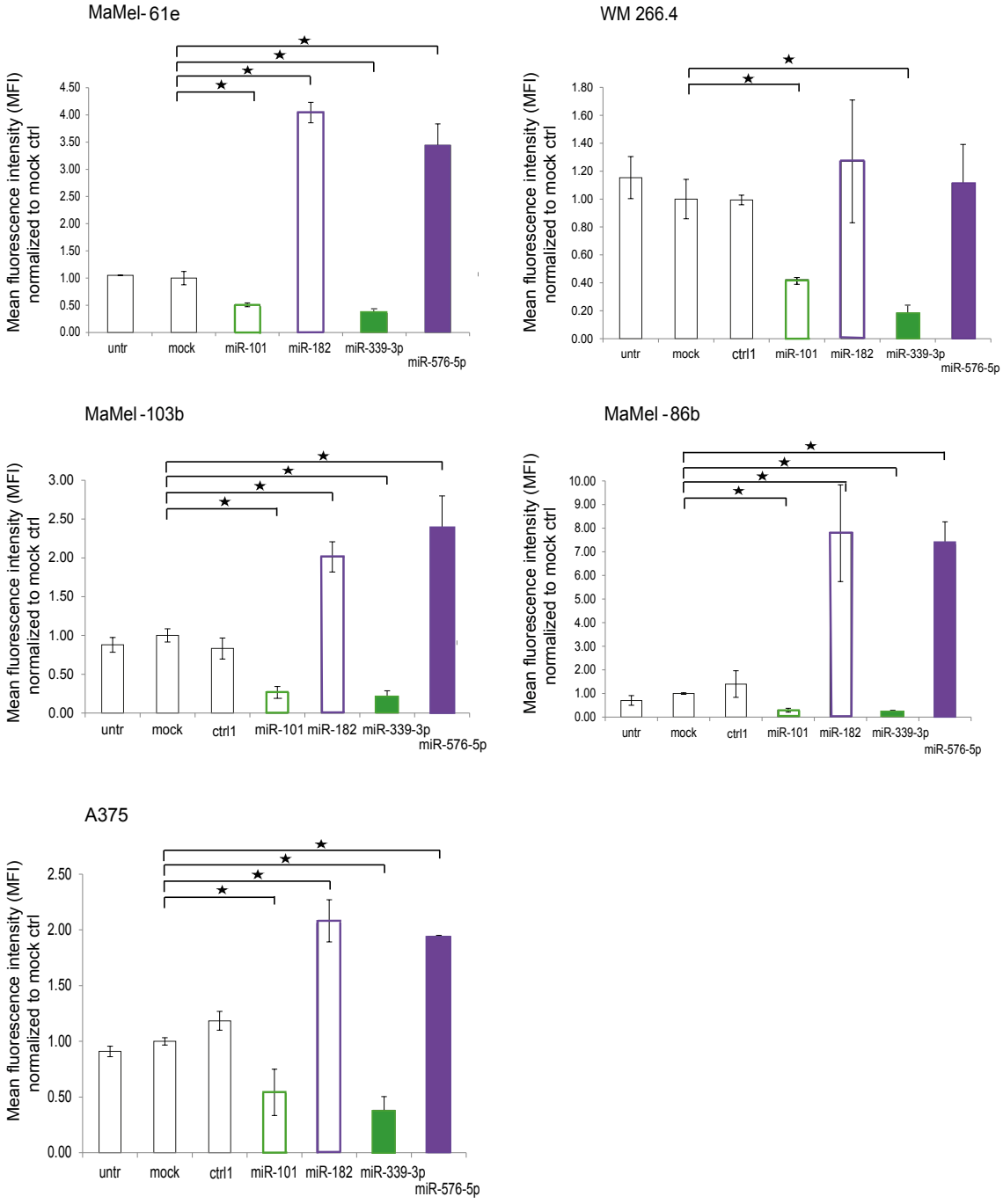

Figure 19: Effects of two miRNA candidates on melanoma cell invasion was tested on five different melanoma cell lines including A375, which was used for screening. miR-339-3p inhibited invasion significantly in all five cell lines and miR-576-5p accelerated invasion significantly in four of five cell lines. Asterisks depict significant differences to mock control (* $p < 0.05$).

5.6.2 miR-339-3p and miR-576-5p do not affect melanoma cell viability

A375, WM 266.4 and MaMel-86b cells were seeded and transfected 24h post with ctrl1 miRNA, miR-339-3p, miR-576-5p and miR-101. To determine the effects of these miRNAs on melanoma cell viability a CellTiter Glo assay was performed 48h post transfection. The mean luminescence intensity (MLI) refers to the amount of viable cells per well as the mean of triplicates normalized to mock treated cells. As it can be observed in Figure 20, no significant viability effects were detectable. Subsequent investigations were focused on miR-339-3p, to identify and validate potential targets genes.

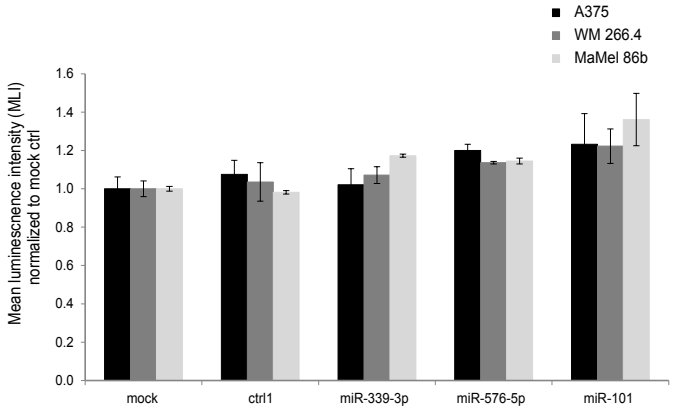

Figure 20: Cell viability effects of miRNA candidates, miR-339-3p and miR-576-5p. Assay control (ctrl1), mock transfection, miR-101 and the two miRNA candidates, miR-339-3p and miR-576-5p were analyzed for their effects on cell viability in the melanoma cell lines, A375, WM 266.4 and MaMel-86b 48h post transfection. Mean luminescence intensity as mean of biological triplicates is displayed after normalization to the mock control treated cells.

5.6.3 miR-339-3p inhibition restores melanoma cell invasion

To clarify the direct role of miR-339-3p on cell invasion, endogenously present miR-339-3p was inhibited by the respective antisense oligonucleotide (anti-miRNA). Subsequently, altered cell invasion was assessed in a Boyden chamber invasion assay. The melanoma cell lines MaMel-61e and MaMel-79b express high levels of miR-339-3p in comparison to A375 cells. Both showed a rather low invasive capacity in comparison to the cell lines A375 or WM 266.4. Altered cell invasion capacity was assessed after miRNA or anti-miR-339-3p

(anti-miRNA against miR-339-3p) transfection according to the protocol applied in Figure 19. As can be seen in Figure 21, anti-miRNA treatment was able to restore melanoma cell invasion in comparison to either control treated cells (untransfected, mock, ctrl1), or miR-339-3p transfected cells. This indicates a direct inhibiting effect of endogenous miR-339-3p on melanoma cell invasion, that can be restored after anti-miR-339-3p treatment.

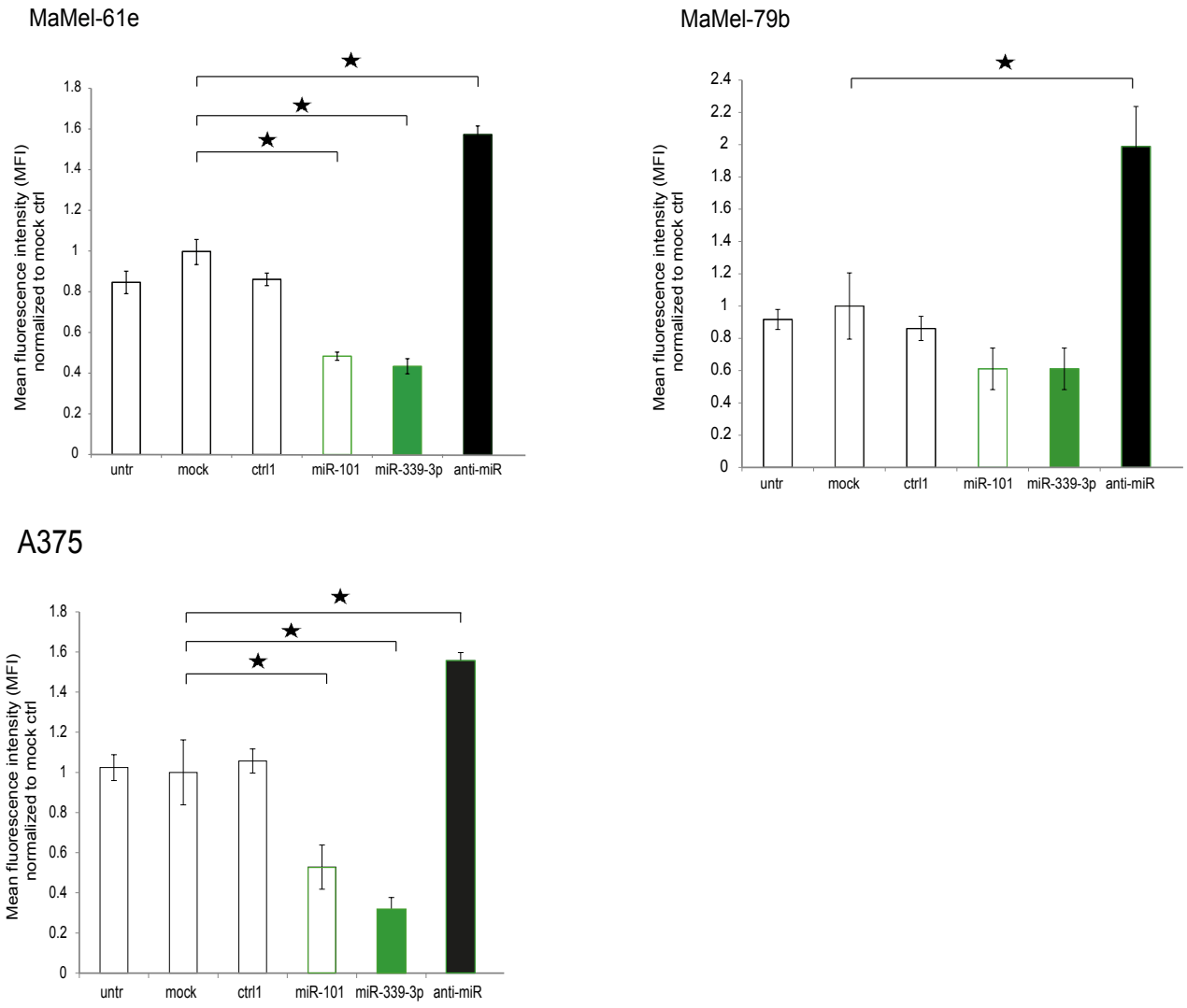

Figure 21: Restoration of the invasive potential of melanoma cell lines by anti-miR-339-3p treatment. The effect of the controls (mock, ctrl1, miR-101), miR-339-3p and the anti-miR-339-3p (anti-miR) on melanoma cell invasion was tested in three different melanoma cell lines, MaMel-61e and MaMel-79b are slow invading cell lines in comparison to A375 cells.

5.7 miR-339-3p targets MCL1

5.7.1 The myeloid cell leukemia protein 1 (MCL1)

In a first step, several potential miR-339-3p targets were identified by a bioinformatics approach using two independent prediction tools (Table 29), miRBase and TargetScan, resulting in several putative targets influencing cell death, proliferation and melanoma cell motility [116, 181, 183, 347]. Focusing on predicted targets with a know function in cancer cell progression and metastasis, oncogenes or tumorsuppressor genes regulated by miR-339-3p were of specific interest for further *in vitro* validation.

One putative target identified for miR-339-3p was the myeloid cell leukemia 1 (MCL1). This oncogene is widely investigated for its role in apoptosis, cell survival and epithelial mesenchymal transformation in several malignancies [16, 25, 62, 188, 237, 264, 289, 325] but not for its role in invasion yet. Three splicing variants are known for MCL1, two short variants the 32kDa MCL1 short (MCL-1S) and MCL1 extra short (MCL-1ES) with only 28kDa and one long version with 40kDa (MCL-1L) [16, 62, 171, 313]. Due to its Bcl-2 homology domains (BH) domains (BH1, BH2 and BH3) MCL1 is able to interact with a variety of proteins to influence cell survival signaling cascades after various cell death stimuli [5, 237]. MCL-1L exhibits three Bcl-2 domains, whereas onyl BH3 is present in MCL-1S and MCL-1ES. The transmembrane domain (TM) enables the association of MCL-1L with the plasma membrane, the mitochondrial membrane but it can also be found non-membrane bound in the cytosol [5, 237, 325]. All three splicing variants exhibit the same 3'UTR (Figure 22) what would indicate a similar regulation of all splicing variants by selective miRNAs.

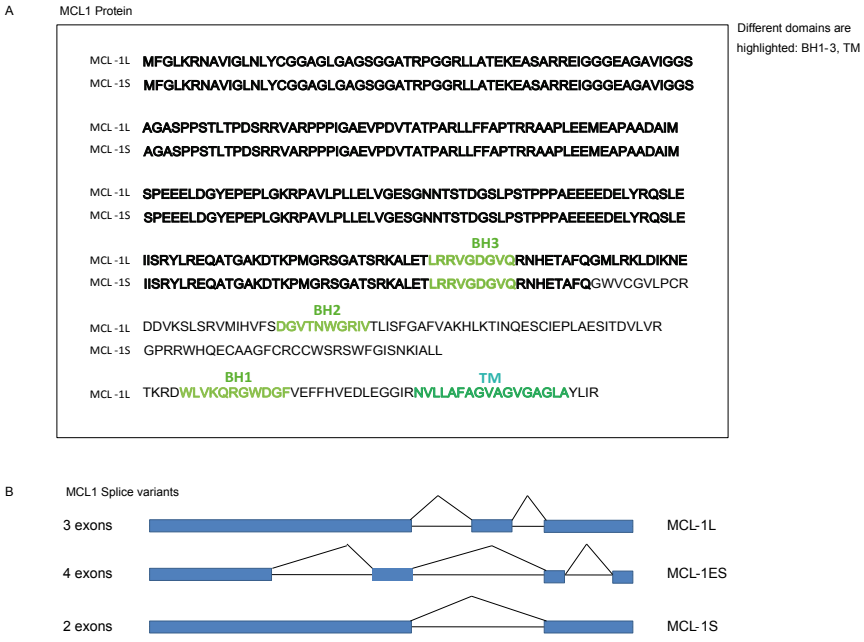

Figure 22: The MCL1 protein. **A:** MCL1 protein domains. The transmembrane domain (TM) for interaction with various membranes, three Bcl-2 homology domains (BH) domains (BH1, BH2 and BH3) mediating its role in cell death and survival. **B:** Three different splicing variants of MCL1, MCL1 long (MCL-1L), MCL1 short (MCL-1S) and MCL1 extra short (MCL-1ES).

5.7.2 The effect of MCL1 knockdown on melanoma cell invasion

As MCL1 was predicted as a target of miR-339-3p the effect of MCL1 knockdown was tested in order to prove, whether MCL1 knockdown can resemble the inhibitory effect of miR-339-3p on melanoma cell invasion. Five different melanoma cell lines were tested for abrogated invasive capacity after siRNA transfection targeting MCL1 (siRNA MCL1) in a Boyden chamber assay. Figure 12 shows the impact of MCL1 knockdown by siRNA transfection, in comparison to the positive control miR-101 and miR-339-3p 23 on melanoma cell invasion.

