Final Report

The role of miRNA-363 in the Regulation of Melanoma Progression

By Hannah Prötsch-Gugerbauer

Marshallplan-Jubiläumsstiftung
Austrian Marshall Plan Foundation

I. ACKNOWLEDGEMENTS

II. STATUTORY DECLARATION

"I declare in lieu of an oath that I have written this bachelor thesis myself and that I have not used any sources or resources other than stated for its preparation. I further declare that I have clearly indicated all direct and indirect quotations. This bachelor thesis has not been submitted elsewhere for examination purposes."

Date: 25.03.2018   Signature:

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IV.   LIST OF ABBREVIATIONS

DMSO   Dimethyl sulfoxide

DNA   Deoxyribonucleic acid

ECM   Extracellular matrix

EMT   Epithelial-to-mesenchymal transition

EtOH   Ethanol

FBS   Fetal bovine serum

GAPDH   Glyceraldehyde-3-phosphate dehydrogenase

GTP   Guanosintriphosphate

HEM   Human epidermal melanocyte

HK   Housekeeping gene

IGF   Insulin-like growth factor

Kb   Kilobase pair

kDa   Kilo Dalton

MAPK   Mitogen-activated protein kinase

miRNA   MicroRNA

MITF   Microphthalmia-associated transcription factor

mRNA   Messenger RNA

Neg   Negative

nt   Nucleotides

PBS   Phosphate buffered saline

PCR   Polymerase-chain reaction

PI3K   Phosphoinositide-3-kinase

pre-miRNA   Precursor microRNA

pri-miRNA   Primary microRNA

PTEN   Phosphatase and tensin homolog

BRAF   Murine sarcoma viral (v-raf) oncogene homolog B1

NRAS   Neuroblastoma RAS Viral Oncogene Homolog

RISC   RNA-induced silencing complex

RTK   Receptor Tyrosine Kinase

UV	Ultra Violet
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
RAS	Rat Sarcoma
RAF	Recombinant Activated Factor
NCC	Neural Crest Cells
AJCC	American Joint Committee on Cancer
IL-2	Interleukin-2
MAP2K4	mitogen-activated protein kinase 4

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VII. ABSTRACT

Melanoma is one of the most common forms of skin cancer and leading cause of death due to this disease. Each year, more than 70,000 patients are diagnosed with melanoma in the USA, and around 9,500 die from this disease. If melanoma is recognized and treated early, it is almost curable, but if it is not, the cancer can advance and spread to other parts of the body, where it becomes hard to treat and can be fatal. Melanomas are genetically complex malignancies characterized by dysregulation of multiple signaling and tumor suppressor/oncogene pathways. DNA damage to skin cells triggers mutations that signal the skin cells to multiply rapidly and form malignant tumors. These tumors originate in the pigment-producing melanocytes in the basal layer of the epidermis mostly in moles. Melanoma is also caused by intense occasional UV exposure, especially in those who are genetically predisposed to the disease. Recently a lot of researchers started focusing on understanding the role of miRNA's in melanoma. miRNA's are small non-coding RNA molecules which are involved in gene expression and regulation. These miRNA bind to their complementary mRNA and therefore interfere in the process of transcription or translation and thus in the production of proteins. miRNAs can be used as biomarkers.

In this project we explore the role of miRNA-363 in the regulation of melanoma progression. We overexpressed miRNA-363 in several melanoma cell lines and performed functional assays to assess the effects of overexpressed miRNA-363 on cell survival, colony formation and proliferation.

miR-363 overexpression significantly reduced cell growth and proliferation

We concluded that miRNA-363 has a significant impact on the cell proliferation in melanoma cell lines.

1. INTRODUCTION

1.1. CANCER IN GENERAL

In 2017, an estimated 1,685,210 new cases of cancer are diagnosed in the United States and 595,690 people will die from the disease [1]. Cancer is an overall definition for cells which carry mutations in their genome. Five Hallmarks define the diagnosis of cancer. First, cancer cells have the ability to produce growth signals by themselves. Secondly cancer cells can manipulate the genome to reproduce infinitely and are insensitive to growth-inhibitory (antigrowth) signals which would stop that process. Furthermore cancerous cells can omit the programmed cell death (apoptosis) and build their own blood vessels (angiogenesis). Lastly, cancer cells are able to perform tissue invasion and travel through the blood system, called metastasis. [2]

1.2. HALLMARKS OF CANCER

To classify malignant cancer growth six essential alterations exist in cell physiology. First, the self-sufficiency in growth signals, secondly the insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis and tissue invasion and metastasis. Those physiologic changes characterize a malignant tumor growth.

Self-Sufficiency in Growth Signals

In a normal cell cycle, cells need mitogenic growth signals to start proliferating. These signals are passed into the cells by transmembrane receptors to which bind signaling molecules such as growth factors, cell-to-cell adhesion molecules or extracellular matrix components.

A lot of oncogenes imitate growth signals for the start of proliferation. Cells are dependent on growth signals to proliferate and only start if the appropriate signal is given.

Tumor cells, in contrast, have a reduced dependence on exogenous growth stimulation because they are producing their own growth signals.

Insensitivity to Antigrowth Signals

In a normal cell cycle several signaling cascades occur that inhibit proliferation to maintain cellular quiescence and tissue homeostasis. Those growth-inhibitory signals are transferred into the cell by transmembrane cell surface receptors coupled with intracellular signaling circuits.

There are two mechanisms which can block cell proliferation by antigrowth signals. The first mechanism forces cells out of the active reproductive cycle into the quiescent state (G0). The second mechanism induces cells to permanently block proliferative potential by forcing them into post-mitotic states.

Evading Apoptosis

Cancerous cells do not only proliferate independently but also evade apoptosis to grow. Apoptosis is the programmed cell death which assures a proper balance of cells in the body.

Cancer cells are resistant to apoptotic signals. Apoptosis is controlled by upstream regulators and downstream effectors.

An important role in apoptosis is played by the Bcl-2 family because it consists of pro- and anti-apoptotic proteins which are responsible for the transportation of signals between regulators and effectors.

The difference in tumor cells is that Bcl-2 pathway is altered and there is an alteration in the pro- and anti-apoptotic protein ratio.

Limitless Replicative Potential

Cancer cells have the ability to replicate infinite due to growth signal autonomy, insensitivity to antigrowth signals and resistance to apoptosis. Furthermore these cells can avoid the cell-autonomous program which would limit their multiplication.

That program ensures in healthy cells that after a certain amount of doublings the cells stop growing and go into senescence.

The ends of chromosomes, telomeres, consist out of thousands of repeats of a short 6 basepair sequence element. After each replication one basepair sequence gets lost. Due to this progressive shortening the DNA polymerases are not able to replicate the 3' ends of chromosomal DNA during each S phase. As a result the cells go into senescence.

This senescence can be circumvented by disabling the Retinoblastoma Tumor Suppressor Protein (pRB) and the p53 tumor suppressor proteins.

Sustained Angiogenesis

The supply of oxygen and nutrients is crucial for cell survival therefore all cells are forced to be within 100μm of a capillary blood vessel to get this supply. Within every formation of a new tissue there must also happen angiogenesis to ensure proper supply for the cells.

Several positive and negative signals encourage or block angiogenesis. In cancerous cells the ratio of positive and negative signals is altered so that only angiogenesis encouraging signals are produced.

Tissue Invasion and Metastasis

In a later stage of the development of most types of cancer the primary tumor cells spread out and invade other tissues and organs. There they form new colonies and form new tumors. These newly formed tumors are called metastases and are the cause of 90% of human cancer deaths.

The capability for invasion and formation of metastasis allows cancer cells to migrate to new parts of the body where new sources of nutrients and space are available.

Invasion and metastasis are very complex processes which are still not completely understood. It is known that cells change their shape, their connection to the extracellular matrix (ECM) and to other cells.

Epithelial-to-mesenchymal transition (EMT) also plays an important role in invasion and metastasis because it enables altered epithelial cells to withstand apoptosis and invade other tissues. [2]

1.3. MELANOMA

Melanoma is the most dangerous form of skin cancer and responsible for 80% of skin cancer deaths. If Melanoma is recognized and treated early it is mostly curable. These tumors originate in the basal layer of epidermis. Mostly a genetically complex mutation happens which leads to dysregulation of several signaling, oncogene and tumor suppressor pathways. [3] Sometimes tumors can also develop directly from moles. When the disease develops to stage IV, cells tend to migrate through tissue and travel through the blood and lymph vessels and form tumors in all parts of the body. Mutations can be caused by intense UV exposure or can also be genetically inherited. [4] Melanomas are genetically complex malignancies characterized by dysregulation of multiple signaling and mutations in key tumor suppressor or oncogene, including BRAF, NRAS and PTEN. DNA damage to skin cells triggers mutations that signal the skin cells to multiply rapidly and form malignant tumors.

Due to the high metastasizing potential melanoma has a very small survival rate as soon as it develops stage IV cancer. The affected organs of metastasizes have the extravasation of melanoma cells regulated by adhesion molecules, matrix metalloproteases, chemokines and growth factors. [5]

Metastases often develop in lymph nodes or in distant organs. Responsible for the migration into different sites of the body are the lymph flow and the chemotaxis.

The global incidence of melanoma continues to rise faster than any other malignancy, and despite considerable research efforts, no curative therapy is available for advanced metastatic melanoma.

One major important signaling pathway in melanoma is the Receptor Tyrosine Kinase (RTK) pathway. The RTK is activated by a growth factor which binds to the receptor pair and induces the cross phosphorylation of the receptor. This phosphorylation further activates the downstream protein RAS via GDP-GTP exchange by RAS-GEF.
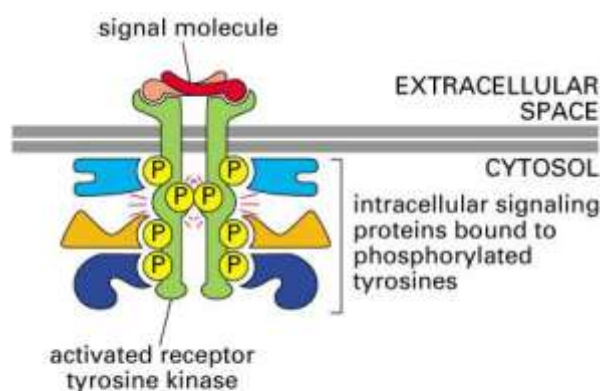
Figure 15–52. Molecular Biology of the Cell, 4th Edition.

