AUSTRIAN
MARSHALL PLAN FOUNDATION
VIENNA | AUSTRIA

California Pacific
Medical Center
Research Institute

Role of microRNA in the regulation of melanoma progression

submitted to the

Austrian Marshall Plan Foundation

by

Claudia RITTSTEUER

Written in the practical training semester at:

CPMC California Pacific Medical Center Research Institute

Degree Program: Medical and Pharmaceutical Biotechnology, IMC FH Krems, Austria

Internal Supervisor: Prof. (FH) Dr. Christoph Wiesner

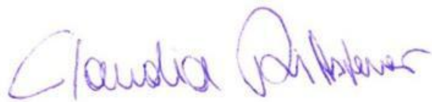
External Supervisor: Altaf A. Dar PhD, Dr. Mohammed Kashani-Sabet

Date: January, 26th, 2012

Statutory declaration

I declare in lieu of an oath that I have written this research paper 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 research paper has not been submitted elsewhere for examination purposes.

San Francisco, January 26th, 2012



Claudia Rittsteuer, Marshall-Plan stipendiary

TABLE OF CONTENT

Statutory declaration ... 2

1. List of Abbrevations ... 4
2. List of figures ... 6
3. Abstract ... 8
4. Introduction ... 8

Melanoma ... 8

microRNA ... 9

MicroRNA and cancer ... 11

5. Methods (and their scientific background) ... 11

5a Cell Culture ... 11

Cell Culture – general ... 12

Growth medium 12

General techniques, used in cell culture ... 13

5b Transfection ... 14

Transfection - Methods ... 16

Transfection - Procedure: ... 17

5c Knockdown verification techniques ... 17

5d Polymerase Chain Reaction (PCR) ... 18

Real time PCR ... 19

Real time PCR - procedure: ... 20

1) Isolation/Purification of RNA from animal cells ... 20
2) Measuring the concentration of the RNA/miRNA. ... 22
3) cDNA synthesis ... 22
4) Micro cDNA ... 24
5) TaqMan® Small RNA Assays ... 24
6) Ct value ... 25

5e Western Blot Analysis ... 26

1) Protein extraction ... 27
2) Yield of extracted protein – Bradford Assay ... 28

Electrophoresis ... 29

3) SDS-Polyacrylamide Gel Electrophoresis ... 29
4) Transfer proteins to membrane ... 33

5) Blocking non-specific sites 34
6) Primary and secondary antibodies 34
7) Detection method 35
8) Outcome Western 36
5f Cell Cycle Assay 37
6. Results 39
7. Discussion 50
8. Conclusion 54
9. Acknowledgements 54
10. References 55

1. List of Abbrevations

miRNA ..... MicroRNA
RNA ..... Ribonucleid acid
DNA ..... Deoxyribonucleic acid
WHO ..... World Health Organization
RGP ..... radial growth phase
VGP ..... vertical growth phase
UTR ..... untranslated region
Ran-GTP ..... RAs-related Nuclear protein - Guanosine-5'-triphosphate
RISC ..... RNA-Induced Silencing Complex
PCR ..... Polymerase chain reaction
ATCC ..... American Type Culture Collection
CO_2 ..... Carbon dioxide
FBS ..... Fetal Bovine Serum
DMEM ..... Dulbecco's Modified Eagle Media
EDTA ..... Ethylenediaminetetraacetic acid
dNTPs ..... Nucleoside triphosphate
cDNA ..... Complementary DNA
Tm ..... melting temperature
AT ..... annealing temperature
Ct ..... cycle threshold
Rmp ..... Rounds per minute

RT-PCR .... Reverse transcription polymerase chain reaction
RNase .... Ribonuclease
RT .... Reverse transcription
qPCR .... quantitative polymerase chain reaction
ΔΔCt .... delta-delta cycle threshold
SDS .... Sodium dodecyl sulfate
RIPA .... Radioimmunoprecipitation assay
PBS .... Phosphate buffered saline
NaCl .... Sodium chloride
SDS-PAGE .... Sodium dodecyl sulfate -Polyacrylamide Gel Electrophoresis
TBST .... Tris-Buffered Saline and Tween
IgG .... Immunoglobulin G
X-ray .... Energetic High-Frequency Electromagnetic Radiation
HEM .... Human Epidermal Melanocytes