Melanoma cell lines transfected with miR-339-3p or siRNA against MCL1 showed significant less invaded cells in comparison to mock control treated cells (Figure 23) in all five melanoma cell lines tested. Thus, MCL1 knockdown by siRNA can mimic the inhibitory effect of miR-339-3p on melanoma cell invasion.

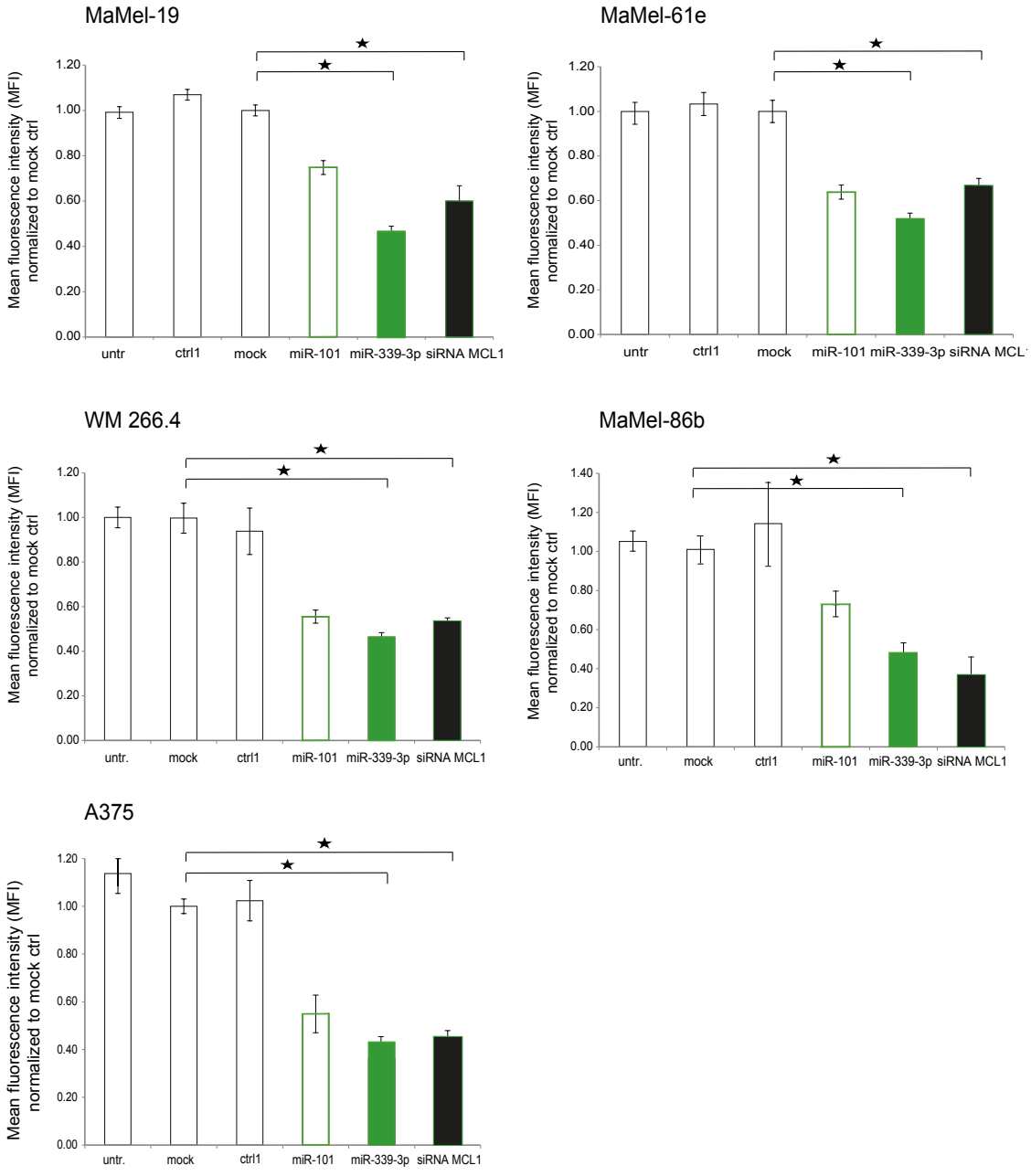

Figure 23: MCL1 knockdown inhibits melanoma cell invasion. The invasion assay was performed with the control miRNA, miR-101, the miRNA candidate miR-339-3p and the siRNA against MCL1 (siRNA MCL1), tested in five different cell lines, MaMel-61e, WM 266.4, MaMel-19, MaMel-86b and melanoma cell line A375.

5.7.3 miR-339-3p downregulates MCL1 protein and mRNA expression

MCL1 protein expression was analyzed in a large cohort of melanoma cell lines by Western Blot. While MCL1 could be detected in all cell lines, a substantial variation in protein levels was observed. The model cell line A375 as well as WM 155, MaMel-103b, MaMel-19 and WM 266.4 showed a strong MCL1 protein expression. Cell lines with a lower level of MCL1 protein expression are for example MaMel-79b, MaMel-20, MaMel-61e, MaMel-86 and MaMel-12 (Figure 24A). Additionally, MCL1 isoform expression varied significantly between different melanoma cell lines. MCL-1S and MCL-1SE were almost not detectable in MaMel-61e and MaMel-12, whereas A375 and MaMel-103 showed a rather high expression level of both short MCL1 isoforms.

The effect of miR-339-3p on MCL1 protein expression as well as on mRNA level was investigated next. As miR-101 was shown to target MCL1 in non-small-cell lung cancer cells [219] it was used as a positive control. Three different cell lines were seeded in 24 well plates, mock transfected or transfected with the positive control miR-101 and with the candidate miRNA miR-339-3p after 24h. After additional 48h or 72h the cells were harvested and either lysed for Western Blot analysis or RNA was extracted for qPCR measurement of MCL1 protein or mRNA expression, respectively.

As it can be seen in Figure 24B, both melanoma cell lines A375 and WM 266.4 showed a decreased expression of MCL1 protein 48h post miR-101 and 48h and 72h post miR-339-3p transfection. The siRNA against MCL1 also decreased the detectable amount of MCL1 protein and RNA in this experiment. Additionally, decreased amounts of MCL1 mRNA 24h and 48h post miR-339-3p transfection in comparison to ctrl1 miRNA transfection was detected by qPCR. This decrease was stronger in the WM 266.4 cell line (Figure 24D) in comparison to the cell line A375 (Figure 24D). The downregulation effect of protein as well as mRNA was partly restored 72h post miR-339-3p transfection. Conclusively, MCL1 protein and mRNA expression is affected by miR-339-3p overexpression in different melanoma cell lines.

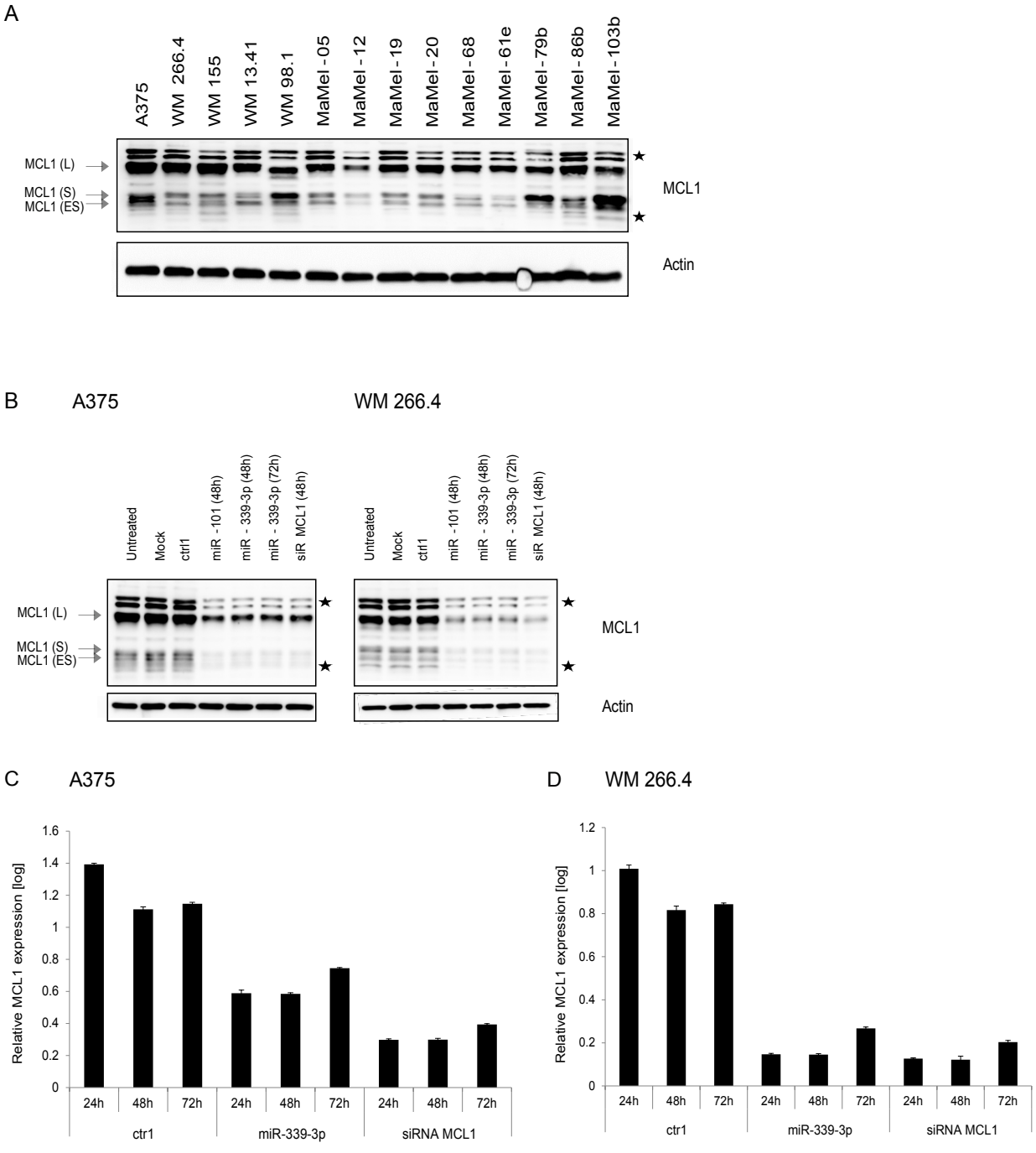

Figure 24: miR-339-3p and MCL1 siRNA down-regulate MCL1 protein and mRNA expression. **A**: MCL1 protein expression levels in 14 different melanoma cell lines. MCL1splicing variants: long isoform MCL1 (L), short isoforms MCL1 (S) and MCL1 (ES). **B:** miR-339-3p abrogates MCL1 protein expression at 48h post transfection in A375 and WM 266.4. **C**: Time course experiment of miR-339-3p effect on MCL1 mRNA expression. miR-339-3p down regulates MCL1 RNA expression 24h, 48h and to a lesser extent 72h post transfection in A375 and WM 266.4 cells. Relative expression of MCL1 mRNA to GAPDH mRNA was calculated and normalized to the untreated control. Asterisks (*) indicate unspecific bands.

5.7.4 MCL1 is a direct target of miR-339-3p

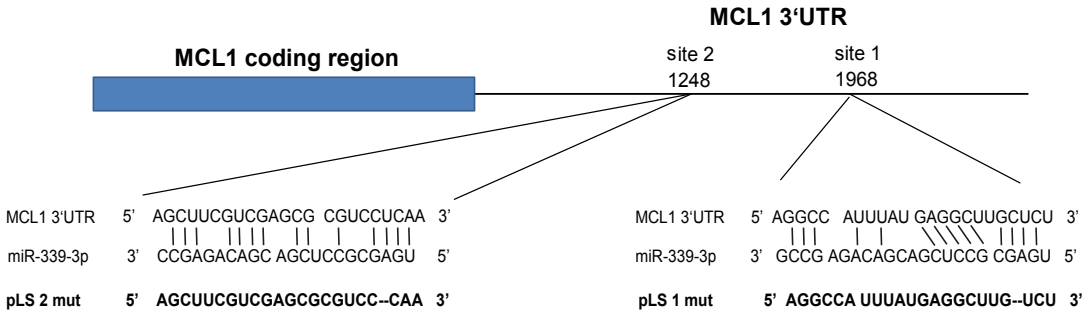

Figure 25: Putative binding sites of miR-339-3p at the MCL1 3'UTR. The 3'UTR is identical for all three splicing variants. The different plasmids were used to investigate these binding sites with the respective mutations.

miR-339-3p interaction with the MCL1 3'UTR was predicted with RNAhybrid at several different interaction sites [184, 286]. A renilla luciferase plasmid with the 3'UTR of MCL1 (pLS 3'UTR MCL1) was used to investigate two putative direct interaction sites of the 3'UTR of MCL1 and miR-339-3p. A375 and WM 266.4 cells were seeded in the appropriate amounts in a glass bottom 96-well plate. The cells were transfected with the luciferase plasmid in combination with the candidate miRNA miR-339-3p or the negative ctrl1 miRNA. The interaction of miR-339-3p with the 3'UTR of MCL1 is indicated by a decreased luciferase signal in comparison to the signal detected after ctrl1 miRNA and pLS 3'UTR MCL1 plasmid transfection. To confirm the direct interaction the two putative binding sites in the 3'UTR of MCL1 were mutated by site-directed mutagenesis, specifically the introduction of a a single deletion within the predictive seed region of miR-339-3p (Figure 25) was intended. The mutation of the first binding site (1968) did not affect the luciferase signal to great extent. In contrary, mutating the second putative binding site (1248, pLS site 2 mut) resulted in an almost complete restored luciferase signal intensity in two melanoma cell lines indicating a direct interaction of miR-339-3p and the 3'UTR of MCL1 at this binding site (Figure 26). It has to be added, that within both plasmids additional mutations can be observed across the whole 3'UTR of MCL1. Despite the signal restoration after mutation of the second binding site, effects of bystander mutations cannot be excluded and have to be further investigated.

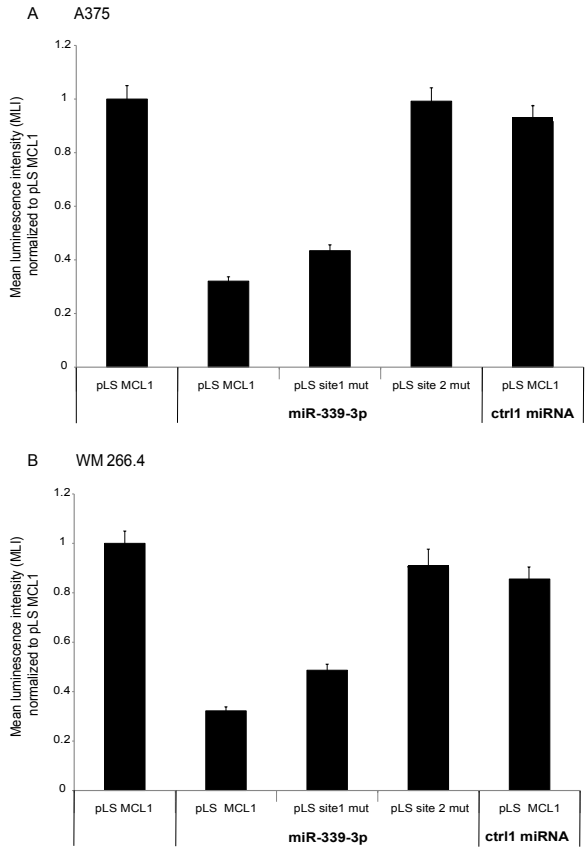

Figure 26: miR-339-3p targets MCL1 3'UTR. Two different melanoma cell lines were transfected with either the luciferase plasmid containing the 3'UTR of MCL1 alone or in combination with the control miRNAs (ctr1 and miR-101) or the candidate miR-339-3p. Interaction of miR-339-3p with the 3'UTR resulted in the decrease of the detected luciferase signal in the cell lines **A:** A375 and **B:** WM 266.4. After site-directed mutagenesis of two putative interaction sites, pLS site 1 mut, pLS site 2 mut, the luciferase signal could be partly (site 1) or completely (site 2) restored.