Figure 1: activated RTK with resulting cross-phosphorylation

The activated RAS is further activating the MAP-Kinase-Kinase-Kinase. There exist three isoforms which belong to the RAF-family, called Raf-1/C-RAF, B-RAF, A-RAF. In melanoma the Ras/Raf/MEK/ERK cascades are often activated by genetic alterations in upstream signaling molecules as receptor tyrosine kinases. Some of these components of the pathways may also be activated by mutations or epigenetic silencing. Mutations in the signaling pathway can alter the sensitivity of the cells to certain inhibitor signals. The dysregulation of components some of these cascades are the reason for the development of resistances to chemotherapeutics or targeted drugs. [6]

BRAF plays an important role in the signaling pathway of melanoma because around 50% of all melanoma cases have a mutation at the BRAF gene. It is a mutation at V600E (at position 600 the gene has the amino acid Glutamate instead of Valine) which enables BRAF to constitutively activate MAP-Kinase-Kinase, also called MEK, without being activated by RAS before. The pathway then continues to activate MAP-Kinase (ERK) and induce transcription. [7]
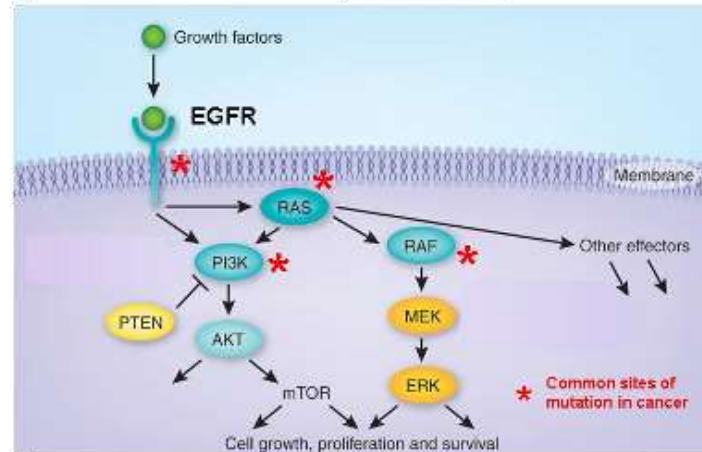
Figure 2: Downstream signaling pathway BRAF

1.3.1. MELANOCYTES

Melanocytes belong to the heterogeneous group of cells present in the human body. Melanocytes do have the ability to produce melanin and originate from embryonic cells which are called neural crest cells [8]. Additionally they have further functions such as they give hair and iris a color of their structures. Furthermore melanocytes are also present in the inner ear, nervous system and heart. Another function is to produce cells of pigmented epithelium of the retina, the epithelia of iris and the ciliary body of the eye, neurons and adipocytes.

The life cycle of melanocytes includes several steps such as the lineage specification of embryonic neural crest cells, migration and proliferation of NCC, differentiation of NCC's into melanocytes, maturation of melanocytes, transport of mature melanosomes to keratinocytes and eventual cell death.

The improvement of the embryonic development of melanocytes is an important step to better understand skin diseases such as melanoma. [9]

1.3.2. DIAGNOSIS AND STAGING

Melanomas are often difficult to diagnose as the first stages of disease do not really affect the body or gives any symptoms. The so called "ABCDE" staging system is used by dermatologists to classify melanoma. Furthermore it can also help an individual person to keep track on moles and recognize any changes early.

	A - Asymmetrical Shape Melanoma lesions are often irregular, or not symmetrical, in shape. Benign moles are usually symmetrical.
	B - Border Typically, non-cancerous moles have smooth, even borders. Melanoma lesions usually have irregular borders that are difficult to define.
	C - Color The presence of more than one color (blue, black, brown, tan, etc.) or the uneven distribution of color can sometimes be a warning sign of melanoma. Benign moles are usually a single shade of brown or tan.

	D - Diameter Melanoma lesions are often greater than 6 millimeters in diameter (approximately the size of a pencil eraser).
	E - Evolution The evolution of moles has become the most important factor to consider when it comes to diagnosing a melanoma. Knowing what is normal for yourself could save your life. If a mole has gone through recent changes in color and/or size, bring it to the attention of a dermatologist immediately.

If there are any changes discovered in moles doctor can differentiate melanoma in different stages to describe the extent of the disease. The tumors will be measured for its thickness and depth and if the cells have spread or metastasized to lymph nodes or other parts of the body. This staging will help to decide which treatment is appropriate and helps to determine a prognosis. [10]

Melanoma Stage	Description
0	Tumor is confined to the epidermis and has not entered the dermis yet. Also called melanoma in situ.
IA	The tumor is less than 1 Millimeter thick and the outer layer of skin does not look cracked or scraped. It has not spread to other body parts yet.
IB	Tumor is either less than 1 millimeter thick and looks cracked or is 1-2 millimeter thick and does not look cracked. It has not spread to other body parts yet.
IIA	The tumor is either 1-2 millimeter thick and looks cracked or is 2-4 millimeter thick and does not look cracked. It has not yet

	spread to other parts of the body.
IIB	Tumor is either 2-4 millimeter thick and looks cracked or is more than 4 millimeter thick and is not ulcerated. It has not spread yet.
IIC	The tumor is more than 4 millimeter thick and is ulcerated. These are aggressive tumors that are most likely to spread.
IIIA IIIB IIIC	The tumor may be of any thickness and may or may not be ulcerated. The cancer cells have spread to either a few nearby lymph nodes or to some tissue outside of the tumor but not to distant organs.
IV	The cancer cells have spread to lymph nodes, distant organs and tissues. This is called metastatic melanoma.

Additionally to this staging system for treatment prognosis there exists another system from the American Joint Committee on Cancer (AJCC), for determining the stage of disease. It is called the T, N and M system.

"T" stands for primary tumor and is based on primary tumor thickness.

"N" is the category for regional lymph nodes and gives information if lymph nodes contain cancer cells or not. And "M" stands for distant metastasis and describes the melanoma metastasis throughout the body. [10]

1.3.3. TREATMENT OF MELANOMA

If the melanoma is staged until IIA, see point 1.3.2 the cancerous tissue can be removed surgically. For every case further than IIA there might be additional treatment with chemotherapeutics or radiation necessary. [10]

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Over 30 years ago the first chemotherapeutic dacarbazine- was approved. Till today this drug is used as standard treatment for advanced melanoma. Unfortunately the percentage of response to this drug is only 10-15%.

Another therapy method is a high dose of interleukin-2 (IL2) which is a cytokine that induces the activation of T-cells and proliferation. This therapy method has a response rate of 15% whereas 6% have complete remissions that are lasting. Due to its toxicity and the absence of randomized phase III trials showing a survival benefit, this therapy is not considered as a standard treatment.

In 2011 a breakthrough was achieved by the approval of the first v-raf murine sarcoma viral oncogene homolog B1 (BRAF) inhibitor, vemurafenib. This drug was co-crystallized with a protein construct which contains the BRAF V600E domain. This enables the preferential binding to the ATP-binding domain of the mutant BRAF.
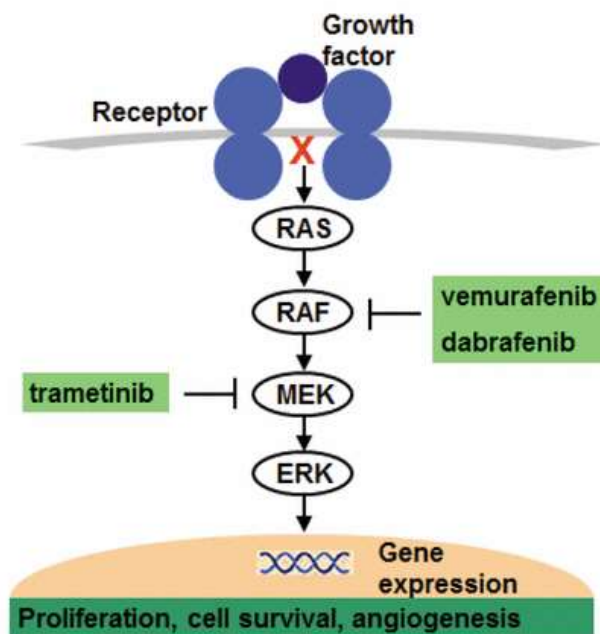
Figure 3: Downstream process of BRAF showing sites of inhibitions through drugs

In 2013 another drug targeting the BRAF V600E mutation was approved, called dabrafenib. Since this breakthrough researchers try to find more ways to inhibit this mutation. [11]

1.4.   MiRNA

In 1993 Rosalind Lee and Rhonda Feinbaum discovered miRNA. They observed that lin-4 gene, which is known to control the timing of C. elegan larval development, does not code for a protein but produces a pair of small RNAs. One was about 22nt long and the other 61nt. The second one was thought to fold into a stem loop and be the precursor for another one. Another investigation made by Ambros and Ruvkun found that these lin-4 RNAs had antisense complementarity to several sites in the 3'UTR of the lin-14 gene. Further demonstrations by Ruvkun showed that the lin-4 gene regulates the lin-14 gene by reducing the amount of lin-14 protein without reducing lin-14 mRNA. As these genes have common roles in controlling the timing of developmental transitions they were first called stRNA which stands for small temporal RNAs. Later on when more genes were discovered which had similar features but unknown functions they decided to use the term miRNAs. [12]
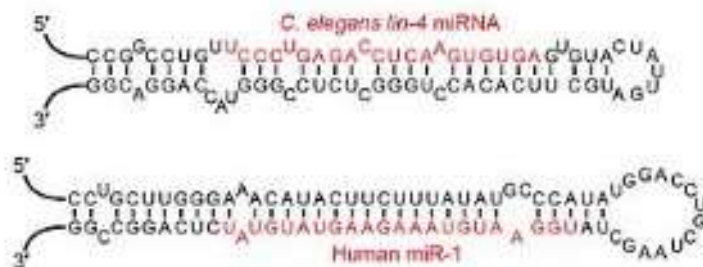
Figure 4: Genome of lin-4 compared to human miRNA-1

1.4.1. MiRNA IN CANCER

miRNA´s are small non-coding genes of a size of 20-25 nt which can act either as tumor suppressor or as oncogene. MiRNAs target its complementary mRNA and inhibits it at its post-transcriptional phase or leads it to degradation post translationally. It generally binds to the 3'UTR end of the mRNA and degrades the targeted gene. This has huge effects on cellular processes like differentiation and proliferation. [13]

miRNA´s have two production sites. Either they are produced by their own promoters of the gene or they are processed out of introns of the pre mRNA. It is thought that the miRNA's processed out of introns are situated in the same orientation as their complimentary mRNA so that the promoters of mRNA act at the same time as promoters for miRNA. This arrangement ensures a convenient mechanism for the controlled expression of miRNA's and proteins. The other production of miRNA is the regular transcription pathway whereas the miRNA holds its own gene sequence and promoters. [12]

miRNA´s are transcribed by RNA polymerase II as a part of capped and polyadenylated primary transcripts which results to either protein-coding or non-coding sequences. Primary transcripts are cleaved by Drosha ribonuclease III enzyme to 70-nt stem-loop precursor miRNA, also called pre-miRNA. This is further cleaved by cytoplasmic Dicer ribonuclease and results in mature miRNA and antisense miRNA star (miRNA*) products. The mature miRNA is integrated into a RNA-induced silencing complex [8] which recognizes target mRNAs by imperfect base pairing with the miRNA and most commonly results in translational inhibition or destabilization of the target mRNA. (RefSeq September 2009)

When miRNA targets an oncogene it is called tumor suppressor or when it targets a tumor suppressor it is called oncogene. An oncogene is a gene which promotes the tumor growth. A tumor suppressor is a gene which inhibits the tumor growth.

miRNA´s have a huge impact in the fundamental processes of cancer. It has been identified in numerous cancers that miRNA expression is deregulated. A lot of data indicates that some miRNA can function as tumor suppressors or oncogenes and are therefore important in cancer development. Dar, A et al describes in his paper that specific subsets of miRNAs have also been shown to be dysregulated in various solid tumors. The discovery of miRNAs at previously identified chromosomal breakpoints, as well as deletion and amplification sites in certain cancers, implies that they may be involved in disease initiation or progression. [14]

1.4.2. MIRNA-363 IN CANCER

miRNA-363 is associated with several types of cancer such as gastric cancer, hepatocarcinoma, neuroblastoma or prostate cancer. In aggressive gastric cancer, miRNA-363 influences the cell adhesion and migration by targeting disintegrin and metalloproteinase 15 (ADAM15). In several studies miRNA-363 is involved in diverse biological processes. In some cancer types miRNA-363 is significantly upregulated for example in human uterine leiomyoma or HPV-positive pharyngeal squamous cancer. [15]

In osteosarcoma cell lines and tissues miRNA-363 is downregulated and leads to smaller formed tumors. It also inhibited cell proliferation, migration and invasion. Xueqin Li et al showed that mitogen-activated protein kinase 4 (MAP2K4) is a direct target of miRNA-363 *in vitro*. [16]

In this project we studied role of miR-363 in melanoma and its effect on cellular proliferation and cell survival.