2. LIST OF FIGURES

Figure 1: Biogenesis of microRNA. 10

Figure 2: Schematic diagrams of two different transfections. 15

Figure 3: General lipid design and proposed mechanism for DNA entry into cells 16

Figure 4: Different knockdown verification methods 17

Figure 5: The characteristic cyles of a polymerase chain reaction 19

Figure 6: Procedural overview of TaqMan® Small RNA Assays 20

Figure 7: RNA purification using RNeasy technology 21

Figure 8: Formation of a cDNA Duplex 23

Figure 9: Preparation of cDNA synthesis 23

Figure 10: cDNA synthesis - parameters 23

Figure 11: Preparation of the RT master mix1 24

Figure 12: Preparation of the qPCR reaction mix 25

Figure 13: Thermal cycling conditions 25

Figure 14: Formula to calculate the ΔΔCt value 26

Figure 15: Western Blotting 27

Figure 16: SDS-polyacrylamide gel electrophoresis 31

Figure 17: Reagents involved in the preparation of the separating gel 32

Figure 18: Reagents involved in the preparation of the stacking gel 32

Figure 19: Assembling of a Semi-dry blot 33

Figure 20: The use of enzyme-linked 2nd antibodies in immunodetection of protein blots 35

Figure 21: The use of enhanced chemiluminescence to detect horseradish peroxidase: 36

Figure 22: Example of outcome of western blotting on a x-ray film 36

Figure 23: Precision Plus Protein 36

Figure 24: fluorescence-activated cell sorter histogram 37

Figure 25: The events of the eukaryotic cell cycle 38

Figure 26: miR-18 expression level in percentage in a normal human melanocyte. 39

Figure 27: qRT-PCR analysis for miR-18b expression in 30 nevus samples 40

Figure 28: qRT-PCR analysis for miR-18b expression in 35 melanoma samples. -. 40

Figure 29: Relative miR-205 expression level in 1205-Lu cells (transient) 41

Figure 30: Outcome of cell survival assay 42

Figure 31: Transient overexpression of miR-18b suppresses colony formation. 42

Figure 32: cell cycle assay of 1205-Lu Neg-miR 43

Figure 33: percentage of cells being in different phases of cell cycle 44

Figure 34: cell cycle assay of 1205-Lu transient transfected with miR-18b 44

Figure 35: percentage of cells being in different phases of cell cycle 44

Figure 36: Relative miR-205 expression level in 1205-Lu cells (stably) 46

Figure 37 Stable over expression of miR-18b in melanoma 46

Figure 38: miR-18b overexpression significantly inhibits the colony formationf 47

Figure 39: cell cycle assay of 1205-Lu Neg-miR 47

Figure 40: cell cycle assay 47

Figure 41: percentage of 1205-Lu Neg-miR cells being in different phases 48

Figure 42: percentage of 1205-Lu miR-18b cells being in different phases 48

Figure 43: Stable overexpression of miR-18b inhibits cell proliferation. 49

3. ABSTRACT

MicroRNA (miRNA) regulate gene expression by repressing translation or directing sequence-specific degradation of complementary mRNA. In melanoma some miRNAs are significantly suppressed (tumor suppressors) and some are overexpressed (oncogenes) (1-2).

In order to study the effect of tumor suppressor miRNAs, melanoma cells are transfected with specific miRNA to investigate their role in suppressing the expression of potential oncogenes.

In this thesis I report that the expression of miR-18b is significantly suppressed in melanoma specimens when compared with nevi and is correlated inversely with melanoma progression. These results demonstrate the role for miR-18b as a tumor suppressor in melanoma.

4. INTRODUCTION

Melanoma

Malignant melanoma is the most aggressive form of skins caner and according to the World Health Organization (WHO), the number of melanoma cases worldwide is increasing faster than of any other type of cancer (3). Although not the most common of skin cancers, comprising only 5% of all skin cancers, it is by far the deadliest, responsible for 75% of skin cancer-related deaths (4).

Traditionally, five distinct steps of melanoma development and progression are distinguished: A dysplastic nevus {2} showing a high level of structural and architectural atypia arises from a common acquired nevus {1}. The subsequent radial growth phase (RGP) primary melanoma {3} is the first recognizable malignant stage in which cells do not possess metastatic potential but are already locally invasive.