5.7.5 miR-339-3p overexpression does not induce apoptosis

MCL1 is reported to play a role in cell death, proliferation and survival. Thus, the effect of miR-339-3p overexpression and MCL1 knockdown on melanoma cell death induction was elucidated. Therefore, an apoptosis and cell death assay was performed with two different melanoma cell lines, A375 and MaMel-86b. Cells were seeded in the appropriate amount in 24 well plates, mock transfected, transfected with ctrl1 miRNA, positive control for cell death induction (cell death siRNA), miR-339-3p or with the siRNA targeting MCL1 mRNA. 48h post transfection cells were harvested and dead cells were detected via double positive staining for PI and YO-Pro.

All treatments are displayed in dot plots in Figure 27A for the cell line A375 and in Figure 27C for the MaMel-86b cell line. YO-Pro positive cells are displayed on the x-axis representing apoptotic cells, whereas the y-axis represents PI positive cells. Double positive cells represent necrotic cells in a transition phase from the early apoptotic to dead cell phase.

Untreated melanoma cells as well as ctrl1 miRNA or mock transfected cells already showed a small double positive dead cell population for both cell lines indicating a naturally occurring baseline cell death induction. However the double positive population was strongly increased 48h after cell death siRNA transfection for the A375 as well as the MaMel-86b cell line (Figure 27A, C). The whole cell population shifted and displayed a high ratio of necrotic cells for the cell line A375, or apoptotic cells for MaMel-86b.

The effect of miR-339-3p on melanoma cell death induction was comparable to the effects detected for cells treated with ctrl1 miRNA in both cell lines. In contrast, cells treated with the siRNA against MCL1 showed an increased amount of double positive, necrotic cells in comparison to miR-339-3p or ctrl1 miRNA treated cells. The effect of MCL1 knockdown might represent a transition state between the slight baseline turnover rate and cell death induction seen in crtl1 or mock transfected cells and strong apoptosis induction after the treatment with cell death siRNA.

These results indicate no significant cell death induction after miR-339-3p overexpression in two melanoma cell lines; however direct MCL1 knockdown by siRNA transfection could be shown to increase cell death rate.

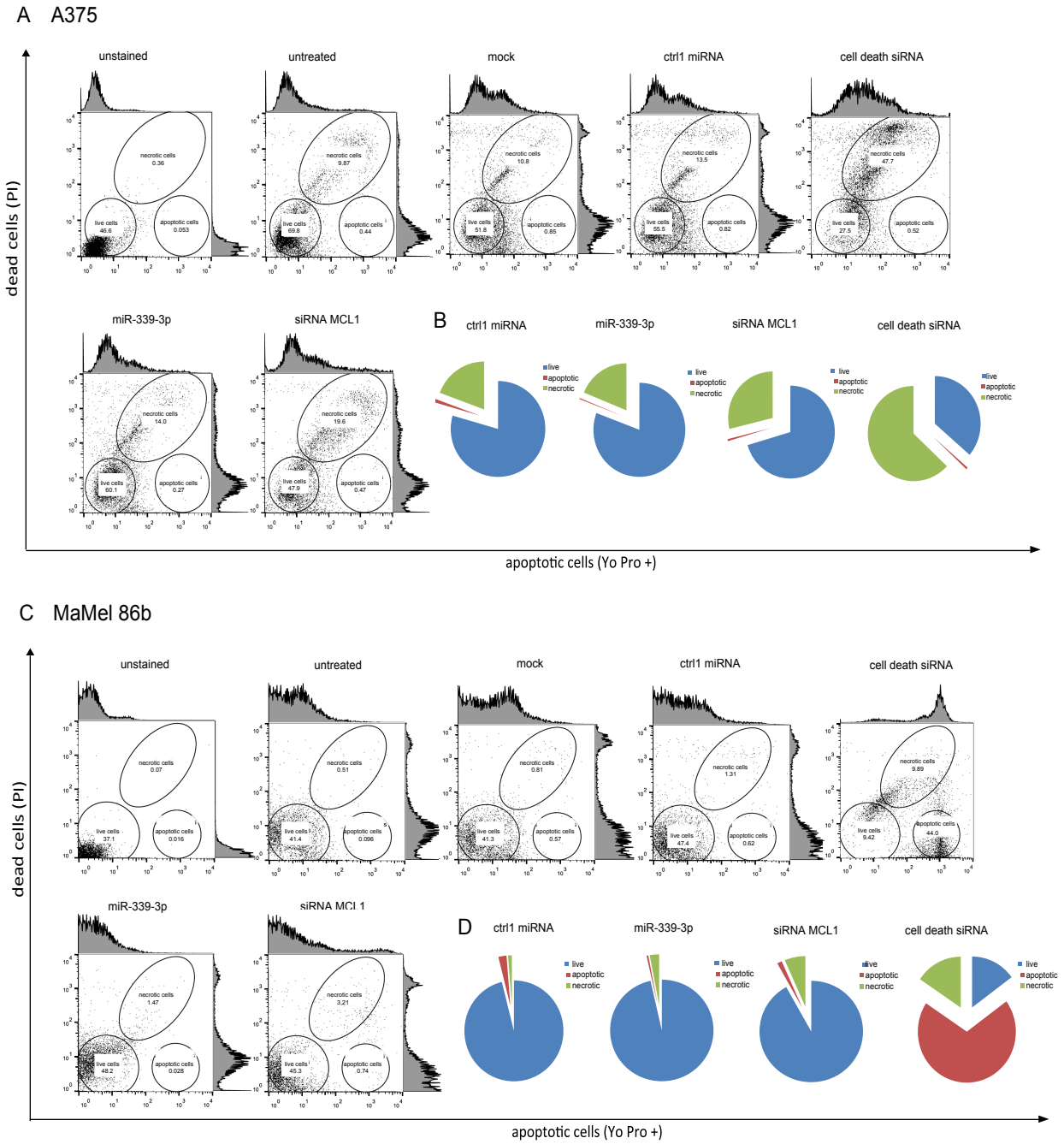

Figure 27: Effect of miRNA candidate, miR-339-3p, on A375 and MaMel-86b apoptosis induction and cell death. A375 **(A, B)** and MaMel-86b **(C, D)** cell lines were mock transfected or transfected with control miRNA (ctrl1 miRNA), miR-339-3p or siRNA against MCL1. 48h post transfection cells were harvested and stained with PI and YO-Pro to determine the percentage of apoptotic and necrotic cells. The respective dot plots are shown for every treatment. The double positive population represents necrotic cells, whereas apoptotic cells are represented by the single YO-Pro positive population. **B, D**: Representation of percentages of live, apoptotic or necrotic cells for A375 **(B)** cells or MaMel-86b **(D)** cells treated with ctrl1, miR-339-3p, siRNA against MCL1 or cell death siRNA.

5.8 Identification of a specific gene expression pattern in miR-339-3p overexpressing melanoma cells

We speculated that miR-339-3p overexpression might induce a distinct gene expression pattern in melanoma cell lines characteristic for the observed phenotype. Therefore, we analyzed the global gene expression pattern after transfection with miR-339-3p. Briefly, A375 and MaMel-86b melanoma cells were seeded and either transfected after 24h with miR-339-3p or kept untreated in biological triplicates. 48h post transfection cells were harvested and whole RNA was extracted and subjected to Illumina Chip gene expression analysis. Melanoma cells 48h post transfection of miR-339-3p were compared to untreated cells of the same cell line. In addition changes and similarities between cell lines were investigated before and after miR-339-3p overexpression.

Figure 28 shows heat maps of two different melanoma cell lines, A375 (Figure 28A, B) and MaMel-86b (Figure 28C, D) untreated or transfected with miR-339-3p and analyzed for differential gene expression in triplicates. The overall representations of genes significantly differentially regulated by miR-339-3p are shown in Figures 28A and C. It can be seen that there is a clear difference in the gene expression profile of melanoma cells untreated or transfected with miR-339-3p for both cell lines. Figure 28B, D shows only differentially expressed genes with an at least two fold difference in untreated to miR-339-3p transfected melanoma cells. Interestingly, both cell lines show more two fold down- than upregulated genes after miR-339-3p transfection (Figure 28 B, D).

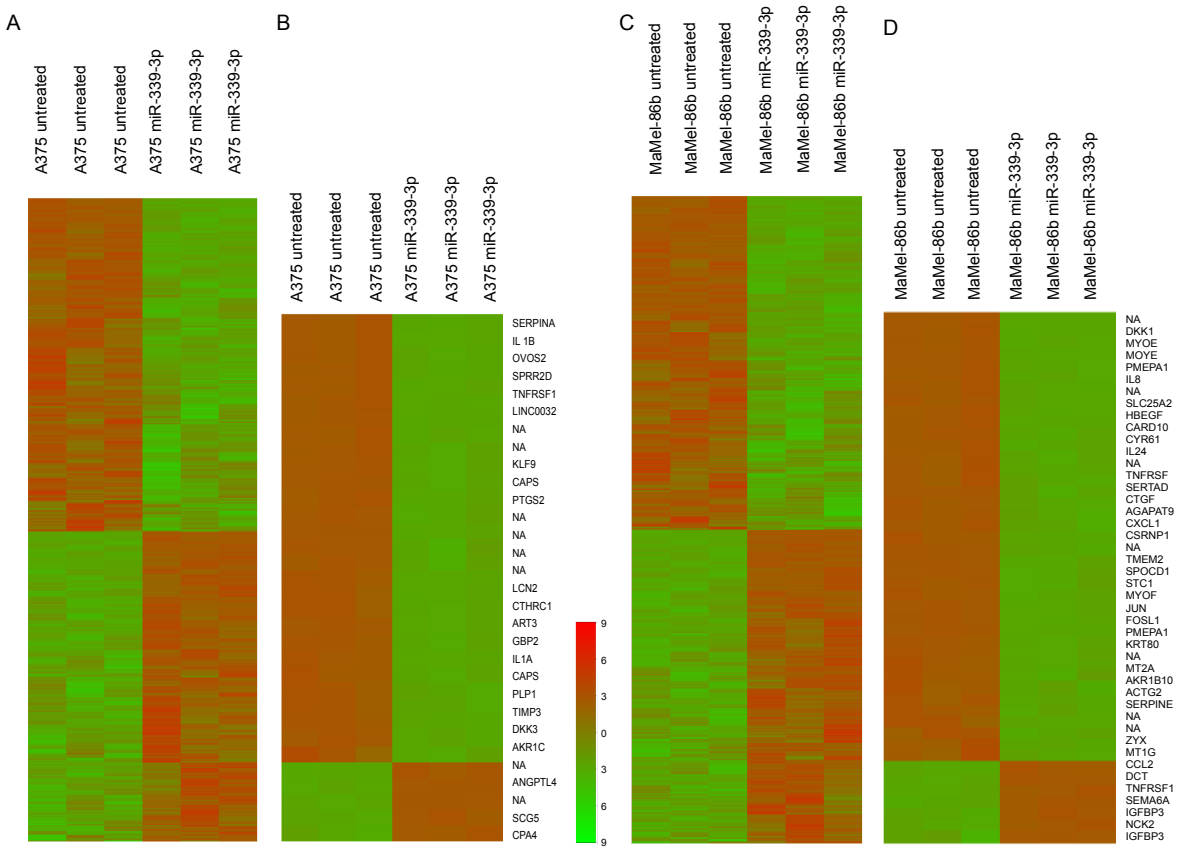

Figure 28: Gene expression analysis in A375 and MaMel-86b cells after miR-339-3p overexpression. **A:** Representation of 5475 genes significantly differentially expressed in untreated or miR-339-3p transfected **(A)** A375 or **(C)** MaMel-86b cells. Display of genes which show an at least two fold difference in mRNA expression levels after miR-339-3p transfection in melanoma cell lines A375, 33 genes **(B)** or MaMel-86b, 46 genes **(D)**. Red represents genes higher, green lower differentially expressed in the respective cells according to scale.

Subsequently, two fold differentially expressed genes were further investigated by gene enrichment analysis in MetaCore to investigate process networks influenced by significant differentially regulated genes after miR-339-3p overexpression. In Table 30 the nine strongest process networks, displaying signaling pathways mostly influenced by this specific gene set, are presented for melanoma cell line A375 and MaMel-86b (Table 30). Despite developmental processes, proliferation in the melanoma cell line MaMel-86b and inflammation in A375 cells, as expected cell adhesion and the cytoskeleton are mainly affected in both melanoma cell lines after miR-339-3p overexpression.

A375	MaMel-86b
Process Networks	**Process Networks**
Inflammation (Amphoterin signaling)	Cell adhesion
Inflammation (Protein C signaling)	(Integrin-mediated cell-matrix dhesion)
Cell adhesion	Developmental (Axonal guidance)
(Integrin-mediated cell matrix adhesion)	Development (Neurogenesis)
Cell adhesion	Cytoskeleton (Actin filaments)
(Endothelium-leukocyte interactions)	Proliferation
Inflammation (anti-inflammatory response)	Cytoskeleton (Rearrangement)
Inflammation (innate inflammatory response)	Inflammation
Inflammation (MIF signaling)	(Amphoterin signaling)
Reproduction	Inflammation (Neutrophil activation)
Cell adhesion (cell-matrix interactions)	Chemotaxis

Table 30: Enrichment analysis with MetaCore to identify process networks influenced by genes at least two fold differentially regulated in the cell lines A375 or MaMel-86b after miR-339-3p overexpression. MIF (macrophage migration inhibitory factor)

Overall, more genes were found to be at least two fold downregulated in miR-339-3p overexpressing melanoma cells, then upregulated (Figure 28B, D). Exemplary, ten of these genes are displayed in Figure 29, e.g. IL1B (interleukin 1β), SERPINA3 (serpin peptidase inhibitor, clade A) and OVOS2 (ovostatin 2) for A375 (Figure 29 A) and IGFBP3 (insulin-like grwoth factor-binding protein 3), NCK2 (NCK adaptor protein 2) and TNFRSF19 (tumor necrosis factor receptor superfamily, member 19) for MaMel-86b (Figure 29B). In addition, molecules with an important role in melanoma cell maintenance and malignant progression, such as MCL1, MITF and DCT (dopachrome tautomerase) are shown for their differential expression. MITF was regulated to different extent in these two melanoma cell lines. In A375 cells MITF expression was not affected, whereas it was downregulated in MaMel-86b cells. Unexpectedley, MCL1 was almost unchanged in MaMel-86b cells, but it was downregulated to -0.64 log 2 fold change in A375 cells after miR-339-3p overexpression.

Furthermore, genes which were significantly regulated in these two melanoma cell lines were compared in Figure 29C. 1624 genes were differentially regulated in both cell lines, thus only 36 of these genes were previously indicated by *in silico* prediction analysis.