1.4.3. MIRNA-363 STRUCTURE AND POSITION

miRNA-363 is located on the Chromosome X on position 134169366 to 134169462 and it belongs to miRNA family. Its sequence is
TCTGTTTTGCTGTTGTCGGGTGGATCACGATGCAATTTTGATGAGTATCATAGGAG
AAAAATTGCACGGTATCCATCTGTAAACCGCAGGACCTTTG

1.5. 1205-Lu

In this project the mainly used cell line was 1205-Lu. This cell line is a metastatic human melanoma cell line which was derived from a metastatic site in the liver. The patient was a 37-year old male patient.

These cells are able to produce xenograft tumors when they get injected into immunocompromised cells.

Furthermore this cell line has a mutation on BRAF V600E as well as a hemizygous deleted PTEN and a CDK4 mutation K22Q. CDK4 is a cyclin dependent kinase 4 which encodes a serine/threonine kinase.

Those missense and silent mutations are observed in several cancer types such as intestinal cancer, endometrial cancer and skin cancer. [17]

Type	Cancer Cell Line
Cell Line Name	1205-Lu
Synonyms	1205-Lu; 1205 Lu; WM 1205LU; WM-1205LU; WM1205Lu; WM1205; LU1205; Lu1205; WC00058
Origin	Human
Disease	Melanoma
Stage	Metastasis
Derived	Lung metastasis
Parent	CVCL_8787 (WM793)
Sex	Male
Mutation	BRAF V600E PTEN Mutated/Hemizygous Deletion NRAS WT CKIT WT CDK4 K22Q
Tumorigenicity	Yes

2. AIM OF THE PROJECT

The aim of the project is to focus on the role of miRNA-363 in melanoma progression which has not been performed by anyone so far. Analyses and screenings of miRNA-363 provide an informative basis that miRNA-363 performs tumor suppressive activities in human cells.

The first part of the project is to determine the expression of miRNA-363 in different melanoma cell lines and compare these results to the expression in normal human melanocyte cells. Additionally we will determine the expression of miRNA-363 in several tissues to provide an informative basis if it acts as tumor suppressor of oncogene in melanoma. To do so we will extract miRNA from samples and cell lines and produce cDNA which will be analyzed using the Taqman method.

As a next step we will observe the effect of miRNA-363 and its modulation on cell proliferation and cell growth. Furthermore we will also observe the effect of miRNA-363 on the cell cycle.

The last goal of this project is to determine possible targets of miRNA-363. We will extract proteins out of wildtype melanoma cell lines and cell lines with upregulated miRNA-363 and perform Western blot analyses.

3. MATERIALS AND METHODS

3.1. CELL CULTURE AND PLASMIDS

The used cell lines LOX (kindly provided by Dr. Oystein Fodstad), HEM, 120-Lu, Mamel-66a, Mamel-103b and A375 (kindly provided by Dr. Boris Bastian) were grown in vitro in RPMI containing 5% FBS. The cell line C8161.9 (obtained from Dr. Danny Welch) was grown in DMEM/F12 with 5% FBS. Plasmids as pMax-GFP (Lonza, Walkersville MD), pMax-E2F1 (Addgene, Cambridge MA), miRNASelect™ pEP-miR Null control vector (pEP Null), miRNASelect™ pEP-hsa-mir-363 expression vector (pEP miR-363) (Cell Biolabs Inc, San Diego CA) were purchased. Furthermore the TaqMan probes for hsa-miR-363 and negative control pre-miRNA were purchased from Applied Biosystems located in Foster City, California.

3.1.1. GROWING CELLS

Different melanoma cell lines and the human epidermal melanocyte cell line were grown in their respective media, as it can be seen in the following table. In general the cells were grown in P100 plates in an incubator at 37°C and 5% CO_2.

Cell line	Corresponding media
HEM	Melanocyte media
MAMEL 66 A	RPMI-1640, 5% FBS+P/S
A375	RPMI-1640, 5% FBS+P/S
1205-Lu	TU 2%
MAMEL 12	RPMI-1640, 5% FBS+P/S
MAMEL 103B	RPMI-1640, 5% FBS+P/S
C8161.9	DMEM-F12 50/50, 5% FBS+P/S
LOX	RPMI-1640, 5% FBS+P/S

Table 1: Cell lines and its corresponding media

When the cells were confluent, the media was aspirated from the plate and the cells were washed with PBS to remove the remaining media containing FBS. After the aspiration of the PBS, roughly 1ml Trypsin EDTA was added for detaching the cells. The plate was put into the incubator for some minutes to give the Trypsin EDTA time to perform, depending on the cell line. After the cells were detached, about 80% of the cells (again depending on the cell line) were thrown away or used for further processes like miRNA or protein extraction. For stopping the Trypsinization and giving the cells the ability to grow again 10ml of the respective media were added as well as additional components like antibiotics. Finally the plate was returned into the incubator.

3.1.2. FREEZING CELLS

The cells were treated according to the procedure "growing cells" in chapter 6.1.1. Then 500µl of FBS and 500µl of the cell in Trypsin EDTA solution were added into the respective labelled Cryovials. Additionally 100µl of DMSO was added into the tubes. This step hast to be done at the end as DMSO is toxic for the cells. It also has to be put on ice immediately. As a last step the tubes get stored in the -80°C freezer or in liquid nitrogen.

3.1.3. THAWING CELLS

The needed cells were taken from the -80°C freezer or from liquid nitrogen and were put into a water bath to thaw them quickly. Then the cell solution is put into a 15ml tube containing 1- ml fresh media and gets centrifuged at 1,000 rpm for 2.5 mins. During the centrifugation a new plate was labelled and filled with 10ml of the respective media. After the centrifugation was completed, the supernatant was aspirated and the remaining pellet was suspended in 1ml of fresh media. The suspended pellet was then put into the new labelled plate and stored in the incubator.

3.2. TRANSFECTION

3.2.1. THEORY

Transfection is an important procedure in studying the function of genes in cells. Generally there are three classes of transfection methods: biological, chemical and physical. The introduced genetic material exists either stably or transiently. Transiently transfected genetic material lasts for a limited period of time and are not integrated into the genome. Transiently transfected genetic materials can be lost due to environmental factors or cell division. [18]
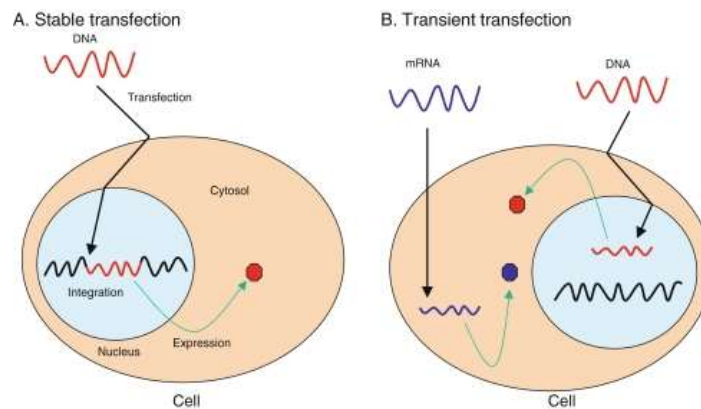
Figure 5: Transient vs Stable Transfection

The most effective way to introduce miRNAs into cells is lentiviral transduction. Lentiviruses are lipid-enveloped particles which comprising a homodimer of linear single-stranded RNA genomes. Lentiviruses build on an active transport of the pre-integration complex through the nucleopore by the nuclear import machinery of the targeted cell. Due to this property lentiviruses are able to infect dividing and non-dividing cells which makes them popular successors in being chosen for various gene delivery applications in basic research.

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In the beginning of the transduction the binding of virus particles to target cells is negotiated by several specific interactions happening between the specific receptors on the cell and the viral envelope. Some recent studies have demonstrated that in the initial step of virus binding not the specific envelope-receptor interactions but receptor-independent binding events take place. [19]
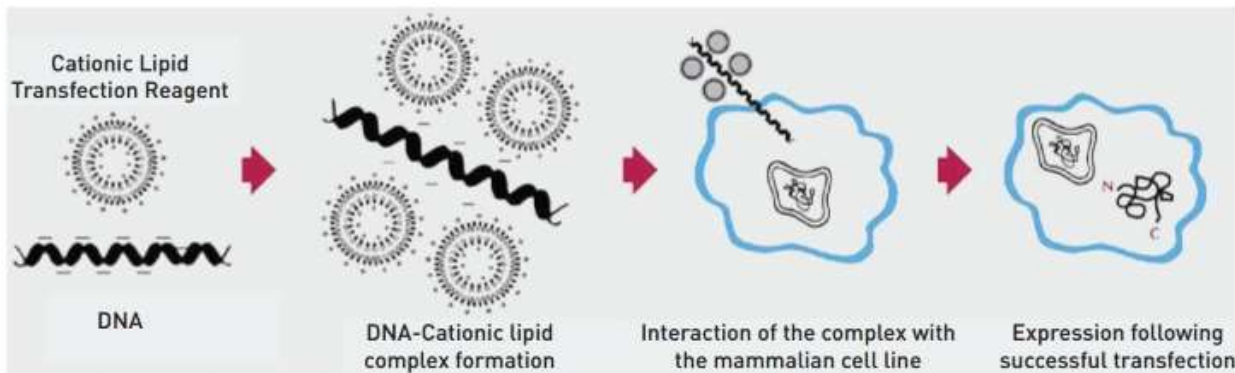
Figure 6: Transfection using Transfection Reagent

3.2.2. PROTOCOL

The transient transfection was carried out with Lipofectamine-2000 purchased from Invitrogen Life Technologies, Carlsbad, California. For the transfection the protocol of Invitrogen Life Technologies was followed.

First of all two Eppendorf tubes per condition were filled with 500µl of media. Important is that the media does not contain FBS because the FBS would interfere with the transfection process. As a next step the vector (miRNA-363, neg miRNA) was added to one of the two tubes per condition. To the second tube per condition 6µl of Lipofectamine were added which supports the formation of micelles and therefore enables the DNA-lipofectamine complex to get into the cell. Depending on cell line the DNA and Lipofectamine amount can vary.

As a next step the tubes were mixed together and incubated for 20 min at room temperature to allow the interaction taking place. Transfection mixture is added on to the cells.

After 3-4 hours the media will be changed and replaced with FBS containing media.

3.3. DETERMINATION OF RELATIVE MIRNA-363 EXPRESSION

3.3.1. RNA AND MIRNA EXTRACTION FROM TISSUES AND CELL LINES

Theory

One crucial method applied in molecular biology is the extraction of DNA, RNA and proteins. These extracted biomolecules are the starting point for a lot of downstream processes and product development. They can be isolated from any biological material provided as tissues, cells or virus particles.