RGP can be followed by VGP (vertical growth phase) primary melanoma lesions {4} in which melanoma cells infiltrate and invade the dermis and show metastatic potential. This process finally results in metastasis to distant organs by an overgrowth of disseminated tumor cells at these sites {5} (3).

Understanding of the gene expression character of early melanocytic transformation and further metastatic processes and their regulatory mechanism is vital for the future of targeted melanoma therapy.

Epigenetic modes of gene expression regulation include:

- DNA Methylation (promoter methylation inhibits transcription factor binding)
- Histone modifications (influences histone binding affinity for DNA) and
- RNA-associated silencing, including microRNA (miRNA) (4)

This family of molecules involved in the genesis and progression of melanoma cells may be the most complex and promising field for future melanoma cancer research and therapy (3-4).

In cancer, miRNAs function as regulatory molecules, acting as oncogenes or tumor suppressors (5).

microRNA

MicroRNAs (miRNAs) are functional RNA molecules that are transcribed from the DNA sequence of RNA genes, but not translated into protein. These small noncoding double-stranded RNA molecules mediate the expression of target genes by directly mediating mRNA degradation. Another way would be the complementary base pairing with the 3'-UTR of target mRNAs, causing their degradation. In addition, miRNAs can also inhibit translation in the event of imperfect base pair matching with the target. miRnas are expressed in a tissue-specific manner and are considered to play important roles in cell proliferation, apoptosis and differentiation.

30% of the human gene set, are implicated as miRNA targets, making miRNA one of the most abundant classes of regulatory genes in humans (1, 5).

Biogenesis of miRNA

Fig.1 shows the biogenesis of microRNA. In the nucleus, miRNA genes are transcribed by RNA polymerase II to give pri-microRNA (500-3000 bases). This molecule is then processed by Drosha, which is an endonuclease of the RNAase III family, giving pre-microRNA (~70 bases). Pre-microRNA is then exported from the nucleus by exportin and Ran-GTP. Dicer, which is another RNAase III, processes the pre-microRNA into double-stranded miRNA duplexes. Each of these duplexes form then a complex with an RNA-induced silencing complex (RISC), leading to unwinding of the duplex to form single-stranded microRNA.