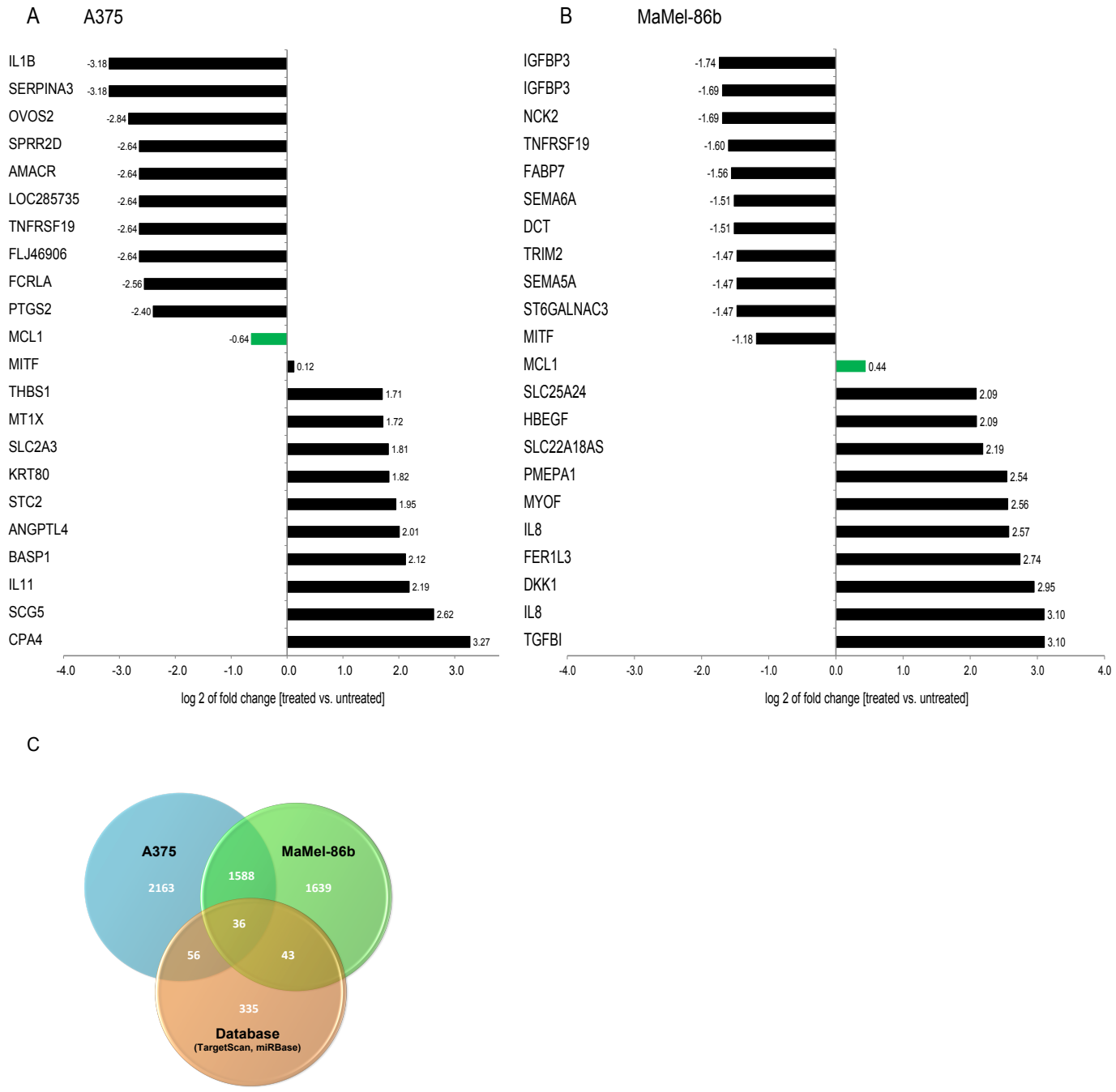

Figure 29: Strongest differentially regulated genes represented in log 2 transformed fold changes of gene expression of treated versus untreated A375 **(A)** and MaMel-86b **(B)** cells after miR-339-3p overexpression. **C:** Comparison of genes significantly differentially regulated in A375 cells, MaMel-86b cells and to *in silico* predicted genes (databases: TargetScan, miRBase).

5.9 Altered lung metastasis formation of A375 cells overexpressing miR-339 *in vivo*

So far, miR-339-3p was shown to inhibit melanoma cell invasion *in vitro*, but the effect of miR-339-3p overexpression on melanoma cell invasion and metastasis *in vivo* still remains elusive. Therefore, A375 cells were stably transfected with miR-339 hairpin pre-miRNA (A375 miR-339) or the empty vector control (A375 empty) and an experimental lung metastasis assay in non-obese diabetic scid gamma (NSG) mice was performed. Due to miRNA processing, A375 cells stably transfected with miR-339 pre-miRNA might overexpress both mature miRNA forms, namely miR-339-3p as well as miR-339-5p. Thus, expression of both forms were analyzed in the stably transfected clones (Figure 30A). While both miRNA mature forms were overexpressed in the stable cell line, miR-339-3p was significantly higher expressed (13 fold) then miR-339-5p (5 fold). Furthermore MCL1 mRNA expression was decreased to 40% in A375 miR-339 cells in comparison to the cell line A375 empty (Figure 30A).

NSG mice were injected intravenously (*i.v.*) with A375 miR-339 cells, A375 empty cells or the parental A375 cells with ten mice per group. 14 days post *i.v.* injection lung metastasis formation was assessed (Figure 30B, C). Lung metastasis formation was lowest in mice injected with the cell line A375 miR-339 in comparison to the A375 empty as well as the parental cell line A375. Most lung metastases were observed in mice injected with the parental cell line A375.

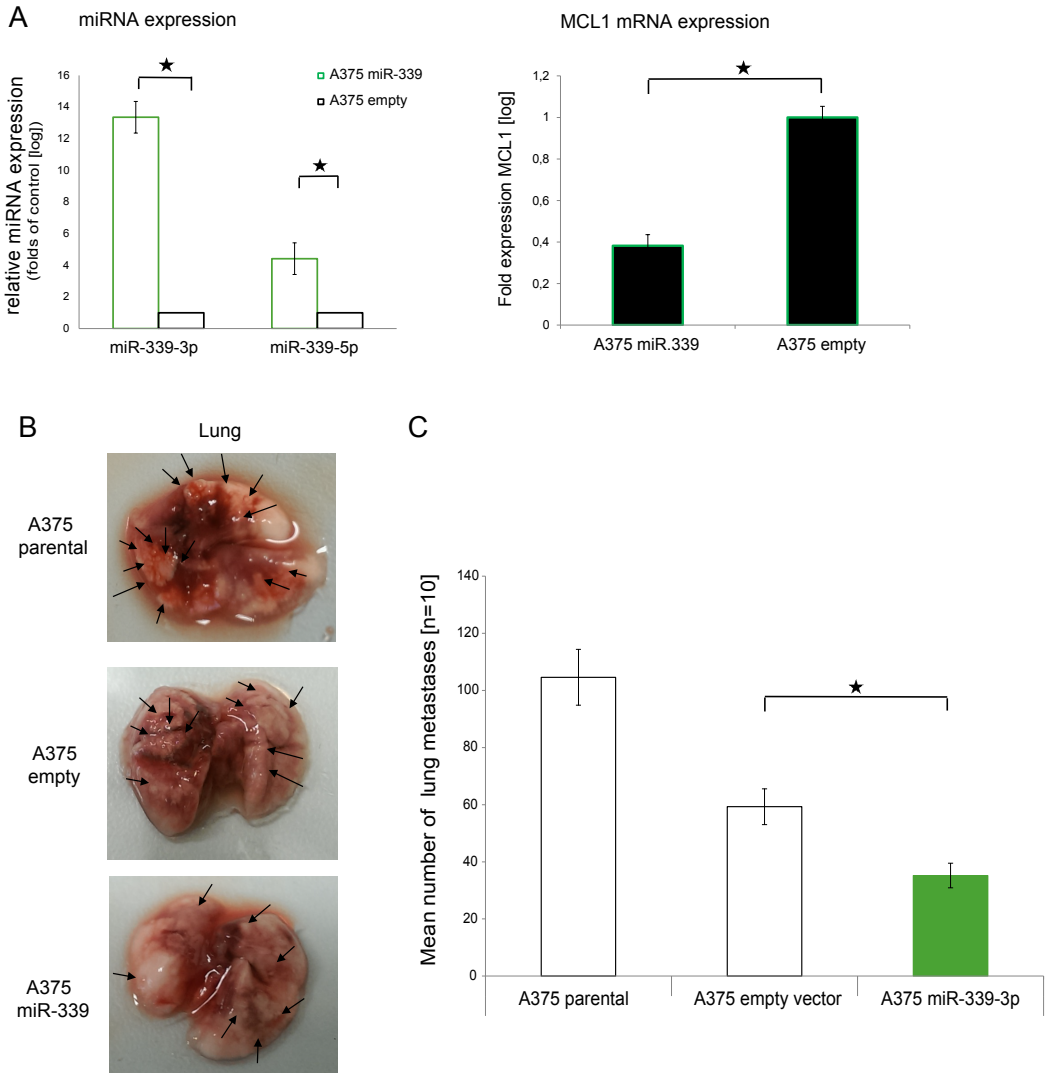

Figure 30: Lung metastasis assay. **A:** miR-339-3p, miR-339-5p expression in the cell line A375 miR-339 in comparison to the cell line A375 empty vector (A375 empty). **B:** Differences in MCL1 mRNA expression in the cell lines A375 miR-339 and A375 empty. Lung metastasis assay in NSG mice 14 days post *i.v.* injection of 5x10^{5}parental A375 cells, A375 empty cells or A375 miR-339 cells. Shown is one representative lung image of each group **(B)** and the accumulated data **(C)** as mean +/- STD. Arrows indicate metastases.

Unexpectedly, liver metastasis formation was observed 14 days post i.v. injection when stably transfected A375 cells were transplanted to NSG mice, but not when parental A375 cells were injected. Similar to the lung colonization, A375 miR-339 cells led to fewer metastasis than A375 empty cells (Figure 31).

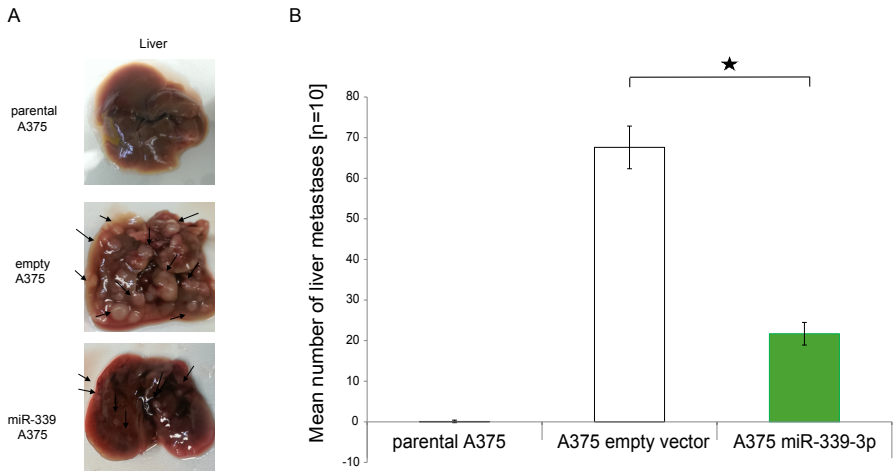

Figure 31: Appearance of liver metastases. **A:** Liver metastasis in NSG mice 14 days post *i.v.* injection of $5x10^5$parental A375 cells, A375 empty cells or A375 miR-339 cells. Shown are images of representative livers **(A)** and the accumulated data **(B)** as mean +/- STD. Arrows indicate examples of metastases.

5.10 Summary of Results

In conclusion, a miRNA library screening to investigate changes in melanoma cell invasion was performed and miRNA candidates for further analysis was be detected. These candidates were validated in independent invasion assays and analyzed for their effect on melanoma cell viability. Finally, miR-339-3p was selected as the candidate of interest and *in silico* prediction analysis revealed oncogen MCL1 as one of its targets. Subsequently, MCL1 was confirmed as miR-339-3p target *in vitro* by Western Blot and qPCR analysis. Furthermore, MCL1 was downregulated melanoma cell invasion significantly, comparable to the effect seen before for miR-339-3p. Additionally, inhibition of endogenous miR-339-3p could restore melanoma cell invasion. A direct interaction of miR-339-3p with MCL1 was shown by 3'UTR binding assays. MCL1 could be identified as a new target of miR-339-3p and overexpression of miR-339-3p led to decreased invasion of melanoma cells *in vitro*.

Part 6
Discussion

6.1 Functional miRNA library screening in A375 cells

Screening procedures are widely used to identify miRNAs with specific impact on cell physiology or pathophysiology. Different approaches are used to detect and validate differentially expressed miRNAs, such as genetic screens to identify changes in the miRNA or gene expression pattern due to malignant growth on the one hand [34, 197, 298, 326], or functional screens to specifically link miRNAs a distinct quantifiable phenotype [75, 150, 157, 188, 277, 301, 178, 382] on the other hand. Genetic screens reveal a global view on differentially regulated genes or miRNA networks. Therefore, these approaches are mainly used to identify differences in the miRNA expression profiles of healthy individuals in comparison to patients for further clinical therapy and prognosis development. Until now, several miRNAs could be identified accordingly, such as miR-19 with its promoting role in development of T-cell acute lymphoblastic leukemia (T-ALL) in a Notch-dependent fashion [225]. Specific blood miRNA expression signatures were shown to be deregulated in breast cancer patients in comparison to healthy individuals, such as miR-125b and miR-200c [129]. Furthermore, distinct miRNA profiles seem to be specific for different acral melanoma subtypes, e.g. miR-214 and miR-142-3p [45]. The differential analysis of miRNA expression pattern in human melanocytes in comparison to melanoma cell lines or melanoma patient samples allowed the identification of possible biomarkers to recognize melanoma onset and progression [276]. Furthermore, Philippidou et al. [276] showed the significance of analyzing patient samples as they see minor correlations in miRNA expression profiles between established melanoma cell lines and melanoma patient samples [276]. miR-155 and miR-146a for example were shown to be upregulated in melanoma patient samples in comparison to melanoma cell lines [276]. miRNA profiling might not only play a role in biomarker identification to detect or prevent malignancies but might also hold prognostic value to predict effectiveness of specific therapies [303]. Overall, miRNA expression analysis has the potential to indicate a certain deregulated miRNA pattern correlating with a certain malignancy or malignant progression. Despite the global information content of miRNA profiling, the specific function of individual miRNAs remains unknown.

Contrarily a functional screening approach as presented in this work aims to identify specific miRNAs, which alter distinct cellular properties. Several miRNAs have already been identified by different functional screening approaches to play role in melanoma cell physiology [150, 157, 188, 269, 271]. Sensitization to apoptosis induction of HCT-116 cells after ABT-263 application was shown for a panel of 19 different miRNAs such as miR-153, miR-892b, miR-582, miR-148b*, miR-876-3p, miR-101, miR-1233 and miR-935 [188]. 12 out of these 19 miRNAs were shown to target MCL1 expression in this cell line [188]. A comparable method was published 2011 by Poell et al. using the melanoma cell line A375 [277]. They investigated the effect of a whole genome lentivral miRNA expression library on melanoma cell viability. Two distinct miRNA clusters could be identified to negatively affect A375 cell viability. They showed that members of the miR-15/16/497 family and the miR-96/182 family were altering melanoma cell viability [277]. The effect they observed for miR-182 is controversially discussed as it is also known for its oncogenic role to induce melanoma cell invasion and metastasis *in vivo* [302, 154]. Several putative targets of miR-182, such as MITF, are currently under investigation to highlight its distinct role during melanoma progression and metastasis formation [211, 282, 302]. Overall, the role of miR-182 in melanoma metastases formation and tumor progression might be directly connected to small changes in the MITF level, favoring melanoma cell invasion [211, 186, 302].