RNAs are very unstable molecules which normally have a very short half-life as soon as they get extracted. Several types of RNAs can be extracted such as messenger RNA (mRNA), transfer RNA (tRNA) or ribosomal RNA (rRNA). It is important to prepare special care and precautions to isolate RNA as they are very susceptible to degradation.

The cause of this problem is the presence of ubiquitous RNases, enzymes present in the blood, tissues as well as most bacteria and fungi occurring in the environment. To inhibit endogenous RNases there have always strong denaturants been used for the intact RNA extraction. To extract successfully RNA good laboratory technique and RNase-free technique have to be performed. Furthermore RNases are heat stable and able to refold after heat denaturation which makes them difficult to inactivate. [20]

Protocol

For the extraction of RNA the RNeasy Mini Kit Qiagen (Valencia, CA) was used, following the manufacture´s protocol.

Samples from patients with primary, metastatic melanoma and benign nevi were obtained under a protocol which was approved by the Institutional Review Board. miRNAs were extracted with the help of the mirVana miRNA extraction kit purchased by Applied Biosystems. The protocol provided by Applied Biosystems was applied for the extraction of miRNA from tissues and cell lines.

Cell lines

Cells were treated with trypsin to detach and then put into a 15ml tube and centrifuged at 1,000 rpm for 2 minutes. The supernatant was removed and the cells were washed with 5ml of PBS and again centrifuged. Afterwards the supernatant was again aspirated and the tube was put on ice if immediately processed or stored in -20°C.

For further processing a master mix of Buffer RLT and β-mercaptoethanol was prepared under the hood. To mix the adequate amount of the master mix the following calculations were used:

Solution	Calculation
Buffer RLT	(number of samples*300)+50 = x µl
β-mercaptoethanol	Volume of Buffer RLT/100 = x µl

Table 2: Calculations for the preparation of the Master Mix

Per sample 300µl were added and mixed well. The cell solution is then transferred into shredder tubes consisting of a collection tube and a purple insert filter which is part of the RNA extraction Kit.

These tubes were then centrifuged at 13,000rpm for 2 minutes. As a next step the insert filter was discarded and 300µl of 100% Ethanol was added to the remaining solution in the tube. This solution was then transferred into pink RNA extraction tubes and then centrifuged for 1 minute at 10,000 rpm. This step was repeated an additional time. Furthermore the RNA extraction tubes were then placed into a new collection tube and centrifuged for 1 minute at 13,000 rpm. After the centrifugation the collection tubes were discarded and the pink insert was put onto a new Eppendorf tube.

Depending on the size of the pellet 25-50µl of RNase free water were added to the pink inserts and incubated for 1 minute at RT. Afterwards the tubes were centrifuged for 1 minute at 13,000rpm.

The pink RNA extraction tubes were discarded and the eluted RNA concentration was determined by using NanoDrop from Thermo Scientific, Rockford, Illinois.

Tissue samples

Tissue samples were extracted out of patients by surgeons and afterwards embedded into paraffin blocks. As a next step the paraffin blocks were cut into micron sections. These sections were stained with hematoxylin-eosin and then evaluated by a pathologist. To determine the cancerous part the samples got micro-dissected under the microscope. The cancerous part was then used for miRNA extraction. For the extraction of the miRNA the mirVana microRNA isolation kit was used.

3.3.2. cDNA SYNTHESIS

Protocol

To synthesize cDNA from miRNA a Master Mix consisting of dNTP, a restriction enzyme, 10x buffer, an inhibitor, 5x primers and miRNA-363 primer were used. RNU44 primer was used as a control primer. Every miRNA sample was probed for miRNA-363 and RNU44. For each condition, in this case two, a separate master mix has to be prepared. The calculations can be seen in the following table.

Reagent	Calculation
dNTP	0.05µl*number of samples
RT enzyme	0.37µl*number of samples
10x buffer	0.55µl*number of samples
Inhibitor	0.07µl*number of samples
H_2O	1.8µl*number of samples

Table 3: Calculations of the preparations of the Master Mix for the synthesis of cDNA

According to the number of samples the two primers were added to one of the two different master mixes. So if there were 5 miRNa samples 5µl of the corresponding 5x primer were added to the mastermix.

According to the number of miRNA samples the number of PCR tubes was chosen. To those PCR tubes 4µl of the mastermix plus primer was added.

To each tube (one containing miRNA-363 primer, one containing RNU44 primer) 200ng of each sample was added.

As a next step the PCR tubes were centrifuged and then put into the preheated PCR machine and the according program was started. After the completion of the program the synthesized cDNA was either further processed or stored at -20°C.

3.3.3. QUANTITATIVE REAL-TIME PCR-TAQMAN

Theory

Real-time Polymerase Chain Reaction (RT-PCR) enables the analysis of products while the reaction is actually in progress. This achievement is due to various fluorescent dyes which react with the amplified product and therefore enables measurement by an instrument. RT-PCR is not only giving information of the identitiy of DNA but also of the quantity of DNA.

In this project we used the Taqman technique which is widely used to analyze gene expression in samples. For the performance of a Taqman run a complimentary DNA (cDNA) has to be produced from RNA of interest. This is carried out with the enzyme reverse transcriptase. Furthermore a primer on the 3' end is necessary to produce cDNA. Due to the combination of reverse transcriptase with qPCR the detection of low levels of target RNA in cells is possible.

For the performance of a Taqman cDNA is combined with two primers and a Taqman probe which is labelled with a quencher and a fluorescent dye which gets excited when separated from the quencher.
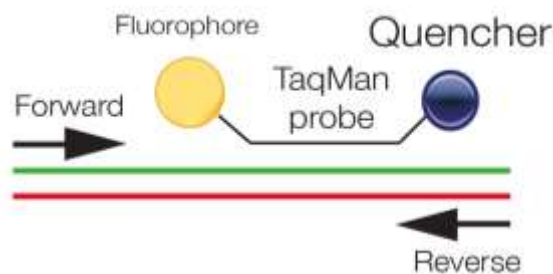
Figure 7: Taqman set up

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The probe will bind to after denaturation to the cDNA. The further reaction will lead the Taq polymerase to cut the probe by the usage of its Exonuclease activity and therefore the separation of the Quencher and the Fluorescent Dye. When light is applied the Fluorescent Dye will get excited and absorbs fluorescent light.

This process will take place for every cycle and every new amplicon of the target sequence. The qPCR machine measures the amount of fluorescent light that gets emitted by each sample and will create a plot showing the fluorescence activity against the number of cycles.
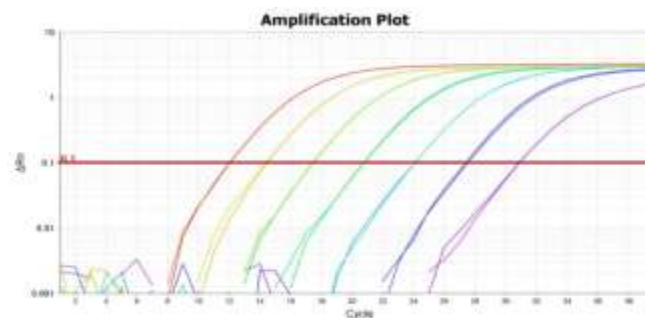
Figure 8: Plot drawn by PCR machine showing the number of cycles against the emitted fluorescent light

The evaluation of this plot is stated as the earlier a sample expresses higher fluorescent intensity, the higher the expression of target RNA is in the cells. The threshold cycle number (Ct) shows at which point the measured fluorescent intensity is increasing above the threshold, it can also be used to calculate the percentage to which the specific gene is present in the cells.

Protocol

All the miRNAs and mRNAs were analyzed by the TaqMan MicroRNA Asays and Gene Expression Assays which were purchased from Applied Biosystems and manufacturer´s instructions were followed. All the RT reactions were run in a 7500 Fast Real Time PCR System which was also purchased from Applied Biosystems.

First of all to carry out a Taqman a cDNA and a Taqman Master Mix have to be prepared as well as the 20x primers RNU44 and miRNA-363. The mixture can be seen in the table below. For each condition (RNU44, miRNA-363) a mixture has to be prepared.

Reagent	Calculation
Master Mix	18µl*number of samples
H_2O	13µl*number of samples
20x primer	1µl*number of samples

Table 4: Calculations for the preparation of the Master Mix for the Taqman

As a next step 5µl of cDNA and 32µl of the Master Mix were pipetted in the PCR tubes. Each sample of the PCR tubes will be pipetted in triplicates per 11µl each in the Taqman plate. The plate is sealed with heat resistant foil and put into the Taqman machine for evaluation.

To quantify gene expression levels the 7500 Fast Real Time Sequence detection system Software (Applied Biosystems) was used. Comparative real-time PCR was always carried out in triplicates, which also includes no-template controls. To calculate the relative expression the comparative Ct method was used.

3.4. CELL VIABILITY ASSAY

3.4.1. THEORY

The ATP assay is currently the fastest cell viability assay used due to its sensitivity and its low susceptibility to artifacts compared to other methods. Another advantage of the ATP assay is that the luminescent signal reaches a steady state and stabilizes within 10 minutes and then lasts for more than 5 hours. ATP has widely been accepted as a valid marker for viable cells.

As soon as cells lose membrane integrity they also lose the ability to synthesize ATP, furthermore the endogenous ATPases rapidly deplete any remaining ATP out of the cytoplasm. In earlier days luciferase has been the reagent mainly used to measure ATP, nowadays recent advances have shown that a single reagent addition in a homogeneous protocol can result in a luminescent signal glowing for several hours. The development of a stable version of luciferase has led to the development of robust assays that can withstand harsh cell lysis conditions. The ATP detection reagent contains solvents to lyse the cells, ATPase inhibitors for the stabilization of released ATP, luciferase as a substrate and a stable form of luciferase for the catalyzation of the reaction to generate photons of light. [21]
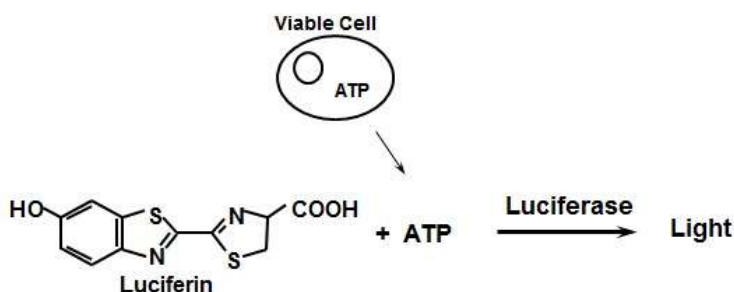
Figure 9: Simplified reaction scheme showing ATP and luciferin as substrates for luciferase to generate light.

3.4.2. PROTOCOL

For the cell viability assay the cells were grown until they were confluent and then trypsinized and counted. As a next step the cells were plated in 96-well plates at a density of $3*10^3$ cells per well. After 24 to 48 hours when the cells had attached to the plate they got transfected with miRNA-363 and control miRNA using the transfection protocol. After 24hr, 48hr, 72hr and 96hr post transfection they were assessed with the help of Cell Counting Kit-8 purchased from Dojindo (Rockville MD). Every day 6-8µl, depending on the confluency, of the Cell Counting Kit-8 was added to 4 wells per condition. After 1-3 hours of incubation time at 37°C and 5%CO_2 the plate was read at 450nm using a Spectrophotometer and data analyzed by SoftMax Pro v5 software.