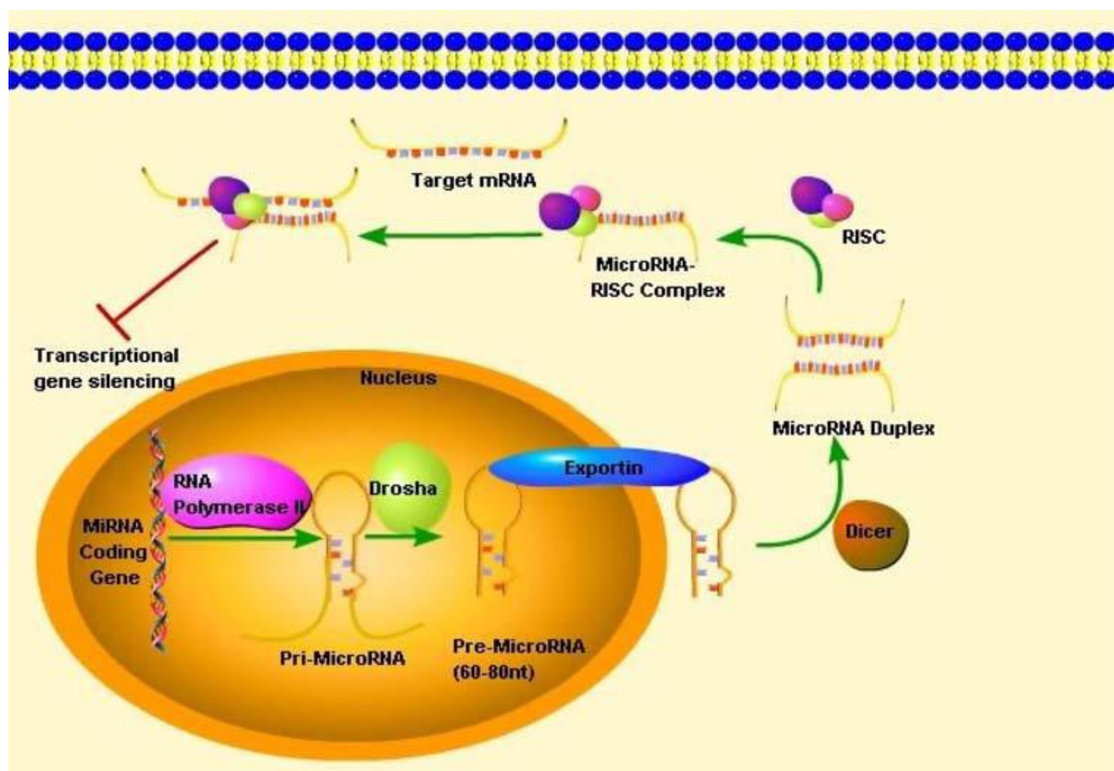


Figure 1: ***Biogenesis of microRNA.***
After transcribing miRNA in the nucleus into pri-microRNA, Drosha (endonueclease of RNAse III) giving pre-mictroRNA with a significantly lower number of bases (~70 compared to ~500-3000). Exportin and Ran-GTP (another RNAase III) export the pre-microRNA which is then converted into double-stranded miRNA duplexes. Each duplex forms a complex with RISC which generates a single-stranded miRNA (5).

MicroRNA and cancer

MicroRNAs provide the cells with a mechanism of gene regulation at the RNA level. Given the important functions of miRNAs in regulation cellular differentiation and proliferation, it is expected that their misregulation is linked to cancer. The action of microRNAs in cancer is dependent on their targets for mRNA, where they act as oncogenes or tumor suppressors (5).

MicroRNA as oncogenes / tumor suppressors

Amplification or overexpression of miRNAs can downregulate tumor suppressors or other genes involved in cell differentiation, thereby contributing to tumor formation by stimulating proliferation, angiogenesis, and invasion. Here they act as oncogenes. Similarly, miRNAs can function as tumor suppressors. They down-regulate different proteins with oncogenic activity.

5. METHODS
(AND THEIR SCIENTIFIC BACKGROUND)

5a Cell Culture

Cell culture has become one of the major tools used in the life science today. In the early 1959 several important developments occurred that made cell culture widely available as a tool for scientists. First, there was the development of antibiotics which made it easier to prevent the cultured cells being contaminated. Second, there was the development of trypsin which allowed adherent cells from culture vessels to be removed and a necessary step to obtain continuously growing cell lines. Third, there was the development of standardized, chemically defined culture media which enabled scientists to make it much easier to grow cells. (Ryan, 2000)

Nowadays cell culture has become one of the major tools used in cell and molecular biology. Some important areas are e.g. model systems, toxicity testing, virology, cell-based manufacturing, gene therapy, genetic engineering and cancer research.
Cell culture is done in order to produce protein, DNA, RNA and transfected proteins product from cultured cells.
Cells isolated from an organism prior to the first subcultivation, called primary cell culture. However, a cell line is a propagated culture after the first subculture. Various cell lines can be purchased from organizations such as ATCC.

Cell Culture – general

Cells are grown and maintained at 37°C at an appropriate gas mixture of 5% CO_2 in a cell incubator. (Wiesner C. P., 2010)
Most of the melanoma cells attach to a surface and appear elongated and bipolar, are grown in different culture mediums.

Growth medium

The WM3211, DO4, 1205-Lu and Lox melanoma cell lines were grown in RPMI with 10% fetal bovine serum. C8161.9 cells are cultured in DMEM/F12 with 5% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) (6).

Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrient components. The growth factors used to supplement media are often derived from animal blood, such as Fetal Bovine Serum.
Growth medium has to be stored for long term use at 4°C, but before adding them to the culture plates, they have to be at room temperature.

Fetal Bovine Serum (FBS)

This serum is containing a high amount of growth factors and hormones and proteins which are needed for cell adherence, trace minerals and inhibitors of cell proliferation. It is low in the amount of antibodies. (Wiesner D. C., 2010)

Dulbecco's Modified Eagle Media (DMEM)

This media, containing glucose, glutamine, sodium pyruvate and essential salts is used for growing cells in vitro. It also contains a a pH indicator (phenol red) which gives the media it's pink/red color at pH 7.2. (Wiesner C. P., 2010)

TU 2%

Containing MCDB153, Leibovitz's L-15, 2% FBS, Insulin, and CaCl2. This medium supports the growth of melanoma cells and it is designed for supporting cell growth in environments without CO_2 equilibration.