Contrariwise to the observations of Poell et al. miR-182 did not show significant effects on A375 cell viability in the work presented here. As miR-182 could significantly increase A375 cell invasion capability, this miRNA was chosen as one of the positive controls throughout the entire screening approach. The discrepancy might be due to different time frames and transfection methods used in both functional screenings. The cell viability was assessed six days post infection by Poell et al. [277], whereas the overall invasion assay screening procedure and cell viability assays presented in this work were performed within three days. Therefore, possible long term effects of miR-182 on cell viability would not affect the presented functional invasion assay screening.

Targeting melanoma cell survival and proliferation is one promising approach to interrogate with melanoma aggressiveness, whereas abrogating melanoma metastasis and invasion might be another promising strategy. To investigate the role of miRNAs in melanoma cell invasion a functional screening approach had to be established to detect changes in melanoma invasion as read-out. Therefore, different melanoma cell lines were investigated for their feasibility and the melanoma cell line A375 was chosen for a functional screening approach. The characteristics of A375 cells, their migrative and invasive potential were requirements for a successful invasion assay screening and the subsequent validation procedure [54, 180, 107]. In a wound healing assay A375 cells were able to show their migrative potential. Furthermore,

A375 cells were able to induce lung and liver metastasis as well as swollen lymph nodes in NSG mice. In accordance with the presented results, the A375 melanoma cell line is reported to show metastatic potential *in vivo* and *in vitro* and its phenotype and genotype are already extensively studied since its establishment by Giard et. al 1973 [180, 107, 54, 378].

First, the feasibility of a 96-well automated wound healing assay was investigated and different possible positive controls were tested. miR-182 [302], miR-221 [103, 192], miR-136 [384] and siRNA targeting SFRP4 [77] are known to induce the invasive behavior of cancer cells. The opposing effect was expected for miR-137 [28, 218], miR-211 [201], miR-206 [23], siRNA against EZH2 [13, 229, 217] and against MMP9 [249, 147] as they are reported to negatively influence melanoma cell invasion or progression. miR-182 as well as miR-137 were able to influence the migratory behavior of A375 cells to a minor extent. As no clear indicative effect for all tested control miRNAs or siRNAs on A375 melanoma cell migration could be detected, another functional screening approach focusing on melanoma cell invasion was investigated. Invasion is a more complex process than cancer cell migration. tumor cell invasion requires the disruption of a complex regulatory network including, the release of cell-cell interactions, remodeling of the ECM and changes in the local microenvironment, such as the surrounding tissue stroma [99, 381]. Investigating cancer cell invasion might explain more complex signaling pathways interfering with cancer metastasis formation than the identification of differences in cancer cell migration can yield.

The matigel-based Boyden chamber invasion assay, allows the identification of miRNAs relevant to influence cancer cell invasion. In breast cancer, miR-373 and miR-520c could be identified to induce a metastasis phenotype *in vitro* and *in vivo* as they were able to promote breast cancer migration and invasion using a transwell migration assay [150]. Additionally, the same system was used in a miRNA library screening approach using the cell line A375M by the group of Levy et al [201].

Thus, a matrigel-based invasion assay was evaluated to investigate the invasive capacity of A375 cells. In this assay the invaded cells were fluorescently stained to directly correlate the amount of invaded cells with florescence intensity. The time-line of the procedure as well as positive and negative controls were successfully validated beforehand. Two positive controls were validated for their effect on A375 cell invasion, miR-182 which increased their invasive potential and miR-101, which could be shown to inhibit A375 invasion, as previously reported by Luo et al. 2013 [217]. In the the functional screening the effect of 988 different miRNAs on A375 cell invasion was investigated.

The screening result is shown in a waterfall plot, representing the homogenous distribution of miRNA candidates with accelerating effect on A375 cell invasion on the left side, miRNA candidates which did not significantly affect A375 cell invasion in the center and miRNA

candidates with inhibiting effect on invasion on the right side of the figure. In summary 50% of all miRNAs tested in this functional screening did not show an effect on A375 cell invasion, however 37% could increase the invasive capacity of the model cell line A375 and 13% were able to inhibit A375 cell invasion.

Due to comparable screenings performed in the here presented work and the approach of Levy et al. [201], it was of additional interest to confirm overlapping miRNA candidates. Levy et al. could identify miR-211, miR-204 as inhibitors of melanoma cell invasion in a 24-well Boyden chamber system [201]. In a second step they were able to validate the observed effect in additional melanoma cell lines (WM1745, WM1716 and WM3314) and computationally investigated the gene regulatory network influenced by miR-211 to identify nuclear factor of activated T-cells 5 (NFAT5), insulin-like growth factor 2 receptor (IGF2R) and transforming growth factor, beta receptor II (TGFBR2) as possible targets [201]. These results support the relevance of the here presented functional screening as the same inhibitory effect of miR-211 on A375 cell invasion could be reproduced, although no significant effect on melanoma cell invasion could be detected for miR-204.

During the functional screening approach miRNAs could be identified which showed either an inhibiting effect on A375 cell invasion or were able to accelerate melanoma cell invasion. miRNAs already published for their inhibition or acceleration for invasion could be confirmed in our assay, e.g. miR-21 [119, 212, 299], miR-214 [274], miR-223 [373], miR-9 [210, 221, 354] and miR-155 [199, 119, 85] are known to influence malignant progression or cancer cell metastasis. miR-21 and miR-155 could be shown to be differentially expressed during melanoma progression from benign melanocytes to metastatic melanoma [276, 119]. The observed strong accelerating effect on A375 melanoma cell invasion mediated by miR-21 overexpression is in accordance to the published oncogenic role of this miRNA in melanoma [119, 212, 299]. miR-214, mR-223, miR-9 and miR-155 showed a comparable strong increase on A375 cell invasion. As mentioned above, especially miR-155 has a multifunctional role ins several malignancies, including cancer, inflammatory processes and immunity [82, 85]. Thus, due to its inhibitory effect on apoptosis induction miR-155 expression is reported to correlate with malignant progression and might yield cancer diagnostic and prognostic potential [199, 186, 85, 82, 276]. As strongest accelerator of A375 cell invasion miR-576-5p could be identified, which so far has not been investigated for its role in melanoma progression or metastasis and therefore was considered an interesting candidate for further analysis.

Several miRNAs which showed an inhibiting effect on A375 cell invasion during the functional screening are likewise reported for their tumor suppressive role. These are for example, two already mentioned miRNAs miR-101[217] and miR-211 [201, 226], as well as additionally miR-126/126* [88, 89, 92, 233] and miR-194 [245]. The tumor suppressive effect of miR-

126/126* was investigated by several groups in different cancer types [88, 89, 92, 233], such as melanoma [88], breast cancer [330] and gastric cancer[89]. In melanoma, loss of the guide (miR-126) strand of this miRNA duplex is frequently reported in comparison to melanocytes [245, 88]. Despite, the reported tumor suppressive function of miR-126, it was additionally correlated to blood vessel stability and formation, thus promoting tumor progression [92]. Overall, miR-126 seems to interfere with cancer progression on several levels influencing cancer cell survival, adhesion, migration and invasion and its downregulation is investigated for its suitability as prognostic biomarker for poor patient prognosis [287, 153, 73].

In contrast, detailed information about the physiological function of miR-194 is still lacking, though it has been reported to be specifically expressed in hepatic epithelial cells with an important role in liver cancer metastasis *in vivo* [235]. Furthermore, it was found to influence intestinal cell differentiation [142] and was shown to be downregulated during melanoma progression [245].

The tumor suppressive or oncogenic effects of the mentioned miRNAs due to their inhibiting or accelerating effect on melanoma cell invasion observed during the functional screening approach could be shown to be in line with previous publications, supporting the significance of the presented screening result.

Nevertheless, the observed effect on cell invasion might not be solely due to decreased or increased cell invasive capacity, but due to changes in cell viability after miRNA transfection. The strongest impact on inhibition of A375 melanoma cell invasion could be observed for miR-325. miR-325 was recently reported to influence autophagy in cadiomyocytes *in vivo* [35], suggesting that the observed inhibition effect on A375 cell invasion might be due to autophagy induction mediated by miR-325 overexpression. To exclude effects on cell viability an auxiliary cell viability assay was performed investigating 98 of the most promising miRNA candidates identified by the functional invasion screening for their role in melanoma cell viability.

6.2 Putative influence on cell viability of miRNA candidates

miRNA overexpression can influence several different signaling pathways and therefore lead to the induction of a variety of intracellular processes as for example cell proliferation, cell differentiation, cell death or changes in cell motility. Therefore, it was of imminent importance to evaluate the influence of the strongest miRNA candidates on melanoma cell survival to exclude effects on cell viability which would abrogate the observed cell invasive phenotype. The CellTiter Glo (CTG) luminescent cell viability assay was used to assess changes in cancer cell viability. Mostly, cell viability differences were determined 48h post treatment with the respective small molecule [377] or after transfection with distinct small RNA molecules [372] to determine alterations due to this treatment. The A375 cell line is frequently used to investigate changes in cell viability and proliferation in response to melanoma therapeutics, such as Vemurafenib or Dabrafenib [374, 358]. Additionally, the CTG assay was used to perform functional RNA interference screenings to discover genes important for cell death or therapy resistance in the model cell line A375 [358]. Therefore, the CTG assay was performed to exclude cell viability effects influencing the functional invasion screening result. The time-line of the functional screening procedure was maintained to solely assess changes in cell viability, that would affect the screening result directly.

Several miRNA candidates which showed an accelerating effect on melanoma cell invasion, as for example miR-576-5p, miR-483-5p, miR-559-5p, miR-575 and miR-595 displayed minor effects on A375 cell viability. For several of these miRNAs, e.g. miR-576-5p, miR-559, miR-575 and miR-595, their exact physiological functions are currently unknown but interesting expression data are reported. miR-595 was found to be upregulated in malignant mesothelioma samples in comparison to healthy mesothelium by microarray analysis [124]. In hepatocellular carcinoma (HCC) the role of miR-595 is controversially discussed as it is reported on the one hand to be deleted in hepatocellular carcinoma (HCC) patients [386]. Whereas, on the other hand, the group of Luedde et al. [216] could detect increased levels in HCC patient samples indicating a more tumor promoting role of miR-595, in line with the accelerating effect of miR-595 on melanoma cell invasion [216]. Another miRNA candidate, miR-575, seems to be upregulated upon radiation in human colon carcinoma cells [312] or human embryonic stem cells [318] and was found to be upregulated in human gastric patient samples in comparison to normal gastric tissue [380]. miR-576-5p was reported to be mainly found in malignant brain tissue, as it could be shown to be overexpressed in carcinomas metastasizing to the brain and in the brain's temporal lobe of the cerebral cortex in

Alzheimer's disease [205, 353]. miR-483-5p identified in adrenal cortical carcinomas (ACC) and might be used as marker for this cancer type [272, 319]. Whereas, in glioma cells miR-483-5p could be shown to inhibit cell proliferation [351]. For all above mentioned miRNAs, miR-576-5p, miR-483-5p, miR-559-5p, miR-575 and miR-595, their specific role in cancer cell proliferation or invasion remains elusive until now, thus they are reported to be either overexpressed or solely expressed in distinct tumor types indicating their potential role for malignant progression and cancer maintenance.

Overall, most miRNA candidates accelerating A375 cell invasion did not significantly influence melanoma cell viability in this setting. This observation might be due to the short time-line of the assay, used intentionally to minimize cell viability alterations as much as possible during the initial screening procedure. Supporting this theory, only minor effects of miR-101 on cell viability could be observed in this setting, despite the published inhibitory effect of miR-101 on cell proliferation by Luo et al. 2013 [217].

The minority, 13% of all miRNA candidates regulating significantly A375 cell invasion was found to exert an inhibiting effect. Several miRNA candidates, such as miR-1302, miR-127-3p, miR-187*, miR-194 and miR-624 were able to decrease A375 cell viability. Additionally, slight effects on A375 cell viability, could be observed for miR-325, miR-30c-1*, miR-339-3p, miR-31* and miR-301b.

miR-127-3p was reported to be downregulated in osteosarcoma cell lines in comparison to osteoblasts [71], indicating a putative tumor suppressive role which would correlate with the effects on cell viability and invasion in the here presented work.

Effects of individual miRNAs observed in different cancer types might be diverse and are not necessarily comparable [277, 154, 302]. miR-187* reported as a promising serum biomarker for gastric cancer in combination with miR-372-5p and miR-378 [208] contradicting the observed anti-cell invasive and viability effect of this miRNA candidate in melanoma observed in this work. The role of miR-194 in malignant development is controversially discussed, as it has already been reported be able to suppress liver cancer metastasis *in vivo* [235], supporting the effect on cell viability that could be observed in this thesis. In contrary, Sundaram et al. [324] report miR-194 to facilitate angiogenesis in a p53-dependent manner supporting colon cancer progression [324]. No role of miR-30c-1* in tumor cell viability or proliferation has been reported so far. Though, this miRNA candidate could be shown to target HMBOX1 in natural killer (NK) cells and thereby enhance NK cell cytotoxicity [108]. The anti-viability effect that was observed for miR-325 could be explained by its role in programmed cell death, namely in autophagy [35], which might not only be induced in cardiomyocytes as reported [35], but might also affect human melanoma cell viability due to miR-325 overexpression. To further clarify the specific observed phenotype another assay

must be used instead of the CTG assay as this assay measures only changes in cell viability but does not further specify the mode of cell death induction. Overall, several of the most promising miRNA candidates are not investigated for their role in melanoma and their effect on tumor cell invasion and proliferation remains elusive until now.

In a next step, it was of importance to investigate, if the observed effect on cell invasion was primarily or only partially due to abrogated cell viability. Therefore, the observed cell viability effect in relation to the strength of cell invasion observed during the functional screening for the 98 most promising miRNA candidates influencing A375 cell invasion was taken into account. As it could be seen for all miRNA candidates under investigation the observed effect on cell invasion during the functional screening could not be explained only due to abrogated cell viability. Some miRNA candidates with a strong inhibiting effect on A375 cell invasion additionally affected A375 cell viability, as for example miR-1302, miR-127-3p, miR-187*, miR-194 and miR-624. The observed impact of these candidates on melanoma cell invasion might be partly due to its negative effect on cell viability. However, also for these candidates the screening result cannot be explained by minor influences on cell viability, therefore indicating an additive role of these miRNAs on A375 cell proliferation and invasion. Conclusively, the impact on cell viability observed for all miRNA candidates under investigation does not explain the observed changes in cell invasion which indicates their importance for further validation and analysis.

Several miRNAs under investigation for their role in cancer cell invasion are reported in literature to concurrently influence proliferation in the respective cancer cells [217, 214, 243, 295]. Moriyama et al. [243] identified miR-21 as potent modulator of cell physiology in pancreatic cancer cells influencing cancer cell invasion and proliferation [243]. This was supported by the finding, that miR-21 seems to simultaneously affect cell invasion and proliferation in ovarian carcinoma [214], non-small cell lung cancer (NSCLC) [123] and squamous cell carcinoma [143]. More recently miR-210 could be identified in breast cancer as a potent indicator for poor patient survival possibly mediated through induction of increased breast cancer cell invasive and proliferative capacity that could be only shown *in vitro* so far [295].

These observations either indicate miRNAs to affect a distinct signaling pathway influencing cell proliferation as well as invasion or emphasize a global impact on gene expression by specific miRNAs influencing several signaling pathways simultaneously. Therefore, further investigation of specific miRNA candidates was required to identify their physiological role, putative targets and highlight their complex regulatory network.

6.3 miRNA candidate expression profiling

miRNA expression profiling is reported to be able to indicate melanoma development [42], melanoma progression [245] and is generally used to differentiate between different cancer types [215, 40]. Distinct miRNA expression profiles are known to indicate melanoma metastasis potential and malignant transformation [245] as it was shown for members of the miR-200 family by several groups [276, 373, 113].