3.5. COLONY FORMATION ASSAY

3.5.1. THEORY

The Colony Formation Assay permits the assessment of the differences in cell viability, means the capacity of cells to survive and divide to form a colony of cells. The comparison takes place in treated vs untreated cells in radiation, cytotoxicity or genetic manipulation as transfection.

The colony formation assay has become one of the most widely used technique to determine the effects of treatment. The normal procedure of the colony formation assay involves the treatment of the cell monolayer in tissue culture flasks, the preparation of single cells suspensions including the plating of an appropriate number of cells in petri dishes and the fixing and staining of colonies after a certain incubation period which is dependent on the cell line. [22]

3.5.2. PROTOCOL

The cells were grown in P100 plates until they were confluent enough to get trypsinized and counted. As a next step they were plated at a density of 80,000 cells per well into a 6-well plate. On the next day when all the cells have attached to the bottom the upper row got transfected with a neg control RNA and the lower row got transfected with miRNA-363. Cells were counted after 48hrs and 400-800 cells were plated in each well of a 6 well plate. Cells for each condition were plated in triplicates. Furthermore the plate was put back into the incubator and observed for a few days until visible colonies have formed. For the evaluation the plate was stained with Giemsa Crystal Violet Dye. To each well 1ml of the stain was added and then incubated at RT for several hours. As a next step the dye was removed and the plate was washed with H_2O until there was no dye left. The plate was airdried and then the colonies were counted and compared.

3.6. CELL CYCLE ANALYSIS

3.6.1. THEORY

Cell Cycle Analysis is commonly obtained by using flow cytometry as this is a very effective and reliable method to measure the cell phenotype. Flow cytometry uses targeted fluorescent stains to measure the cell phenotype and is therefore very useful in modern biological laboratories. These fluorescent stains are used to label cellular processes and components, which exposes specific cell phenotypes in the population and furthermore quantifies the particular state of each cell. For the Analysis of the Cell Cycle the exposure is performed with multiple stains. Typically for the evaluation of G1, S and G2 phases a stoichiometric fluorescent stain is used. For mitotic cells some additional stains are required. [23]
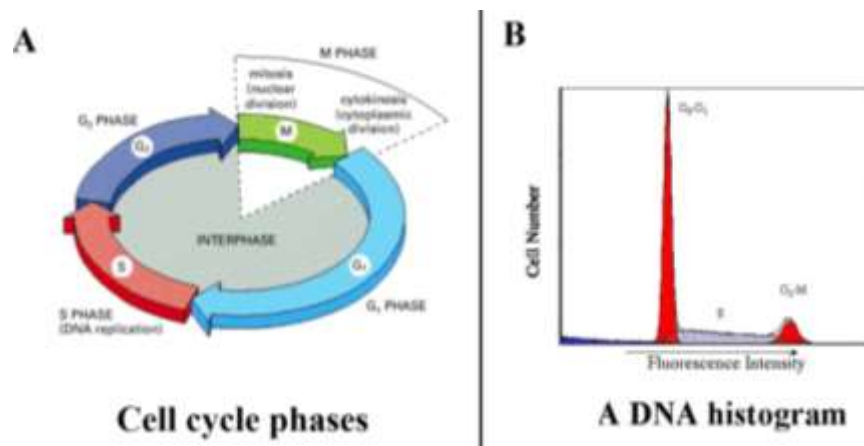
Figure 10: A: Cell Cycle with its Phases B: Histogram after Cell Cycle Analysis

3.6.2. PROTOCOL

The Cell Cycle Analysis is performed to determine at which stage of the cell cycle the cells are. This analysis can be performed as soon as the plates of cells were confluent. The cells were plated in a 6-well plate and transfected with miRNA-363 and negative control miRNA as described above. After 48 hours the cells can be analyzed.

First of all the cells were trypsinized and pelleted into a 15ml tube. To ensure that no remaining media is in the tube it got washed with PBS. Furthermore the supernatant was aspirated but a few microliters were left in the tube. As a next step the pellet was resuspended in 100% Ethanol by vortexing the pellet and then adding the Ethanol dropwise to avoid the formation of clumps. Then the pellet was kept in the freezer at -20°C for at least an hour. Afterwards the pellet is centrifuged and washed with PBS. Then 200μl of the Muse Cell Cycle Kit purchased from Millipore (Billerica, MA) solution was added to the 15ml tube and resuspended and transferred into an Eppendorf tube. To allow the cell cycle kit solution to react the Eppendorf tube was stored in the dark for 30 minutes. After the 30 minutes the cell solution was passed through a filter to remove any clumps that might have formed. Finally, the samples were run for analysis. For the evaluation of the cells the Muse Cell Analyzer was used following the protocol of the Manufacturer EMD Millipore Corporation.

3.7. WESTERN BLOT ANALYSIS

3.7.1. THEORY

Western Blots are an important technique applied in cell and molecular biology. This method enables to identify specific proteins out of a protein mix which was extracted from cells. It permits researchers to separate and identify proteins based on their molecular weight and their type using the first step of Western Blotting, Gel Electrophoresis.
The result of this separation is furthermore transferred onto a membrane which produces a band for each protein. The third step of Western Blotting is the incubation of the membrane with labeled antibodies which target specific proteins. The unbound antibodies are washed off and the bound antibodies are then detected by Chemiluminescence imaging.

The antibodies only bind to one protein of interest and therefore expose only one band. The thickness of the detected band gives information about the amount of protein present in the cells. [24]
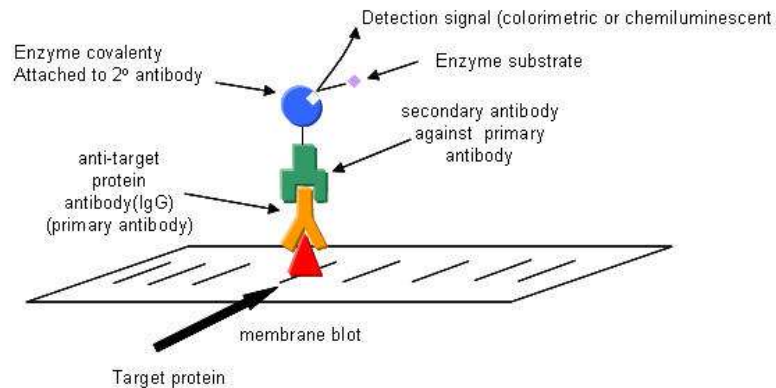
Figure 11: Complex performed during Western Blotting

3.7.2. PROTOCOL

Protein extraction

To generate the proteins that are necessary for the Western blot analysis the plate of cells has to be confluent. First of all the cells were washed and trypsinized and pelleted into a 15ml tube by centrifugation at 1,000 rpm for 2 minutes. The pellet has to be kept on ice afterwards to avoid the degradation of proteins. Depending on the size of the pellet between 60µl to 300µl of RIPA buffer was pipetted into an Eppendorf tube. For every 100µl of RIPA 1µl of a protease-phosphatase solution (Pierce, Rockford) was also added. The mixture was then resuspended with the pellet and transferred into a new Eppendorf tube. To allow the RIPA-protease-phosphatase mix to disrupt the cell membranes the resuspended pellet was kept on ice for 20 minutes. Afterwards the solution was sonicated and then centrifuged at 14,000 rpm for 10 minutes at 4°C. As a next step the supernatant was transferred into a new tube. It was important not to have any precipitant (cell debris) in the supernatant.

Determination of the Protein Concentration-Bradford Assay

To determine the concentration of a protein solution there needed to be a mastermix containing HBS and BioRad reagent. The calculations can be seen in the table below.

Educt	Calculation
Volume of stock solution	(number of samples*3)+9=x
HBS	x*160µl
BioRad	x*40µl

Table 5: Calculations for the preparation of the Mastermix for the determination of the Protein Concentration

These two components are mixed together in a 15ml tube. It has to be carried out under a Bio Safety Cabinet because of the BioRad solution. As a next step 500µl of this mix were pipetted into a new Eppendorf tube. 20µl of BSA (1µg/µl) were added to this mix to generate a concentration of 40µg/µl which acted as stock for the standard curve. Furthermore 200µl of the HBS/BioRad solution was pipetted into 6 wells of the first row of a 96 well plate. The second well was left empty. Furthermore into the next row was for every sample being analysed 3 wells were filled with 200µl of the HBS/BioRad solution.

The empty second well of the first row was then filled with 200µl of the 40µg/µl stock solution prepared before. Afterwards another 200µl of the stock solution were pipetted into the third well of the first row and resuspended several times. 200µl of this well were then put out and pipetted into the next well and again resuspended. The serial dilution was continued until the sixth well. This was the standard curve used for the analysis. Furthermore 1µl of each protein sample was added into their respective 3 wells and then mixed. An important fact to be aware of was to not create bubbles because those would lead to false results.

For the evaluation of the unknown protein concentrations SoftMax Pro was used to compare the standard curve with the unknowns by performing the program of Bradford Assay.

Performing a SDS-PAGE gel

According to the size of the proteins of interest a SDS-PAGE gel was prepared. In this project all proteins of interest could be evaluated by the use of a 10% gel. The preparation protocol is outlined in the table below.

Separating Gel (10%)		Stacking Gel (4%)	
H$_2$O	4.6ml	H$_2$O	3.1ml
40% Acrylamide	2.25ml	40% Acrylamide	0.5ml
1.5M Tris	2ml	0.5M Tris	2ml
10% SDS	80µl	10% SDS	55µl
APS	50µl	APS	50µl
Temed	8µl	Temed	5µl

Table 6: Preparation of a10% separation gel and a 4% stacking gel

First of all normally 30µg of the protein was loaded on the gel and the total volume loaded on the gel was 20µl. Therefore the amount of protein was calculated by $\frac{wanted\ concentration}{actual\ concentration}$, and then added 4µl of loading dye. The remaining volume is filled with RIPA buffer. Each sample was prepared in a separate Eppendorf tube. After mixing all ingredients into an Eppendorf the tubes were heated at 95°C for 5 minutes to denature the proteins. Afterwards they got spun down and put back on ice again.

As a next step the samples were loaded on the gel. Each sample was loaded into one well and the corresponding protein ladder was also loaded onto the gel.

The electrophoresis chamber is filled with "running buffer" which consists of 1x Tris/Glycine/SDS buffer and then a voltage of 90 is applied to the chamber as long as the samples are in the stacking gel. Afterwards the voltage was increased to 120V. After the marker was properly separated either a semi-dry transfer or a wet transfer could be performed depending on the size of the proteins of interest. The semi-dry transfer was carried out in a TransBlot machine.

First of all two filter papers were soaked in Transfer buffer which consisted of 100ml Tris/Gycine/SDS, 200ml Methanol and 700ml distilled water. Secondly a nitrocellulose membrane was soaked in the transfer buffer and placed on top of the two filter papers. On top of the membrane the gel was placed followed by another two filter papers. Between the additions of the next layer the layers were rolled to avoid the formation of air bubbles which would affect the result. The transfer was then run on 25V and 2.5 Ampere for 25 minutes.

A wet transfer is performed for high molecular weight proteins. The chamber was set up and filled with cold transfer buffer, additionally to that a tray ice was also put in the chamber to maintain the temperature of the transfer buffer. Then the tray containing a sponge is soaked in transfer buffer, followed by one soaked filter paper and the nitrocellulose membrane. Afterwards the gel is put on top and then again one filter paper. Same as for the semi-dry transfer the layers have to be rolled in between to avoid the formation of air bubbles. A magnetic stirrer was added into the tray and the transfer ran at 22V at 4°C overnight.