RPMI

The RPMI 1640 is an enriched media formulation with extensive applications for melanoma cells. It contains 4.5 g/L D-glucose, 1.5 g/L Sodium Bicarbonate, 1 mM Sodium Pyruvate, 10 mM HEPES and 300 mg/L L-glutamine. L-Glutamine is an essential amino acid, supporting the growth of cells which have a high energy demand and synthesize large amounts of proteins and nucleic acids. (Life Technologies Corporation)

General techniques, used in cell culture

In a regularly base, the morphology of the cells should be examined. The growth medium is changed frequently which is usually two times a week or at 80% cell confluence. At this time, a subculturing of the cells should be done. Furthermore to prevent bacterial contamination, the cells are treated with antibiotics (5% Penicillin/Streptomycin).

Splitting cells (Sub culturing)

This process involves transferring a small number of cells into a new vessel in order to decrease the cell density. To avoid senescence associated with prolonged high cell density, the cells are split every second or third day.

As melanoma cells belong to adherent cultures, the cells first need to be detached from the surface. (Wiesner C. P., 2010)

For monolayer culture systems, the culture medium is sucked off and the cells are washed with PBS in order to remove dead cells and DMEM which inhibits the action of trypsin. (Life Technologies Corporation) Trypsin-EDTA solution is used for dissociation of cell monolayers because the enzyme trypsin is hydrolyzing the intracellular junctions. After a five minute incubation time of the dissociation solution, the cells are detached from the surface. Next, growth medium is added to inhibit the function of trypsin any further. Now, a small number of these detached cells (dilution rate 1:20) can then be used to seed a new culture in a fresh culture flask or plate. (Invitrogen, Life Technologies)

Avoiding contamination

When working with cells it is necessary to take special care to avoid the possibility of contamination.

There are chemical sources like endotoxins, disinfectants, metal ions or plasticizers. Yeast, bacteria, viruses, fungi and mycoplasma belong to the biological resources of contamination. To maintain an aseptic work space, a laminar flow, which is regularly sterilized, is used. The bottles of culture medium are sprayed with 70% ethanol and the tips of the pipettes are autoclaved.

In order to prevent bacterial contamination, the melanoma cells are treated with 5% Penicillin/Streptomycin. This antibiotic is necessary to inhibit bacterial growth. (Invitro, Life Technologies)

5b Transfection

Transfection is the process of introducing foreign DNA or RNA into eukaryotic cells so that it is taken up by the nucleus for the purpose of expressing a protein or RNA species. (Bruce Alberts, 2002) The use of transfection is to study the role and

regulation of proteins encoded by a cloned cDNA, to study the miRNA knockdown of gene function, to study the function of non-coding regions of mRNA or to understand the mechanisms of a pathway.

In our case miRNAs are introduced to understand specific genes. MicroRNAs are short ribonucleic acid molecules found in eukaryotic cells. MiRNAs regulate gene expression by repressing translation or direction sequences-specific degradation of complementary mRNA (1).

Transfection can be either transient (for rapid analysis) or stable (for stable induction of expression).