Since miRNA overexpression within a functional screening approach can lead to the identification of false positive miRNA candidates due to observed artificial, non-physiological effects, it was of interest to investigate the endogenous expression of our miRNA candidates in melanoma cell lines in comparison to melanoncytes.

The expression analysis of miR-325 in melanoma cell lines in comparison to NHEMs showed no significant difference between the two groups. Additionally, miR-325 was almost not detectable in either melanoma cell lines or NHEMs. Therefore, although miR-325 showed the strongest inhibitory effect on A375 cell invasion and it additionally affected A375 cell viability to more than 20%, it was not further investigated. Nonetheless, miR-325 might still be an interesting candidate for cell viability and autophagy investigations as mentioned above [35].

The role of miR-193b in melanoma cells is controversially discussed. Chen at al. [47] reported miR-193b downregulation in metastatic melanoma in comparison to benign nevi and its potent inhibitory effect on cell proliferation *in vitro* [47]. However contradictory, high miR-193b expression in patient melanoma samples was reported to be indicative for poor melanoma patient survival [42]. miR-193b showed a significant difference in expression in NHEMs in comparison to melanoma cell lines, but in very low levels in both cell types.

miR-559-5p, one of the strongest miRNA candidates accelerating A375 cell invasion during the functional screening, did not show a significant different expression in NHEMs in comparison to melanoma cell lines and was only marginally detected by qPCR analysis in both groups. All other miRNAs, miR-30c-1*, miR-339-3p, miR-576-5p, miR-483-5p showed a significant different expression in NHEMs in comparison to melanoma cell lines and were endogenously expressed in either the melanoma cell lines of the NHEMs. miR-576-5p and miR-483-5p were significantly higher expressed in melanoma cell lines in comparison to NHEMs. Additionally, both miRNAs were able to strongly increase A375 cell invasion during the functional screening indicating their possible oncogenic potential in melanoma cell lines. Both miRNAs are reported to be mainly found in brain metastases but a clear correlation with their potential to accelerate metastasis remains elusive [205, 319]. Thus, a potential phys-

iological relevance for both miRNAs in cancer progression and metastasis is indicated and they were considered as interesting candidates for further analysis.

Additionally two miRNA candidates, miR-339-3p, miR-30c-1* with an inhibiting effect on A375 cell invasion shown by the functional screening were significantly higher expressed in NHEMs in comparison to melanoma cell lines. This observation indicates a putative tumor suppressive role for both miRNAs *in vitro*. Neither miR-339-3p, nor miR-30c-1* are currently reported to play a role in cancer metastasis or malignant progression.

To further support the importance of the identified miRNAs on melanoma cell invasion a correlation analysis was performed. Correlation analyses are frequently performed to estimate the relation of gene or miRNA expression to the observed phenotype under investigation. In this respect, miR-10b expression was shown to correlate with glioma cell [322] and human esophageal cancer cell invasion [334]. miRNA expression analyses are often performed to assess miRNA expression profiles with a distinct physiological role in malignant progression. This was shown, for three miRNAs, miR-125b, miR-199a, and miR-100, which could be correlated to gastric cancer progression and are within a set of 22 previously identified miRNAs to be upregulated in gastric cancer [338]. Correlation analysis of miRNA expression and the respective physiological phenotype of interest further elucidates and increase the significance of the miRNA candidates under investigation.

Therefore, the invasive capacity of a penal of melanoma cell lines was determined and correlated to miRNA candidate expression. Melanoma cell lines under investigation either showed a high invasive potential, such as MaMel-19 or A375, an intermediate invasive potential, MaMel-86b or MaMel-68, or almost no invasive potential e.g. MaMel-79b. Nine miRNA candidates with the strongest effect on A375 cell invasion were analyzed to estimate their potential influence on melanoma invasion. Significant correlations were observed for miR-339-3p, miR-30c-1* and miR-576-5p with a a correlation coefficient of 0.24 for miR-576-5p, 0.47 for miR-30c-1* and 0.59 for miR-339-3p. As miR-576-5p and miR-339-3p showed a significant different expression in melanoma cell lines in comparison to NHEMs and were within the strongest miRNA candidates influencing A375 cell invasion both miRNA candidates were considered for further investigation.

Overall, the outlined work-flow, starting with a functional screening approach, followed by cell viability analysis, miRNA expression and correlation analysis, which finally allowed to expand the data set under investigation as additional melanoma cell lines were included, was shown to be a powerful tool to analyze high-throughput data sets and select putative miRNA candidates for further target gene analysis *in silico*, *in vitro* and *in vivo*. Despite the strong effects of the presented miRNA candidates on melanoma cell invasion, supported

by additional miRNA candidate expression analysis, this effect had to be validated and their distinct role in cell physiology or pathophysiology had to be further clarified by identification of potential target genes.

6.4 miRNA target identification

To further validate the effect of specific miRNA candidates, their putative target genes had to be identified. As indicated before, a single miRNA can regulate more than hundreds of different target genes leading to the observed phenotype, e.g. cell proliferation, invasion or cell death [204, 120, 101]. Therefore, the identification of one target gene regulated by one individual miRNA might correlated with the observed functional changes seen during the screening procedure but might not indicate the overall complexity of miRNA induced gene regulation. To understand miRNA networks and complexity of gene regulations by a single miRNA the global impact on gene expression has to be considered [304].

Several miRNA target predictions tools are available at the moment for *in silico* prediction analysis, which mainly try to correlate mRNA:miRNA expression and sequencing data to predict possible regulations [184, 307, 345, 347, 286]. Two databases were combined by Dr. Agnes Hotz-Wagenblatt to define possible target genes, miRBase and TargetScan [117, 182, 204]. These two target prediction programs use different algorithms to identify mRNA:miRNA binding possibility not only in the target genes 3'UTR. Moreover, miRBase additionally indicates mRNA:miRNA interaction sites in the 5'UTR, whereas TargetScan indicates binding sites within the open reading frame of putative target genes as well [204, 116, 117, 181, 182]. Despite the wide range of possible prediction tools and constant development of more precise algorithms based on high-throughput sequencing data many putative target genes remain unidentified by *in silico* prediction analysis or can not be confirmed *in vitro*. Therefore, putative target genes identified by computational algorithms and gene enrichment analysis indicate the direction for subsequent *in vitro* and *in vivo* validation analysis, but do not substitute for it.

The two most interesting miRNA candidates, miR-339-3p and miR-576-5p, were further considered for investigation and several proposed target genes for both miRNA candidates were identified by target prediction analysis. Subsequently the putative target genes were subjected to the Database for Annotation, Visualization and Integrated Discovery (DAVID) [148, 149]. miRNA-339-3p and miR-576-5p were found to substantially influence genes which regulate signaling pathways involved in cell viability, proliferation and cell motility, sup-

porting their observed potency on melanoma cell invasion during the functional screening approach. As shown before, miR-339-3p and miR-576-5p have a slight effect on melanoma cell viability and a strong increasing, or decreasing impact on A375 cell invasion, respectively. The *in silico* identification of putative target genes with a role in cell death, proliferation and cell motility reflects the so far observed phenotype after miRNA candidate overexpression in melanoma cells.

Several of the *in silico* identified putative target genes with a role in apoptosis are targeted by miR-576-5p as for example cell cycle regulators, p35 and p21, or the co-factor of the tumor suppressor p53. p53 requires the co-factor help to function in a tumor suppressive and apoptosis inducible fashion [333]. Therefore, downregulation of the p53 cofactor might indicate a favorable situation for tumor progression and metastasis formation mediated by miR-576-5p overexpression.

Putative targets regulated by miR-576-5p seem to influence distinct biological pathways, as miR-576-5p was predicted to interrogate with apoptosis induction as well as to target Cullin 3 and thereby abrogate cell proliferation. Interleukin 2 as well as Neurofibromin 1 are known to play diverse roles in cell viability and maintenance [223, 198, 132, 11]. Caspase 8 (CASP8) Fas-associated death domain protein (FADD)-like apoptosis regulator (cFLIP, CFLAR) is another overlapping putative target gene, which is reported to have a divergent role in cell death and proliferation [297, 270]. cFLIP molecules are able to determine cell survival or cell death dependent on the activation of either pro-apoptotic or NF-$\varkappa$B-inducing signaling pathways mediated due to different expression levels of the different cFLIP isoforms [270, 97]. Several cytoskeletal components as for example actin, moesin, myosin and tropomyosin 3 are possible targets for miR-576-5p indicating a complex regulation of cell motility and a putative role for this miRNA candidate in cancer metastasis [84, 131, 195]. These putative target genes of miR-576-5p influencing cell motility are of most interest in further analysis as their regulation might directly translate into the observed phenotype during the functional screening. However, putative targets reported to influence cell viability might also play an so far unknown additional role in cell invasion or promote invasiveness by altering intracellular stability towards a more favorable environment for malignant progression.

A similar distribution could be observed for miR-339-3p and its predicted potential target genes. Several genes were indicated by *in silico* analysis, which regulate components of the cytoskeleton, but controversially to miR-576-5p, mainly the tubulin and tubulin polymerization seems to be affected by miR-339-3p overexpression. Actin and tublin-interactions are reported to be important for cell morphology and motility, thus both filament systems

have distinct roles intracellularily [292, 110]. The actin cytoskeleton is mainly responsible for directed cell migration whereas microtubuli are important for bipolar spindle formation and chromosomal segregation [292, 110]. However, the role of microtubuli in maintenance of the polarized actin cytoskeleton could be proven in migrating fibroblasts [343] indicating a combined role in cell physiology and functional motility [110]. The regulation of distinct components of the cell cytoskeleton by miR-339-3p or miR-576-5p might translate into a more or less invasive phenotype. Furthermore, both miRNAs affect expression of cytoskeleton remodeling proteins, namely non-catalytic region of tyrosine kinase (NCK) adaptor protein 2 is targeted by miR-339-3p, whereas miR-576-5p downregulates NCK adaptor protein 1. NCK2 is known to support melanoma invasion *in vitro* and proliferation *in vivo* [187]. Furthermore, NCK2 was found to be primarily upregulated in metastatic melanoma cell lines in comparison to primary melanoma cell lines or melanocytes [187]. Contradictory, NCK2 up-regulation was additionally correlated with down-regulation of specific cell adhesion molecules [187]. However, no significant difference could be seen for NCK1 expression in different melanoma cell lines or melanocytes [187]. In carcinoma cell lines NCK1 influences actin remodeling required for invapodia formation [266]. On the one hand NCK2 seems to play a more promoting role in melanoma cell invasion that might be abrogated by miR-339-3p overexpression, supporting its observed inhibiting impact on melanoma cell invasion. On the other hand miR-576-5p might induce a complex actin skeleton rearrangement in melanoma cell lines by targeting various actin remodeling proteins, namely NCK1, tropomyosin, tropomodulin, moesin and actin accelerating melanoma cell invasion [83, 343, 195].

In addition to processes influencing cell motility miR-339-3p might also impact on cell proliferation and survival. Several known mediators of cellular life and death decisions as for example cFLIP, tumor necrosis factor (TNF) receptor-associated factor 3 and the oncogene pim-2 are listed as putative targets [270, 297, 247, 202]. Furthermore, protein kinase C (PKC) might be affected which plays an important role in signal transduction influencing a variety of intracellular signaling pathways to induce cell proliferation and survival [259, 368]. Interestingly, another putative target of miR-339-3p, the myeloid cell leukemia sequence 1 (MCL1), a known oncogene in melanoma is already investigated for its potential for antisense therapy in the clinics. MCL1 overexpression was reported to be indicative for a poor prognosis in melanoma patients and might counteract therapies targeting BRaf and MEK. Therefore, not only MCL1 downregulation, but targeting anti-apoptotic molecules in general is highly investigated in clinics with small molecule inhibitors as for example Obatoclax [254]. The role of MCL1 in cancer initiation and progression makes it an interesting candidate for further analysis and validation in respect of miR-339-3p target analysis.

This variety of genes potentially regulated by a single miRNA already indicates the com-

plexity and difficulties of miRNA target identification and the importance of individual evaluation and *in vitro* validation. Therefore, in a next step the observed invasion effects during the functional screening were validated in an independent invasion assay as well as putative targets for miR-339-3p were confirmed *in vitro,* respectively.

6.5 miR-339-3p and miR-576-5p affect melanoma cell invasion

The functional screening and the additional cell viability assay was solely performed in the melanoma cell line A375. To validate and highlight the physiological importance the effect of the respective miRNA candidates in additional cell lines *in vitro* as well as *in vivo* were of further importance. Five different melanoma cell lines with different invasive potential could be shown to be influenced by miR-339-3p or miR-576-5p overexpression. Without transfection of any miRNAs A375 and MaMel-103 showed a high, MaMel-86b an rather intermediate and MaMel-61e a low invasive potential. WM 266.4 is a highly invasive melanoma cell line derived from a skin metastasis of the same patient with the primary tumor WM 155 [273, 291]. The accelerating or inhibitory effect on melanoma cell invasion upon miRNA candidate overexpression could be validated for all five cell lines. Overall, the invasive potential influenced was accelerated the most in less invasive cell lines, e.g. MaMel-61e and MaMel-86b, whereas invasion inhibition was more pronounced in the high invasive melanoma cell lines A375 and WM 266.4. These physiological differences might account for the distinct context specific physiological effects in these melanoma cell lines.

While accelerated invasion could be observed in all melanoma cell lines after miR-576-5p overexpression in the independent assay, it was not as strong as during the screening. This might be due to two small changes within the protocol: an exact number of cells was seeded into the Boyden chamber inserts and a prolonged invasion time of 24h was used for this independent invasion assay. miR-339-3p, the miRNA candidate with inhibitory effect on melanoma cell invasion could be shown to reduce invasive behaviour of all cell lines under investigation. Its inhibitory effect could be shown to be strongest in the melanoma cell lines WM 266.4, MaMel-103b and A375, the cell lines with a more potent invasive potential.

So far, miR-339-3p could be shown to be a potent inhibitor of melanoma cell invasion, after miR-339-3p overexpression. To further support its significant role in melanoma cell invasion, endogenous miR-339-3p was knocked down in three different cell lines, MaMel-61e and

MaMel-79b, which showed a rather high endogenous miR-339-3p expression in comparison to A375. In all three cell lines an increased invasion in the miR-339-3p inhibitor (anti-miR) transfected cells could be observed in comparison to mock treated cells, thus confirming the physiological relevance of miR-339-3p.

Overall miR-339-3p inhibited melanoma cell invasion significantly in five different melanoma cell lines and transfection with antagomiR in cell lines with high endogeneous miR339-3p levels accelerates cell invasion. Moreover miR-339-3p does not significantly influence melanoma cell viability in the here presented experimental approach. To further highlight the importance of miR-339-3p on melanoma cell invasion, its target genes had to be identified and verified *in vitro* and *in vivo*.

6.6 MCL1, a novel target of miR-339-3p

Several putative miR-339-3p targets were already mentioned above as a result of the *in silico* database analysis. Due to its importance as oncogene MCL1, the myeloid cell leukemia sequence 1, was further investigated for its role in melanoma cell invasion and as putative miR-339-3p target gene *in vitro*. In an independent invasion assay MCL1 knockdown was able to inhibit melanoma cell invasion in all five melanoma cell lines under investigation to different extent. The inhibitory effect on melanoma cell invasion was comparable to the effect of miR-339-3p overexpression in the respective cell line, suggesting an influence of MCL1 on melanoma cell invasion.