After the transfer the membrane was put on "milk" which consisted of 40ml 1xTBST (100ml Tris buffered Saline, 900ml dH$_2$O, 1ml Tween-20) and 2g of milk powder, for one hour. Furthermore the desired primary antibody was diluted in milk in a ratio between 1:300 and 1:1000, dependent on the used antibody.

After the one hour incubation period the primary antibody solution is sealed together with the membrane in a pouch and stored on a shaker at 4°C over night.

On the next day the membrane was washed three times with TBST for five minutes each. After the three washes the membrane was incubated with the secondary antibody consisting of 3-5ml TBST and depending on the antibody 1-5µ of the secondary antibody. The incubation takes place on a shaker at room temperature. As a next step the membrane is again washed three times with TBST for five minutes each.

Luminal reagents were mixed together in a ratio of 1:1 and applied on the membrane. The membrane was developed using the BioRad Transluminator machine.

The used antibody GAPDH was purchased by Santa Cruz Biotechnology (Santa Cruz, CA). The other antibody BCL-2 was purchased by Cell Signaling Technology (Danvers, MA).

3.8. STATISTICAL ANALYSIS

All quantified data represents an average of at least triplicate samples or as indicated. Error bars represent standard error of the mean. Statistical significance was determined by the Student's *t*-test and two-tailed p values <0.05 were considered significant.

4. RESULTS

For the performance of project the first part was the determination of the expression of miRNA-363 in melanoma cell lines, tissues and normal human cells. To get those results we analyzed the samples by the Taqman method.

After we performed the necessary experiments the next part was to observe the effect of miRNA-363 and its modulation on cell growth and proliferation and the effects on the cell cycle. Therefore we overexpressed miRNA-363 in cells by transfection.

The last part of the project was to find out possible targets of miRNA-363. Therefore we extracted proteins from wildtype melanoma cell lines and transfected cell lines and performed several Western Blot analyses to compare those and find the targets.

4.1. MIRNA-363 EXPRESSION IN MELANOMA CELL LINES

First of all the cells that were analyzed originated from 6 different melanoma cell lines which we compared with human epidermal melanocyte cell line (HEM) which acted as control. To achieve that miRNA was extracted out of the cells and cDNA was synthesized. Afterwards several Taqman analyses were performed to see the relative expression of miRNA-363. The table below shows the Fold Level and the calculated Standard Deviation of those different cell lines.

Cell Line	Fold Level	STED
HEM	1	0.112929
LOX	0.025687	0.052675
1205-Lu	0.733534	0.018636
Mamel-66a	0.155279	0.14066
Mamel-103b	0.046082	0.028173
A375	0.373293	0.052151
C8161.9	0.042395	0.056594

Table 7: Expression of miRNA-363 in melanoma cell lines

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The graph below shows that in a panel of melanoma cell lines the relative expression of miRNA-363 is clearly downregulated as compared to normal melanocyte. The downregulation varies from cell line to cell line. Overall it is clearly evident that in melanoma samples the miRNA-363 is downregulated.
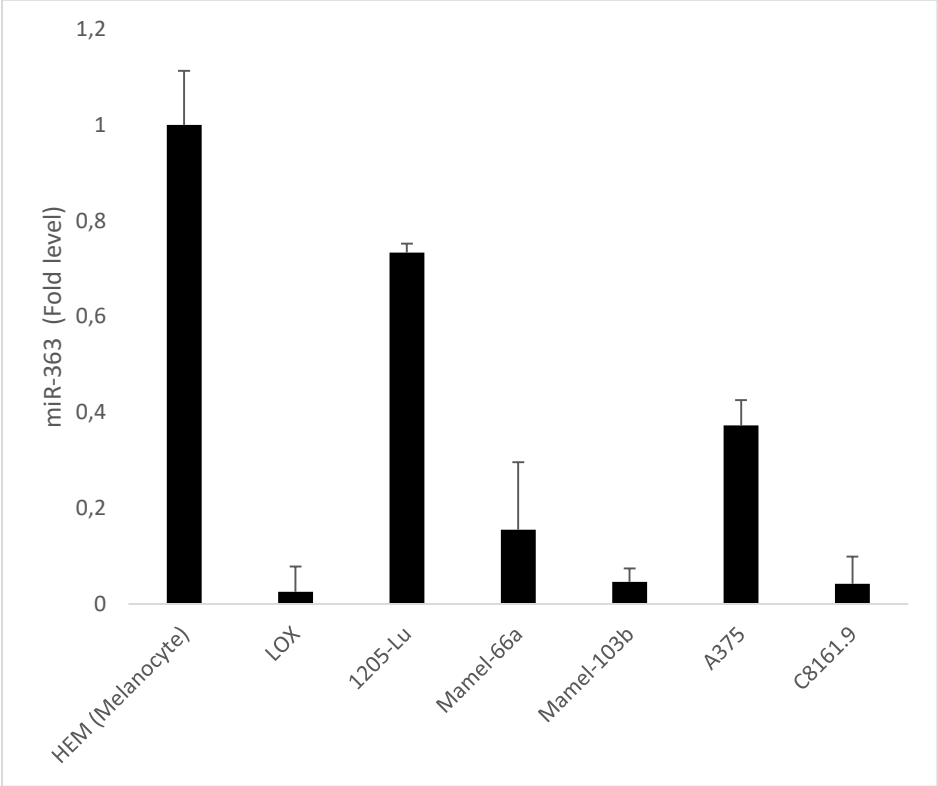
Figure 12: Graph showing the relative expression of miRNA-363 in 6 different melanoma cell lines compared to Human Epidermal Melanocyte

4.2. MIRNA-363 EXPRESSION IN NEVUS AND MELANOMA SAMPLES

As a next step several nevus samples and melanoma samples were analyzed to determine expression of miRNA-363. The table below shows clearly that in regular nevus samples of non-cancerous patients (N) the miRNA-363 is higher expressed than in Primary Melanoma samples (P). All primary tumor patient samples were obtained via surgical procedures. Primary tumors are defined as tumors which rise at one site of the body. They do not have the ability to metastasize and migrate and invade to other parts of the body yet.

Nevus Samples	Relative Expression	Melanoma Samples	Relative Expression
N1	4.031276	P1	0.00138894
N2	2.033458	P2	0.02659638
N3	5.651565	P3	0.02842737
N4	0.33022	P4	0.04352392
N5	0.33022	P5	0.04555517
N6	0.438348	P6	0.04723265
N7	0.083389	P7	0.05139057
N8	0.125837	P8	0.0556539
N9	0.276164	P9	0.0576281
N10	0.671085	P10	0.05947929
		P11	0.06855103
		P12	0.07968805
		P13	0.08316708
		P14	0.14892315
		P15	0.16540009
		P16	0.16735112

Table 8: Relative expression of miRNA-363 in nevus samples and melanoma samples

The graph below shows the Fold level of miRNA-363 in nevus samples where it can be clearly seen that it is highly expressed in nevus samples compared to the melanoma samples where in all 16 samples the miRNA-363 expression was clearly downregulated.
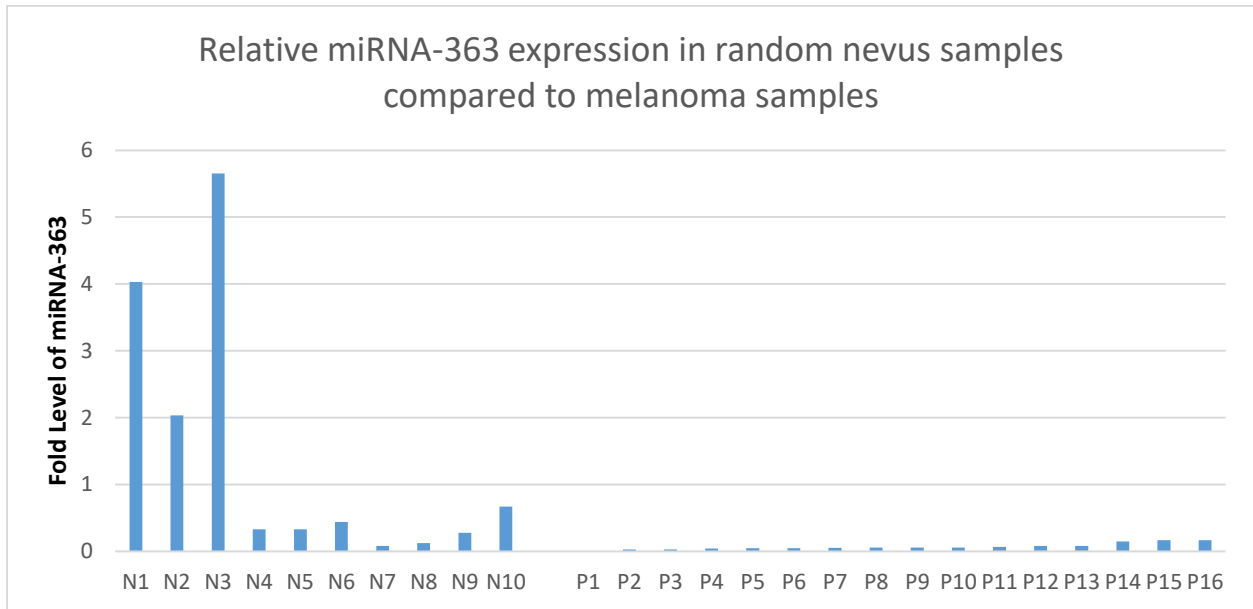
Figure 13: Relative expression of miRNA-363 in nevus samples compared to melanoma samples

For the next experiments, colony formation, cell survival, cell cycle analysis and Western blots, transient transfections were carried out.

In aim to guarantee significant differences between the control cell line and the miRNA-363 upregulated cell line, the cell line which showed the highest transfection efficiency was used to carry out further experiments.

The determination was carried out by transfecting a melanoma cell line with control miRNA and miRNA-363 and then comparing the expression levels of both. This experiment showed that 1205-Lu was the cell line with the highest efficiency of transfection.

4.3. CELL SURVIVAL

1205-Lu cell line got plated into a 96-well plate at a density of 500 cells per well. As soon as the cells were attached to the bottom they got transfected with neg-miRNA and miRNA-363. The cells were evaluated after 24, 48 and 72 hours by measuring the absorbance level at 450nm.