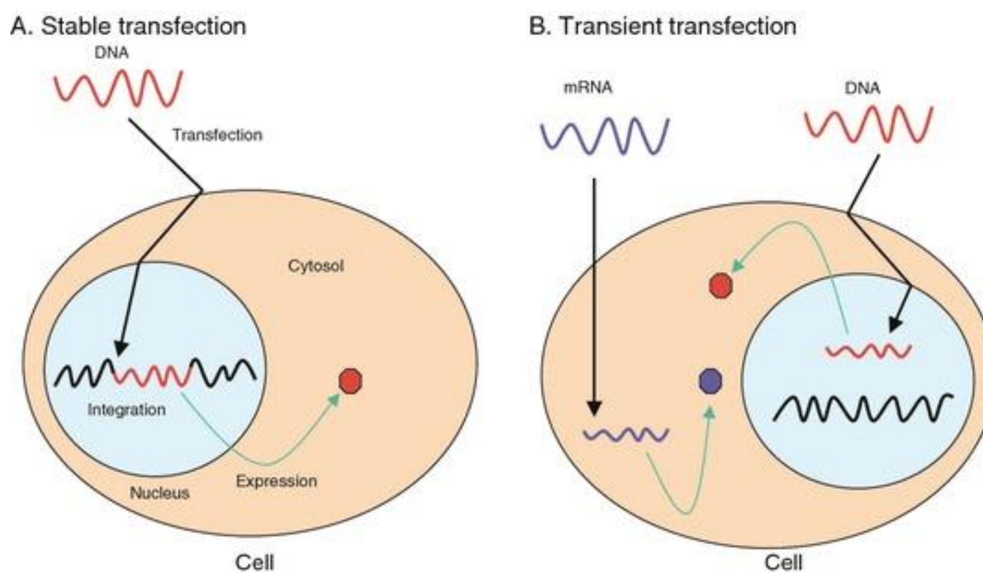


Figure 2: Schematic diagrams of two different transfections. *(a) Stable transfection. Foreign DNA (red wave) is delivered to nucleus by passage through the cell and nuclear membranes. Foreign DNA is integrated into the host genome (black wave) and expressed sustainably. (b). Transient transfection. Foreign DNA is delivered into the nucleus but is not integrated into the genome. Foreign mRNA (blue wave) is also delivered into the cytosol, where it is translated. Hexagons are expressed proteins from transfected nucleic acids. Black arrows indicate delivery of foreign nucleic acids*

For *stable transfection*, introduced genetic materials that usually have a marker gene for selection (transgenes) are integrated into the host genome and sustain transgene expression even after host cells replicate. Stable transfections require a clonal selection, which is an antibiotic resistance. (Kim Tae Kyung, 2010) In our case, we use puromycin. This is antibiotic, which acts a protein synthesis inhibitor inhibiting

translation. In order to keep cells stable, this antibiotic is added to the transfected cells when adding the growth media. (Berg JM, 2002)

Transiently transfected genes are only expressed for a limited period of time and are not integrated into the genome. (Invitro, Life Technologies)

Transfection - Methods

For performing the foreign introducing of genetic material into cells, there are various methods available. For example, some methods are physical transfection methods (electroporation, particle-gun, micro-injection of DNA), chemical delivery methods (dendrimeres, poly-ethylene-imine, transferrinfectin, DEAE-dextran and calcium phosphate). Other methods would be DNA-calcium precipitates, polycations-mediated transfection, viral vectors or lipid-mediated transfection in mammalian cells. The choice of transfection depends on the cell type used. The most desirable technique is the one which gives high efficiency of nucleic acid transfection with less interference to the cells physiology and high reproducibility. (Markus R. Wenk, 2007)
The lipid-mediated gene delivery (lipofection) is the most widely used transfection method:

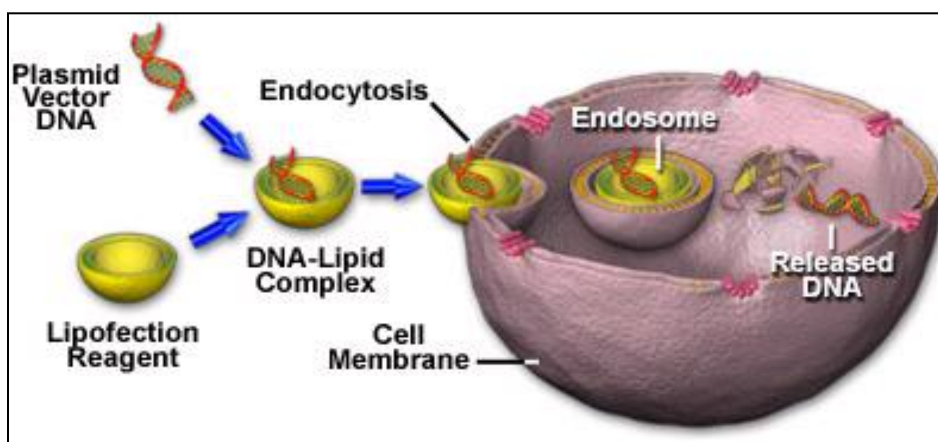


Figure 3: General lipid design and proposed mechanism for DNA entry into cells *(Wiesner C. P., 2010) (1) Cationic Lipid Transfection reagent (2) Cationic and neutral lipids form lipid bilayer structures (3)Ionic interactions between liposome and DNA form a complex, containing the foreign genetic material (4) This complex interact with the negatively charged cell membrane and via endocytosis the complex gets into the cell, resulting of successful expression. (Life Technologies, 2011)*