Until now, MCL1 was reported to play an anti-apoptotic role in melanoma cell survival, cell death induction, proliferation and EMT [230, 62, 189, 288]. Except cell survival and apoptosis MCL1 is reported to influence several intracellular signaling pathways which play a promoting role during cancer progression and invasion, as it is thought to additionally interfere with anoikis induction inducing a favoring environment for cancer cell dissemination [37, 364]. Anoikis, one specific program for cell death induction, is activated once a cell lost contact to its surrounding cellular network, the extracellular matrix or does appear in an unphysiological position in the body [175]. Therefore, anoikis resistance is known to be one of the hallmarks of cancer as it enables migrating and invading cancer cells to avoid cell death induction [175, 133]. MCL1 degradation in combination with increased Bim expression, a pro-apoptotic member of the Bcl-2 family [260], is thought to be crucial for anoikis induction and prevention of metastases formation in breast cancer cells [364]. Additionally MCL1 could be shown to prevent BRaf mutated melanoma cells from anoikis induction, that was restored

after MCL1 knockdown [37].

MCL1 is one anti-apoptotic member of the Bcl-2 family of proteins which is known to induce pro-survival pathways intracellularily [331, 237, 325]. As Bcl-2 family members regulate apoptosis and cell survival, an overall balanced level of pro- as well as anti-apoptotic members of this family is required for a physiological situation within cells [237, 362, 388]. MCL1 expression is important for functional development and immunity as for example during embryogenesis and lymphocyte development [289, 264]. Due to its anti-apoptotic role, MCL1 is widely investigated for clinical therapies to target cell survival pathways which interfere with cancer cell progression and metastasis induction [25, 258, 362]. Several therapeutic applications, which aim to inhibit oncogenic BRAF and MEK, seem to additionally require MCL1 downregulation, as it seems to be crucial for melanoma cell resistance to cytotoxicity [362, 388, 189].

MCL1 exists in three different splice variants, two short variants, MCL1 short (MCL-1S) and MCL1 extra short (MCL-1ES) and one long version MCL1 long (MCL- 1L) [331, 171, 172, 310, 16]. All three splice variants exhibit the same 3'UTR (Figure 22), however possible protein:protein interaction domains, e.g. two of three Bcl-2 homology domains (BH1 and BH2) and the transmembrane domain (TM) that allows it localization to intracellular membranes [5, 331] are solely present in MCL-1L. Additionally, MCL-1L and MCL-1S proteins consist of one domain rich in proline, glutamic acide, serine and threonine amino-acids, called a PEST sequence [5] that is missing in the MCL-1ES isoform. This sequence mediates protein degradation and might therefore account for the short half life that is reported for MCL-1L and MCL-1S isoforms, of only a few hours [331, 5, 63, 237]. All three splicing variants of MCL1 inherit the same 3'UTR, indicating a simultaneous regulation of all isoforms by one miRNA with a binding site within this 3'UTR [5]. The two short splicing variants of MCL1 are reported to have pro-apoptotic functions whereas MCL-1L is know for its pro-survival effect in various cell types, including cancer cells [172, 171, 237]. Therefore a thight balance of MCL1 isoform expression might be indicative for cell survival or cell death decisions. As MCL1 expression could be linked to melanoma progression it was of no surprise to find the pro-survival isoform, MCL-1L, upregulated in melanoma cell lines dependent on the BRaf mutational status in comparison to primary human melanocytes [230]. As most reports do not distinguish between the different MCL1 isoforms, the anti-apoptotic isoform, MCL-1L is referred to as MCL1.

A high MCL1 expression could also be observed in most melanoma cell lines under investigation in this work. In more invasive melanoma cell lines, e.g. A375, WM 266.4, MaMel-103b, MaMel-19 and MaMe-86b higher MCL1 protein levels in comparison to less invasive cell lines as for example MaMel-79b or MaMel-61e. All cell lines express the pro-survival

MCL-1L and show different protein expression levels of the short MCL-1S and MCL-1ES isoforms. Melanoma cell lines WM 98.1, MaMel-79b and MaMel-103b seem to express high endogenously both MCL1 short isoforms. Pro-survival MCL-1L was higher expressed in cell lines with a more invasive phenotype, A375, MaMe-103b and WM 266.4, however some of these cell lines, namely A375, MaMel-103b, also express the short MCL1 isoforms indicating a thightly regualted balance of MCL1 in cell survival and apoptosis. 48h after miR-339-3p overexpression all three isoforms present in the melanoma cell lines A375, WM 266.4 were downregulated on protein and mRNA level. Furthermore, MCL1 mRNA levels were partially restored 72h post transfection. This might result from the short half life of MCL1 and the necessity to tightly balance its intracellular expression in respect of cell physiology, cell survival and apoptosis [237, 331]. These results indicate a cooperation between miR-339-3p overexpression and subsequent MCL1 downregulation, with direct targeting of MCL1 3'UTR by miR-339-3p as the most likely regulatory mechanism. To further support this hypothesis, miR-339-3p could be shown to downregulate MCL1 protein and mRNA expression in different melanoma cell lines.

The influence of MCL1 downregulation by miRNAs is widely investigated due to its potential in sensitizing malignant cells to apoptosis. This was shown by a functional screening for cell death induction upon Bcl-2 inhibitor (ABT-263) treatment, where several miRNAs, as for example miR-29b, miR-101, miR-153, and miR-193, were identified to induce apoptosis after ABT-263 treatment in HCT-116 cells resistant to Bcl-2 family inhibitor treatment [188].

Several miRNAs are reported for their role in MCL1 regulation and modulation of cancer cell death and survival [48, 109, 219, 244, 365, 198]. miR-139b overexpression could be shown to sensitize melanoma cells to Bcl-2 inhibitor (ABT-737) to undergo apoptosis, due to the downregulation of MCL1 [48]. Recently, miR-139b knockdown was reported to significantly inhibit head and neck squamous cell carcinoma (HNSCC) progression and invasion by directly targeting neurofibromin 1 in these cells [198]. Both results indicate that one single miRNA is potent enough to regulate several signaling pathways influencing cancer survival, progression and metastasis.

Additionally, miR-125b could be shown to influence MCL1, IL-6R and an another Bcl-2 family member, Bcl-w, expression leading to decreased hepatocellular carcinoma (HCC) survival and progression [109]. Controversially, the group of Wu et al. [365] could show that miR-125b is a marker of poor patient prognosis due to its accelerating effect on gastric cancer cell invasion [365]. These contradictory findings might indicate a complex regulatory network, where small changes direct complex intracellular signaling cascades. miRNA alterations might therefore result in completely different phenotypes depending on cell type, differentiation or malignant state of the respective cell. These reports would support the

hypothesis, that miR-339-3p might inhibit melanoma cell invasion via a complex network of signaling events that require MCL1 downregulation as an intermediate step in the downstream signaling cascade. Thus, a direct regulation of MCL1 by miR-339-3p was anticipated as all isoforms, if present in the respective cell lines, were regulated simultaneously.

6.6.1 MCL1 as direct target of miR-339-3p

MCL1 was predicted as target of miR-339-3p by database analysis and supported by the finding, that miR-339-3p is able to regulate MCL1 protein and mRNA expression. Therefore, the mode of interaction of miR-339-3p and MCL1 3'UTR was of immanent interest and investigated by a luciferase binding assay. Two putative binding sites in the MCL1 3'UTR were mutated to abrogate putative miR-339-3p binding at the respective 3'UTR region, which would be translated into a restored luciferase signal. It could be shown that miR-339-3p decreased the observed luciferase activity indicating an interaction of miR-339-3p with the 3'UTR of MCL1. In contrast, the direct interaction can only be proven by restoration of the observed decreased luciferase signal after MCL1 3'UTR mutation at the respective predicted binding sites of miR-339-3p.

Mutation of binding site one did not result in a significant change in luciferase activity indicating no direct interaction of miR-339-3p and this respective binding site. Thus, the attenuated luciferase activity was restored to almost 100% after mutation of the second binding site in two different melanoma cell lines, WM 266.4 and A375 indicating a direct functional interaction of miR-339-3p with this region of the MCL1 3'UTR. Unfortunately, due to side-effects during the process of site-directed mutagenesis, several additional mutations were introduced ancillary to the intended mutation. Therefore, the luciferase restoration effect observed, cannot be clearly contributed to the abrogated binding on the predicted binding site as it may also be due to mutations in neighboring regions of the MCL1 3'UTR. Overall, it can be hypothesized that miR-339-3p does directly bind to the 3'UTR of MCL1 indicated by the strong luciferase restoration effect that can be observed after introduction of several distinct mutations.

6.6.2 miR-339-3p does not promote melanoma cell death induction

miR-339-3p could be shown to inhibit melanoma cell invasion and target MCL1 expression. As MCL1 is known to play a role in intracellular cell death and survival decisions mediating cancer cell resistance to therapies [325, 364, 372, 389], the role of miR-339-3p on melanoma cell death induction was investigated. miR-339-3p did not strongly induce melanoma cell death in the melanoma cell lines, WM 266.4 and A375. In contrast, consistently with previous reports, MCL1 knockdown did result in increased cell death observed in both melanoma cell lines [325, 364, 372, 389]. MCL1 downregulation, might be one important regulatory mechanism among others, to mediate the observed miR-339-3p dependent invasion suppressive activity.

Several studies focusing on tumor suppressive miRNAs could correlate MCL1 downregulation with an increased cell death induction in the respective cancer cells [371, 372, 219, 48]. However, successful cell death induction could not be mediated by MCL1 downregulation alone, additional death receptor activation or outside stimuli were required [244, 257, 63, 109]. This could be shown on the one hand by Mott et al. for miR-29b, which directly targets MCL1 and therefore sensitizes cholangiocarcinoma cells to trail-mediated apoptosis induction [244]. One the other hand MCL1 knockdown did require additional ultraviolet irradiation to successfully induce apoptosis in HeLa cervical cancer cells reported by Nijhawan et al. [257].

Supported by these reports our findings suggest a specific interplay of several signaling pathways regulated by miR-339-3p, which on the one hand affect MCL1 expression and therefore provide a less favorable intracellular environment for melanoma cell invasion and on the other hand might influence melanoma cell invasion directly by abrogating expression of currently unknown target genes. Therefore, a more global impact of miR-339-3p on melanoma cell intracellular signaling had to be assessed.

6.7 miR-339-3p induces specific gene expression profiles in melanoma cell lines

miR-339-3p could be shown to influence melanoma cell invasion and deregulate MCL1 expression. In order, to assess global mRNA expression changes a microarray assay was used to compare gene expression of melanoma cells transfected with miR-339-3p to untreated

melanoma cells. Overall, more genes were significantly downregulated than upregulated in response to increased miR-339-3p expression in both cell lines, correlating with miRNAs known potency in target gene knockdown [9, 10]. No genes could be found to be more than two fold deregulated overlapping in both cell lines after miR-339-3p overexpression. However, overall more than 3000 genes could be found to be significantly differentially regulated after miR-339-3p overexpression in both melanoma cell lines. In respect of target gene selection for miR-339-3p, only 36 genes identified by database analysis could be confirmed as being deregulated by miR-339-3p overexpression via microarray gene expression analysis simultaneously in both different melanoma cell lines, including MCL1. This might be due to specific algorithms of the databases used to identify miRNA targets, that are mainly focusing in miRNA:mRNA duplex formation due to seed paring, whereas seedless pairing gains increased attention and seems to be as important in miRNA-target interaction [290]. While a wide range of additional miRNA:mRNA interaction possibilities are not covered by these algorithms yet, additional putative targets cannot be predicted *in silico* that can be detected by genexpression analysis [61, 379, 182, 290]. Thus, miR-339-3p dependent gene expression analysis indicates the global pattern of target regulation independent of its direct and indirect effects.

MCL1, which was predicted by database analysis, was differentially regulated in both cell lines after miR-339-3p overexpression. In the model cell line A375 miR-339-3p overexpression resulted in a significant decrease of MCL1 gene expression, whereas in the MaMel-86b the opposite effect was observed. This observation can be explained by substantial differences in the invasiveness of these melanoma cell lines, while A375 shows a strong invasive capacity, MaMel-86b invades rather weakly. A higher level of endogenous miR-339-3p expression might abrogate its intracellular impact in MaMel-86b cells in comparison to the cell line A375. Overall, a distinct context specific regulation giving rise to a high complexity, includes both direct and indirect miRNA target effects. Different intracellular signaling pathways influenced by miR-339-3p overexpression might mediate the observed inhibition of cell invasion.

The performed gene enrichment analysis indicated comparable signaling networks being influenced by increased miR-339-3p overexpression in both melanoma cell lines. Supporting our *in vitro* findings of miR-339-3p inhibiting invasion in melanoma cell lines, networks influencing developmental processes, inflammation, proliferation but also cell adhesion processes and chemotaxis are particularly regulated by miR-339-3p overexpression in MaMel-86b cells whereas inflammation, developmental processes and cell adhesion are mainly affected in A375 cells. However, no cell viability or cell death effects of miR-339-3p were observed in our setting, which might indicate distinct physiological phenotypes induced after miR-339-3p overexpression maybe at later time points post transfection. Furthermore, inflammatory

processes which might favor melanoma progression and might be inhibited by miR-339-3p overexpression were not investigated so far. On a more long-term basis miR-339-3p might influence melanoma cell viability and inflammation processes, whereas a more immediate mechanism induces alterations in melanoma cell adhesion and therefore inhibits melanoma cell invasion as it could be observed during the functional invasion screening. For further clarification, time-lapse experiments should be conducted to unravel possible time dependent effects of miR-339-3p on melanoma cell viability. Inflammation processes deregulated by miR-339-3p can be further investigated by identification of additional targets genes to elucidate the exact mechanisms and physiological changes of miR-339-3p influencing cell survival and inflammatory stress responses.

These results reflect the complexity of miRNA regulation where identical pathways are regulated by distinct genes and on different levels, possibly leading to a similar physiological outcome. miR-339-3p might interfere on different levels with expression of various genes which alone might not induce certain phenotypes but altogether are potent enough to mediate the here presented invasion inhibiting phenotype.

Most studies at the moment are focusing on miRNA profiles to distinguish cancer types or detect malignant progression [36, 45, 129, 197, 205]. Therefore, studies comparing gene expression profiles after specific miRNA deregulation to further highlight differences within the same cancer type, are still lacking. However, different melanoma subtypes were investigated for their distinct miRNA profile, indicating significant differences in the physiological gene expression as well as miRNA expression profile between these subtypes [45]. Additionally, as it could be seen in the microarry data presented here, that every melanoma cell line exhibits a distinct gene expression profile after miRNA transfection indicating a distinct physiological status even before miRNA treatment. The analysis of additional melanoma cell lines as well as mock transfection as proper control would further clarify specifically deregulated signaling pathways induced by miR-339-3p overexpression, since even small changes in the miRNA profile might induce distinct phenotypes dependent on the physiological state of the respective cells before treatment. Finally, *in vivo* analysis of miR-339-3p overexpression impact on melanoma metastasis was required to clarify its potency to inhibit melanoma cell invasion.

6.8 Abrogated lung metastasis formation by miR-339-3p overexpression *in vivo*

All results presented so far indicate a tumor suppressive role, induced by inhibition of invasion, for miR-339-3p in melanoma. To further support this hypothesis, the effect of miR-339-3p on melanoma metastasis formation was elucidated in a lung metastases assay in immunodeficient NSG mice *in vivo*.

Lung metastasis assays in NSG mice, performed with patients melanoma samples, could be shown to be clinical relevant as the metastasis rate and spontaneous metastasis formation in the lungs correlated with clinical outcome in these melanoma patients [283]. Therefore, a lung metastasis assay in NSG mice was performed with A375 cells stably overexpressing miR-339 (A375 miR-339), the control A375 cell line transfected with the empty vector (A375 empty) and parental A375 cells. Fourteen days post injection all mice showed lung metastasis formation. However, mice injected with A375 miR-339 showed significantly less lung metastases then control mice receiving A375 parental or A375 empty cells. Decreased lung metastasis formation in mice after A375 miR-339 *i.v.* injection correlated with the observed decreased invasive potential of this cell line due to miR-339-3p overexpression in the functional invasion screening supporting our hypothesis for miR-339-3p as a putative tumor suppressor in melanoma.