1205-Lu Transfected with miRNA-363

	0hr	24hr	48hr	72hr
Neg-miRNA	0	0.248	0.661633	0.8021485
miRNA-363	0	0.2096	0.5504	0.64865

Table 9: Absorbance at 450nm after transfection with neg-miRNA and miRNA-363 after 24hr, 48hr and 72hr

Standard Deviation

	0hr	24hr	48hr	72hr
Neg miRNA	0	0.050612	0.018707	0.00770534
miRNA-363	0	0.079065	0.040274	0.03180865

Table 10: Calculated Standard Deviation of 1205-Lu transfected with neg-miRNA and miRNA-363 after 24hr, 48hr and 72hr

The Absorbance level at 450nm of Neg miRNA in the graph below is clearly higher than of the cells transfected with miRNA-363. This means that miR-363 transfected cells survived less than Neg miR cells.
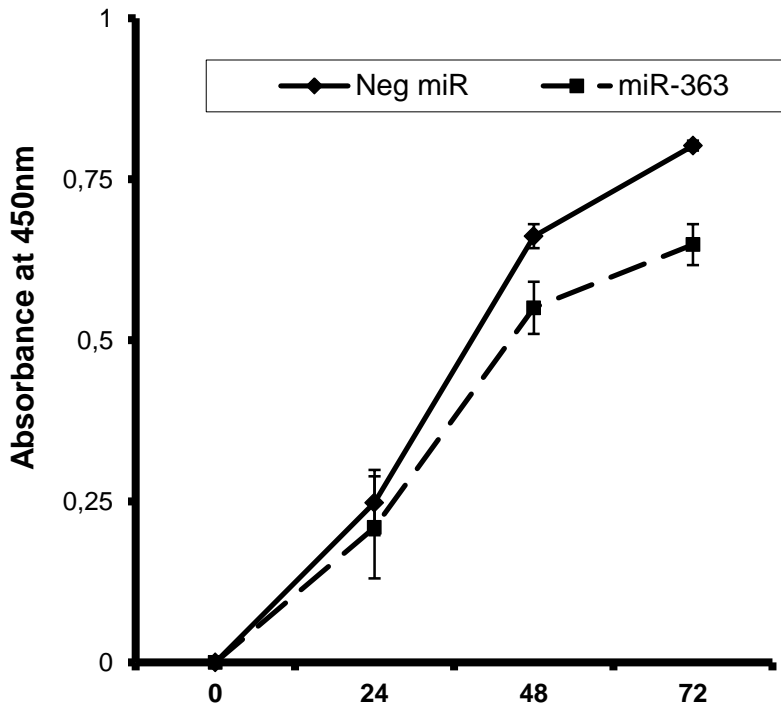
Figure 14: Difference of the absorbance level at 450nm between 1205-Lu transfected with neg-miRNA and miRNA-363

4.4.   COLONY FORMATION FOR 1205-LU MIRNA-363

For the determination of the effect of miRNA-63 on cell growth and proliferation a colony formation assay was performed. 1205-Lu got transfected with neg-miRNA and miRNA-363. An equal number of cells were plated in 6-well plates and three wells were transfected with neg-miRNA and the other three with miRNA-363. The plate was kept in the incubator for some days until visible colonies were formed. Afterwards the plate was stained and the colonies were counted.

	Control miRNA	miRNA-363
Amount of colonies	47	17
	47	18
	39	11
Mean	44.33333	15.333333
Standard Deviation	4.618802	3.785939

Table 11: Table showing the amount of colonies counted after they were grown and stained. The mean and the standard deviation were calculated to show comparable results.

The plate was stained using Giemsa Crystal Violet Dye. The stain was left several hours and was then washed until all the residual dye was removed.
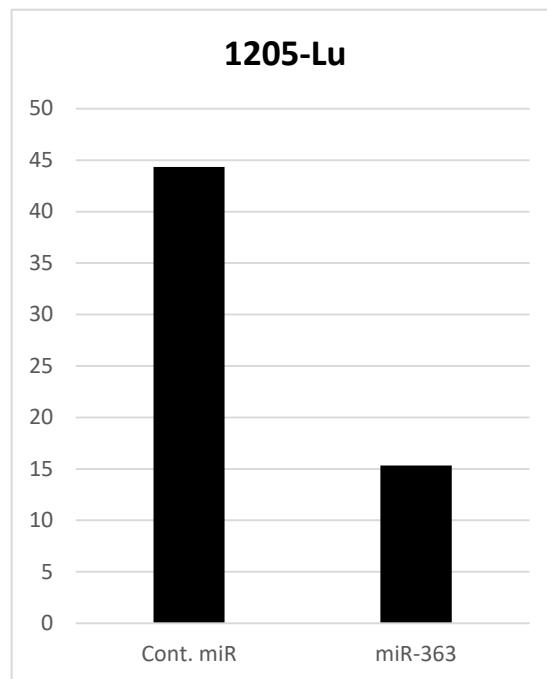
Figure 15: Transfection efficiency of melanoma cell line 1205-Lu. The cells in which the miRNA-363 level was upregulated via transient transfection showed approximately 3 times more colonies of than the cells which were just transfected with the control miRNA

The result in Figure 16 shows clear and countable colonies in all wells. There is a certainly difference between the upper three wells which were transfected with neg-miRNA and the lower three wells which were transfected with miRNA-363. A significant decrease in the colony formation ability was observed in cells transfected with miR-363 as compared to Neg miR transfected cells. In order to ensure a statistical significance the experiment was repeated several times.

Figure 16: Image that was taken after the cells were stained. It clearly shows a higher number of cells in the upper three wells which include the cells that were transfected with the neg-miRNA. The lower three wells which contained the cells that were transfected with miRNA-363 show a clear reduction of cell growth and proliferation.

4.5. CELL CYCLE ANALYSIS

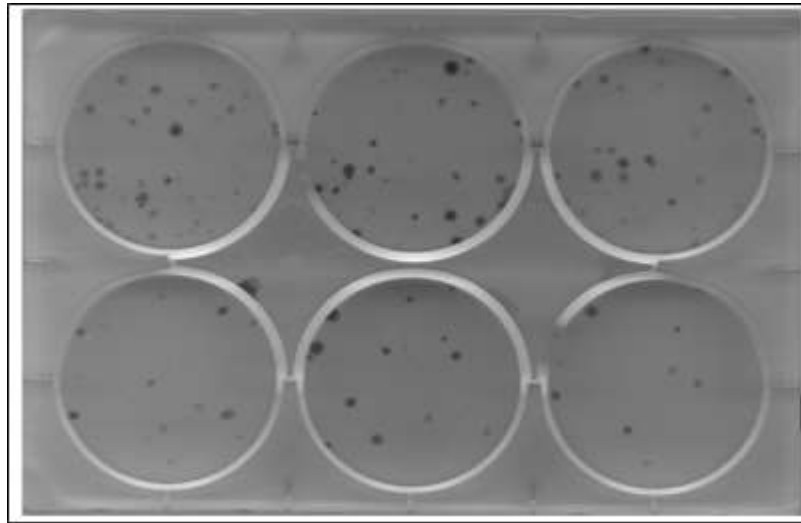In order to determine the effect of miRNA-363 on the cell cycle, 1205-Lu cells were transfected with neg-miRNA and miRNA-363. As soon as the cells were confluent enough they were harvested and analyzed using the Muse Cell Analyzer as it was described in point 6.6.2. The analyzed data gives information about the percentage of cells in the different phases of the cell cycle. In this case the special focus emphasizes on the S phase as it is the phase responsible for the replication of DNA and determines the speed of the cell division.

1205-Lu transfected neg-miRNA
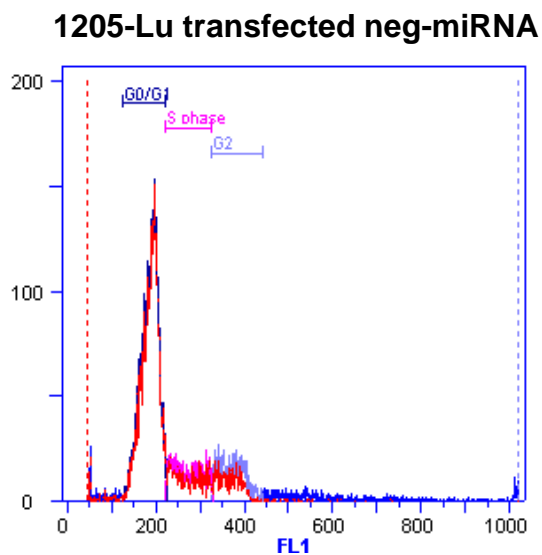
Figure 18: Figure shows the result of the cell cycle analysis of 1205-Lu which got transfected with neg-miRNA.
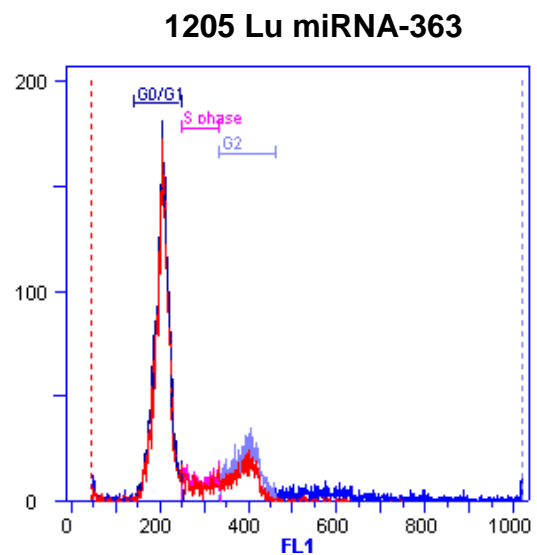
1205 Lu miRNA-363

Figure 17: Figure shows the analysis of the cell cycle after the transfection of 1205-Lu with miRNA-363.

The results of the analysis of the cell cycle of 1205-Lu transfected with neg-miRNA and miRNA-363 shows that overexpression of miRNA-363 resulted a blockage of G0/G1 phase. Therefore fewer cells went into the S phase and less DNA was synthesized. To show the difference more clearly two pie charts were created for the direct comparison see Figure 19 and 20.

	Neg-miRNA		miRNA-363	
Region	Count	Pct Total	Count	Pct Total
G0/G1 Phase	6,025	60.25%	5,872	58.72%
S Phase	1,516	15.16%	808	8.08%
G2 Phase	1,507	15.07%	1,990	19.90%

Table 12: Table showing the percentage of cells being in each phase of cell cycle at the time point they were harvested
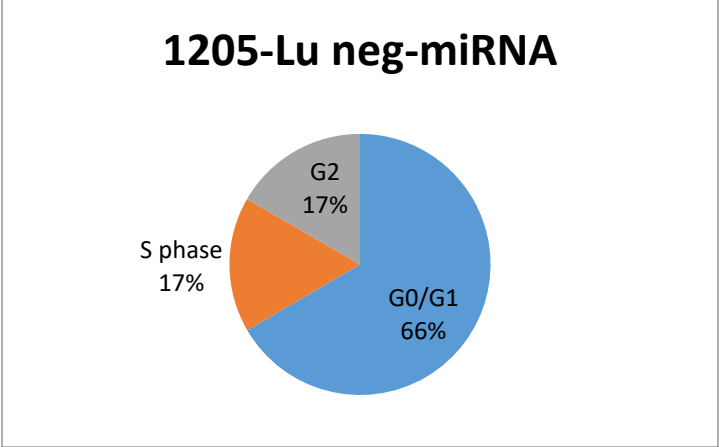
Figure 19: Result of Cell Cycle analysis of 1205-Lu transfected with neg-miRNA
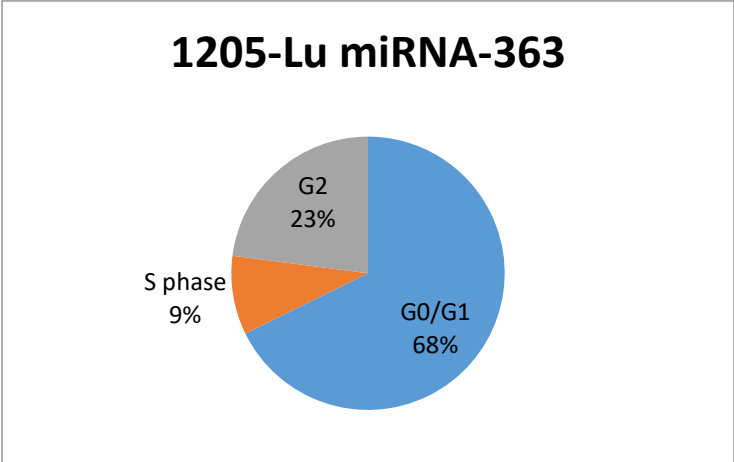
Figure 20: Result of Cell Cycle Analysis of 1205-Lu transfected with miRNA-363

4.6.   WESTERN BLOT ANALYSIS

The last goal of the project was to find at least one functional target of miRNA-363. In order to determine the target 1205-Lu melanoma cell line got transfected with neg-miRNA and miRNA-363. When the cells were confluent enough they got harvested and proteins were extracted. With the freshly processed proteins a Western blot analysis was performed. The proteins were loaded on a 10% Acrylamide gel and further processed as described in the method section. As a first step the nitrocellulose membrane got incubated with the antibody GAPDH in order to determine if it is equally expressed and therefore acts as a control. One tested protein was the anti-apoptotic protein Bcl-2 because it is an anti-apoptotic member of the Bcl-2 family and it is overexpressed in about 90% of all melanoma cases. This overexpression is a result due to a mutation in the BRAF gene. In the figure below it can clearly be seen that BCL-2 is downregulated in the lane where protein from 1205-Lu transfected with miRNA-363 was loaded.