Additionally, mice injected with A375 miR-339 and A375 empty cells showed liver metastasis, which were not observed in livers of mice injected with parental A375 cells. A375 cells are reported to form lung metastasis in NSG mice but the appearance of liver metastasis was not reported so far [43, 180].

Recently, a paper was published proposing a newly identified immature NK cell subset which resides in the liver and interferes with hepatic metastasis formation of B16 melanoma cells after *i.v.* injection into wild type black 6 (B6) mice [22]. Depletion of this specific NK cell subset enabled liver metastasis formation in this murine B16 melanoma model. Lung metastasis formation was not affected, as this specific NK cell subset was found to be restricted to the liver. Conclusively, they could show that hepatic NK cells need to be present to prevent liver colonization by B16 melanoma cells and propose a suppressive role in B16 melanoma metastasis formation for distinct organ-specific NK cell subsets, so far mainly investigated in the liver [22]. These results suggest an important role of immune cells in modulating melanoma metastasis formation. Thus, NSG mice used in the reported lung metastases assay, lack NK cells or any other immune cells which could influence melanoma cell dissemination and metastases formation. Nevertheless, the appearance of hepatic colonization

might be due to the lack of this specific immature NK cell subset [22] and in combination with altered surface molecule expression favor hepatic colonization in these mice.

Irrespective, of the so far not described mechanism of liver metastasis formation in mice injected with A375 cells, A375 miR-339-3p cells induced the formation of viewer metastasis and of smaller size than mice receiving A375 empty cells. Consequently, A375 cells stably transfected with this specific vector seem to acquire the capability to form liver metastases. The question arises, if due to vector integration tumor suppressor genes are silenced inducing a more aggressive form of A375 cells or altered cell surface molecule expression patterns enforce A375 cells to colonize preferentially to the liver at an earlier time point. The organ specificity of a distinct tumor system is mediated by its own mutagenenic properties and the host microenvironmental influences. Subsidiary metastases might occur due to stochastic events and molecular modulation of A375 cell adhesion and invasion properties. As liver metastases were observed in NSG mice three weeks post *i.v.* injection of $1x10^6$ A375 cells, liver colonization might be a secondary time dependent phenomenon in this model system. The integration site of the empty vector needs to be further elucidated to clarify the observed effect. This would indicate either a preferred integration site for this vector at one specific position or a selective advantage of specific clones *in vitro* as well as *in vivo*.

Overall, a complex regulatory mechanisms post miR-339-3p upregulation inhibits metastasis formation in the lung as well as in the liver of immunosuppressed mice. Despite, the decreased metastasis formation in the lungs and livers observed in NSG mice after injection with A375 miR-339-3p overexpressing cells, the mechanism of liver metastasis formation has to be further clarified.

6.9 Conclusion and outlook

In conclusion, a high throughput functional miRNA invasion screening was performed successfully and miRNAs inhibiting or accelerating melanoma cell invasion were identified. Specifically, miR-339-3p was validated as new tumor suppressor miRNA and was found to be downregulated in melanoma cells. Furthermore, MCL1 was unraveled as a new specific target of miR-339-3p and MCL1 knockdown by siRNA was able to mimic the phenotype of miR-339-3p overexpression in melanoma cell lines.

To understand the full range of miR-339-3p regulation in melanoma, global changes in gene expression patterns after miR-339-3p overexpression should be extended by using additional melanoma cell lines. These results will help to depict the most prominently occurring

alterations in gene expression in response to miR-339-3p activity. As single miRNAs might impact on complex intracellular signaling networks, direct and indirect effects determine their physiological influence and have to be considered. Subsequently, newly identified target genes by gene expression analysis can be validated by AGO2 immunoprecipitation for their direct interaction with miR-339-3p in melanoma cells.

Overall, a more comprehensive understanding of the miR-339-3p:target network might lead to future therapeutic applications of miR-339-3p in melanoma, especially in respect of the cellular context and the malignant state. Despite promising *in vivo* data presented here, indicating abrogated metastasis formation in NSG mice after *i.v.* injection of miR-339-3p overexpressing A375 melanoma cells, the appearance of unexpected liver metastases has to be further investigated. Clones of the stable miR-339-3p overexpressing and especially control vector transfected A375 cell lines should be established and used for future in vivo experiments. These clones can subsequently be analyzed for genomic insertion of the insert which might help to understand the unexpected phenotype of the control cell line resulting in increased hepatic colonization in NSG mice.

Conclusively, miR-339-3p is a new promising tumor suppressive miRNA candidate, thus further preclinical studies are required to determine its therapeutic potential in melanoma progression and invasion *in vivo.* Since the beginning of miRNA research miRNAs have emerged as essential regulatory molecules in various malignancies which might yield promising therapeutic and diagnostic potential in the future.

Part 7

Supplement

50 of the most promising miRNA candidates accelerating or inhibiting A375 cell invasion are listed here, the complete Table S1 and S2 for all 988 miRNA candidates will be presented in digital form attached to the here presented work.

Table S1: Significance test for the invasive effects of 50 miRNA candidates in the A375 cell line in comparison to the irrelevant control cel-miR-342 (ctrl1 miRNA).

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	882	902.0977567	1.0227866	40.58	<0001
Error	2121	53.4573924	0.0252049		
Corrected Total	3003	955.5551492			

Note: This test controls the Type I experiment wise error for comparisons of all treatments against a control

Comparisons significant at the 0.05 level are indicated by ***				
miRNA Comparison	**Difference Between Means**	**Simultaneous 95% Confidence Limits**		
hsa-miR-576-5p - cel-miR-243	2.06983	1.68985	2.44980	***
hsa-miR-554 - cel-miR-243	2.00980	1.62983	2.38977	***
hsa-miR-483-5p - cel-miR-243	1.9707	1.59073	2.35068	***
hsa-miR-559 - cel-miR-243	1.94073	1.56076	2.32070	***
hsa-miR-206 - cel-miR-243	1.92378	1.54381	2.30375	***
hsa-miR-2054 - cel-miR-243	1.75757	1.37760	2.13754	***
hsa-miR-578 - cel-miR-243	1.74991	1.36993	2.12988	***
hsa-miR-575 - cel-miR-243	1.72654	1.34657	2.10651	***
hsa-miR-202 - cel-miR-243	1.70262	1.32265	2.08259	***

hsa-miR-1200 - cel-miR-243	1.69209	1.31212	2.07207	***
hsa-miR-200b - cel-miR-243	1.68529	1.30532	2.06526	***
hsa-miR-20b - cel-miR-243	1.67454	1.29457	2.05451	***
hsa-miR-507 - cel-miR-243	1.66553	1.28556	2.04551	***
hsa-miR-595 - cel-miR-243	1.65485	1.27488	2.03482	***
hsa-miR-2052 - cel-miR-243	1.62927	1.24930	2.00925	***
hsa-miR-611 - cel-miR-243	1.61860	1.23863	1.99857	***
hsa-miR-205* - cel-miR-243	1.60648	1.22651	1.98645	***
hsa-miR-202* - cel-miR-243	1.56418	1.18421	1.94415	***
hsa-miR-21 - cel-miR-243	1.55159	1.17162	1.93156	***
hsa-miR-518b - cel-miR-243	1.53961	1.15964	1.91958	***
hsa-miR-498 - cel-miR-243	1.53842	1.15844	1.91839	***
hsa-miR-661 - cel-miR-243	1.53428	1.15431	1.91426	***
hsa-miR-632 - cel-miR-243	1.53245	1.15248	1.91242	***
hsa-miR-640 - cel-miR-243	1.52985	1.14988	1.90982	***
hsa-miR-2053 - cel-miR-243	1.51840	1.13843	1.89837	***
hsa-miR-301b - cel-miR-243	-0.75754	-1.13751	-0.37757	***
hsa-miR-139-5p - cel-miR-243	-0.76758	-1.14755	-0.38761	***
hsa-miR-129* - cel-miR-243 1	-0.77213	-1.1521	-0.39216	***
hsa-miR-125a-3p - cel-miR-243	-0.81255	-1.19252	-0.43258	***
hsa-miR-194 - cel-miR-243	-0.82678	-1.10310	-0.55046	***
hsa-miR-127-3p - cel-miR-243	-0.84504	-1.22501	-0.46507	***
hsa-miR-196b* - cel-miR-243	-0.88194	-1.26191	-0.50197	***
hsa-miR-523 - cel-miR-243	-0.89000	-1.26997	-0.51003	***
hsa-miR-660 - cel-miR-243	-0.89769	-1.27767	-0.51772	***
hsa-miR-1248 - cel-miR-243	-0.89838	-1.27835	-0.51841	***
hsa-miR-198 - cel-miR-243	-0.90694	-1.28692	-0.52697	***
hsa-miR-192* - cel-miR-243	-0.91787	-1.29784	-0.53790	***
hsa-miR-659 - cel-miR-243	-0.92156	-1.30153	-0.54159	***
hsa-miR-331-3p - cel-miR-243	-1.00843	-1.38840	-0.62846	***
hsa-miR-31* - cel-miR-243	-1.02517	-1.40514	-0.64519	***
hsa-miR-193b - cel-miR-243	-1.05052	-1.43049	-0.67055	***
hsa-miR-187* - cel-miR-243	-1.07600	-1.45597	-0.69603	***
hsa-miR-302d - cel-miR-243	-1.08544	-1.46541	-0.70547	***
hsa-miR-211 - cel-miR-243	-1.10715	-1.48712	-0.72718	***
hsa-miR-549 - cel-miR-243	-1.11725	-1.49722	-0.73728	***

hsa-miR-342-3p - cel-miR-243	-1.13354	-1.51351	-0.75357	***
hsa-miR-624 - cel-miR-243	-1.15976	-1.53973	-0.77979	***
hsa-miR-339-3p - cel-miR-243	-1.51124	-1.89121	-1.13127	***
hsa-miR-30c-1* - cel-miR-243	-1.51997	-1.89994	-1.14000	***
hsa-miR-325 - cel-miR-243	-1.91813	-2.29810	-1.53815	***

Table S1: Significance test for all miRNA mimics tested for their effect on A375 cell invasion. Each MFI value, normalized to the mock control, was logarithmized to control for the normal distribution. Then a one way anova was performed in the first step and a Dunnetts test in the second step to calculate significance of specific miRNA candidates to the control (cel-miR-243). Comparisons significant at the 0.05 level are indicated by ***.

Table S2: 50 strongest miRNA candidates tested for their effect on A375 cell invasion

miRNA Candidates	**Mean Fluorescence Intensity (MFI)**	**Standard Deviation (STD)**	**z-score**
cel-miR-243	*0.96*	*0.08*	*-0.46*
ath-miR-416	*1.01*	*0.08*	*0.41*
hsa-miR-182	*1.76*	*0.06*	*0.38*
hsa-miR-101	*0.45*	*0.05*	*-0,99*
hsa-miR-576-5p	**7.60**	**0.24**	**6.48**
hsa-miR-554	7.16	0.07	6.02
hsa-miR-483-5p	6.95	1.15	5.80
hsa-miR-559	6.68	0.12	5.52
hsa-miR-206	6.57	0.17	5.40
hsa-miR-19b	5.87	0.40	4.67
hsa-miR-2054	5.57	0.30	4.36
hsa-miR-578	5.52	0.10	4.31
hsa-miR-575	5.39	0.17	4.17
hsa-miR-202	5.26	0.05	4.04
hsa-miR-1200	5.21	0.12	3.98
hsa-miR-200b	5.17	0.04	3.94
hsa-miR-20b	5.12	0.28	3.89
hsa-miR-507	5.07	0.10	3.84
hsa-miR-595	5.02	0.24	3.78
hsa-miR-2052	4.91	0.59	3.67
hsa-miR-611	4.85	0.35	3.61
hsa-miR-205*	4.80	0.50	3.55
hsa-miR-518a-3p	4.67	0.06	3.42
hsa-miR-202*	4.60	0.44	3.35
hsa-miR-21	4.54	0.40	3.28
hsa-miR-518b	4.47	0.07	3.21
hsa-miR-498	4.47	0.09	3.21

hsa-miR-632	4.45	0.40	3.19
hsa-miR-661	4.45	0.16	3.19
hsa-miR-640	4.44	0.37	3.18
hsa-miR-2053	4.38	0.25	3.12
hsa-miR-301b	0.45	0.05	-0.99
hsa-miR-139-5p	0.45	0.05	-0.99
hsa-miR-129*	0.44	0.03	-0.99
hsa-miR-1302	0.43	0.01	-1.00
hsa-miR-125a-3p	0.43	0.02	-1.01
hsa-miR-127-3p	0.41	0.01	-1.01
hsa-miR-196b*	0.40	0.01	-1.03
hsa-miR-523	0.39	0.03	-1.04
hsa-miR-660	0.39	0.02	-1.05
hsa-miR-1248	0.39	0.02	-1.05
hsa-miR-198	0.39	0.01	-1.05
hsa-miR-192*	0.38	0.01	-1.05
hsa-miR-659	0.38	0.01	-1.06
hsa-miR-331-3p	0.35	0.06	-1.06
hsa-miR-31*	0.34	0.01	-1.10
hsa-miR-193b	0.34	0.01	-1.11
hsa-miR-187*	0.33	0.03	-1.12
hsa-miR-302d	0.32	0.01	-1.13
hsa-miR-194	0.32	0.01	-1.13
hsa-miR-211	0.32	0.02	-1.13
hsa-miR-549	0.31	0.01	-1.14
hsa-miR-342-3p	0.31	0.05	-1.14
hsa-miR-624	0.30	0.01	-1.15
hsa-miR-339-3p	**0.21**	**0.01**	**-1.24**
hsa-miR-30c-1*	0.21	0.03	-1.24
hsa-miR-325	0.14	0.02	-1.31

Table S2: 50 strongest accelerating or inhibiting miRNA candidates in the functional invasion assay screening. Mean fluorescence intensity (MFI) directly correlates with the amount of invaded cells 48h post miRNA transfection The irrelevant controls (cel-miR-243, ath-miR-416) and positive controls miR-101 and miR-182 are listed on top, indicated in *italic* letters. Data is represented as mean of technical replicates (MFI) and standard deviation (STD). The z-score represents the distance between the raw score and the

population mean in units of the standard deviation. miRNA candidates, miR-339-3p and miR-576-5p are highlighted in the **bold** letters.

Part 8

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Part 9

Publications and Presentations

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Publications

1. Weber CEM, Luo C, Hotz-Wagenblatt A, Wolfram O, Holland-Letz T and Eichmüller SB. *Functional invasion assay identifies miR-339-3p as potential new tumorsuppressor in melanoma.* (in prep.)

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Presentations

1. Weber C, Luo C, Hotz-Wagenblatt A, and Eichmüller SB (2014) *Functional invasion assay identifies miR-339-3p as potential new tumor-suppressor for melanoma.* 2. International Symposium "Trends in Melanoma Research"

2. Weber C, Luo C, Hotz-Wagenblatt A, and Eichmüller SB (2014) *Funktioneller Invasions-Assay im malignen Melanom identifiziert miR-339-3p als möglichen neuen Tumorsuppressor.* 24. Deutscher Hautkrebskongress (Frankfurt, Germany)

3. Weber C, Luo C, Wiemann S, and Eichmüller SB (2014) *A functional microRNA screen to identify specific microRNAs with a role in melanoma cell invasion.* RNA@dkfz (DKFZ, Heidelberg, Germany)

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Teil 10

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