BCL-2

GAPDH

1205-Lu   1205-Lu

Neg miRNA   miR-363
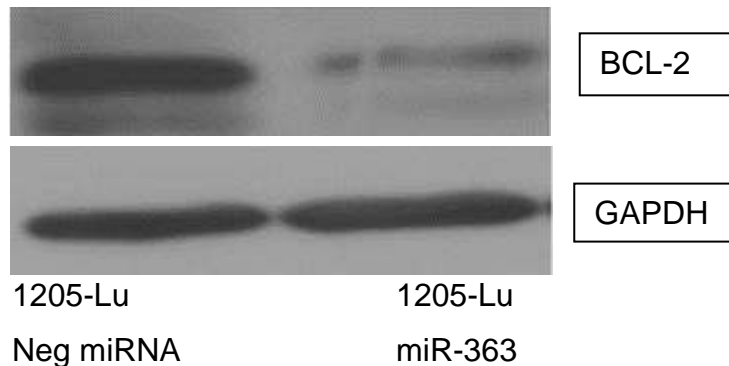
5. DISCUSSION

miRNA-363 expression in Melanoma cell lines

First of all several samples of melanoma cell lines got analyzed to determine the expression levels of miRNA-363. This was performed by extracting miRNA from melanoma cell lines and analyzed by Taqman analysis.

In all known melanoma cell lines the relative expression of miRNA-363 was downregulated when compared to the Human Epidermal Melanocytes. As these are different Cell lines the relative expression is not downregulated the same fold level in all. Fact is that all of those cell lines have lower miRNA-363 levels if compared to the control cell line HEM. This means that there must happen some genetic changes in healthy melanocyte cells which turn them into melanoma cells.

Those genetic changes most likely affect the cell's defense mechanism so it is prone to behavioral changes.

miRNA-363 expression in nevus and primary melanoma samples

After the analysis of melanoma cell lines we wanted to investigate if this downregulation of miRNA-363 also happens in nevi and primary melanoma samples. Therefore nevi and primary melanoma samples where obtained from patients during surgical procedures. The tissue samples where embedded into paraffin blocks and cut in small micron slides. As a next step the micron slides were stained with hematoxylin-eosin and furthermore were examined by a pathologist. As soon as it was clear which part was the tumor and which part was normal healthy tissue, the cancerous tissue was micro-dissected. The obtained samples where then used again to extract miRNA, synthesize cDNA and analyzed via Taqman.

When having a look at Figure 13 it can be seen that the miRNA-363 expression in nevus samples is higher than in primary melanoma samples. When comparing P1 and N3 there is a difference of almost 6 fold levels. This shows that in cells which develop into primary melanoma there must happen a downregulation of miRNA-363. The downregulation is mostly caused by mutations which lead to the deletion of the gene expressing miRNA-363.

Once again it is not expected that all samples of primary tumors have the same relative expression of miRNA-363 as there can happen different mutations which cause a different effect on miRNA-363.

This investigation also forms the question if certain mutations cause a special type of cancer. It is already known that in over 50% of all melanoma cases a mutation on the BRAF gene is responsible. This knowledge can help to improve the treatment and expand the borders of personalized medicine.

For further experiments several transfections were performed to determine the melanoma cell line with the highest transfection efficiency. All melanoma cell lines were transfected with miRNA-363 and neg-miRNA. After a few days we extracted miRNA of all of them and analysed its expression by performing a Taqman analysis.

The result of this experiment was that the cell line 1205-Lu had the highest transfection efficiency. Therefore this cell line was used for further experiments.

Cell survival

After the determination of the relative expression of miRNA-363 in several samples the next step was to obtain the effect of miRNA-363 on cell proliferation. To analyse the effect on cell growth a cell survival assay was performed.

To begin with the cells were plated in a 96-well plate, when they were attached to the surface the upper row got transfected with neg-miRNA and the lower row with miRNA-363. Therefore the cells transfected with miRNA-363 had a lot higher expression of miRNA-363. They were evaluated after 24hr, 48hr and 72hr.

When having a look at Figure 14 it can be seen that the wells containing cells which had overexpressed miRNA-363 show a lower absorbance at 450nm which means that there were less cells in the wells at the time of measurement compared to the wells with cells that had no overexpression of miRNA-363.

Both rows had the same amount of cells plated therefore the result suggests that when melanoma cells have overexpressed miRNA-363 those grow slower than the cells which do not have the overexpressed miR-363.

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Reasons for that is that miR-363 acts as tumor suppressor and therefore target oncogenes. Oncogenes are responsible for a faster cell growth in tumor cells. When miRNA-363 is overexpressed it surpresses those oncogenes and therefore slows down the cell growth.

Colony Formation Assay

After determining the cell line with the highest transfection efficiency and the observation of the growth behavior of cells which have an overexpression of miRNA-363, another experiment observing the proliferation behavior was performed.

First of all the same amount of cells was plated into 6-well plates and after the attachment of the cells to the surface they got transfected with neg-miRNA and miR-363 in triplicates. As a next step the cells were kept in the incubator for several days until they formed visible colonies. As a next step they got stained and washed to make the colonies better visible. The result can be obtained in Figure 16.

As it can be seen in Figure 16 the wells which overexpressed miRNA-363 formed less colonies than the control wells which got transfected with neg-miRNA. In the wells with overexpressed miRNA-363 there are almost three times less colonies than in those wells which got transfected with neg-miRNA. This result supports the result of the cell survival assay shown in the point above, that if cells overexpress miRNA-363 they suppress the oncogenes and therefore slow down the cell proliferation.

So if the level of miR-363 is upregulated in a cell, this has an impact on the cancerous behavior of the cell. It slows down the growth and also the colony formation of these cells and activates the cell own defense mechanism again. The more the miRNA-363 level increases within the cells, the slower they will grow and proliferate.

Cell Cycle Analysis

After the dissection of miRNA-363 on the growth behavior of cell the next part of the project was to study the effect on the cell cycle if miRNA-363 is upregulated to show at which part of the cell cycle miR-363 is active and to prove the hypothesis that miR-363 slows down the cell proliferation which resulted of the cell survival and colony formation assay.

For the performance cells got plated into 6-well plates and were transfected with neg-miR and miR-363. After 48 hours the cells were harvested and analysed using the Muse Cell Cycle Analyser. The machine determined which cells were in which phase of the cell cycle at the time point when they were harvested.

The cell cycle starts with the G1-phase where the cell starts to grow and expand. As a next step it enters S-phase which is also called synthesis phase and is responsible for DNA replication. The second growth phase is called G2-phase, in which the cell continues growing and prepares for Mitosis. In between those phases there are several checkpoints which ensure a perfectly working mechanism. Those checkpoints are often targets for cancer cells to be blocked.

First of all it can be seen in Figure 17 and 18 that in cells with upregulated miR-363 the cell cycle was blocked in G0/G1 phase as less cells were in the S phase. The main focus of the analysis of the cell cycle was the observation of the S phase. The S phase offers valuable clues about the replication status of cells. The more DNA is synthesized the more cells replicate.

As a consequence, also less cells are in G2 and M phase which means that cells divide less and therefore the cell proliferation is slowed down.

Western Blot Analysis

The last aim of the project was to determine possible targets of miRNA-363. In order to guarantee the correctness of the results, they were compared to a so called housekeeping gene. In this case the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase, short GAPDH, was used. GAPDH is one of the most commonly used housekeeping genes used in comparisons of gene expression data. (http://www.physiology.org/doi/abs/10.1152/physiolgenomics.00025.2005)

Housekeeping genes maintain constant expression levels in all cells. Furthermore it does not depend on the condition or the stage of cell cycle a cell is. Housekeeping genes help to expose the cellular infrastructure and help to gain more knowledge about the various structural genomic features. [25]

Due to that constant expression GAPDH was used in this project to compare it to the expression of other proteins.

As a first step the cells were grown in P100 plates until they were confluent. Secondly they got transfected with neg-miRNA and miRNA-363 following the protocol described in Step 3.2. After 48 hours post transfection period the cells were harvested and protein were extracted as described in Step 3.7.2. For the Analysis the proteins were loaded on a 10% Acrylamide gel and blotted on a Nitrocellulose Membrane. After one hour blocking the membrane was incubated with several antibodies to determine the effect of overexpressed miR-363 in cells compared to the cells transfected with the control neg-miR and find possible targets.

We were able to identify BCL-2 as direct target of miR-363 after database analysis. BCL-2 is an anti-apoptotic gene which means it is upregulated in about 90% of all melanoma cases which means it inhibits cell death.

When having a look on the bands shown in Figure 21 it can be seen that if miR-363 is upregulated the amount of BCL-2 is significantly suppressed.

BCL-2 overexpressed in melanoma due to a very common mutation on the BRAF gene. This mutation is the reason for 60% of all melanoma cases.

When this mutation appears it leads to a 90% overexpression of anti-apoptotic genes such as BCL-2, BCL-XL or MCL-1.

Therefore we arrived to the conclusion that if miR-363, which acts as a tumor suppressor, is overexpressed it decreases the cells ability of proliferation. Additionally one part of this tumor suppressor role also includes the reduction of BCL-2 levels.

6. CONCLUSION

The aim of the proposed project was to study the role of miRNA-363 in the regulation of melanoma progression. During this project we were able to determine relative expression of miRNA-363 in melanoma cell lines and patient samples. Furthermore we determined the effects of miR-363 to melanoma cells in cell proliferation and colony formation. Another significant result we were able to proceed was the effect of miRNA-363 on the cell. Additionally we were able to find one direct target of miR-363. miRNA's are known to act either as a tumor suppressor or an oncogene. In this case we were able to achieve significant results to prove that miRNA-363 acts as a tumor suppressor in melanoma cells.

A future goal to achieve in this project would be to determine more direct targets of miR-363 which I was not able to identify due to the limited time period of my work. Furthermore the next step in this project would be to study the role of miRNA-363 *in vivo*. To do so melanoma cells would be transfected with neg-miR and miR-363 and then implanted into athymic nude mice. This procedure would give an insight on the behavior of those cells in a living organism.

These steps are already planned and will definitely carried out in the future to get more information about miRNA-363.

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