Transfection - Procedure:

In our case we use the transfection reagent "Lipofectamine® 2000" (Invitrogen).
In order to reach a 60 – 70 % cell confluence at the time of transfection, the cells are plated in growth medium without antibiotics and FBS one day before the experiment is performed because this will interfere with building the miRNA-lipid-complex. The cells are plated out on a 24-well-plate. DNA or miRNA along with Lipofectamine® 2000 Transfection Reagent (50 µl) is added to each well containing the melanoma cells. As cells get stressed when they are treated with Lipofectamine® so the medium is changed after five hours. Afterwards, the cells are incubated at 37°C in a 5% CO_2 atmosphere for 24- 48 hours until they are ready to assay for gene knockdown.

5c Knockdown verification techniques

There are a lot of knockdown verification techniques available (see figure 4). In our case we test the up- or down-regulation of a specific gene two different ways; either performing a western blot or using the advantages of a quantitative Real-time PCR.

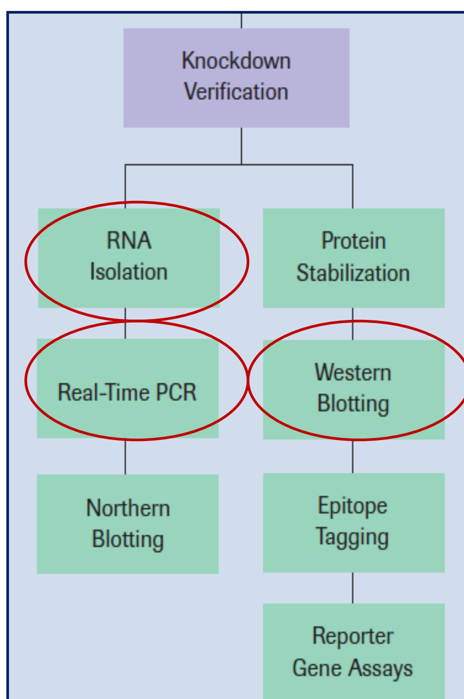


Figure 4: Different knockdown verification methods (Biochemica)

As RNA is the expressed form of a gene, it is possible to determine which genes are expressed or knocked down by isolating RNA and afterwards use quantitative real time PCR.

5d Polymerase Chain Reaction (PCR)

The PCR is a powerful tool for amplifying specific regions of DNA by the use of sequence-specific primers and multiple cycles of DNA synthesis, each cycle being followed by a brief heat treatment to separate complementary strands. (Bruce Alberts, 2002)

Template, primer, dNTPs and the polymerase are the main requirements for a PCR. Only DNA can act as template. RNA has first transcribed into cDNA before using the PCR. Therefore the reverse transcriptase synthesizes a single-stranded DNA molecule which then can act as a template for double stranded DNA synthesis.

The primer is a critical factor for running a successful PCR.

The primers (forward and reverse primer) bind to their complementary template sequence and the region between the primers gets amplified. The melting temperature (Tm) and annealing temperature (AT) are playing an important role. Double stranded DNA dissociates into single strands by heating.

The polymerase is a naturally occurring enzyme that copies DNA molecules. To attach to the DNA which should be synthesized it needs a primer which guides the polymerase to the right place. Then it reads the template strand and adds complementary dNTPs to the 3´OH end which results in an elongation of the new strand in a 5´-3´direction of the DNA. (Winkler, 2008)

The three main steps in the PCR are:

Denaturation: DNA fragments are heated at high temperatures, which reduce the DNA double helix to single strands. These strands become accessible to primers.

Annealing: The reaction mixture is cooled down. Primers anneal to the complementary regions in the DNA template strands, and double strands are formed again between primers and complementary sequences.

Extension: The DNA polymerase synthesizes a complementary strand. The enzyme reads the opposing strand sequence and extends the primers by adding nucleotides

in the order in which they can pair. The whole process is repeated over and over. (Griffiths, 1996)

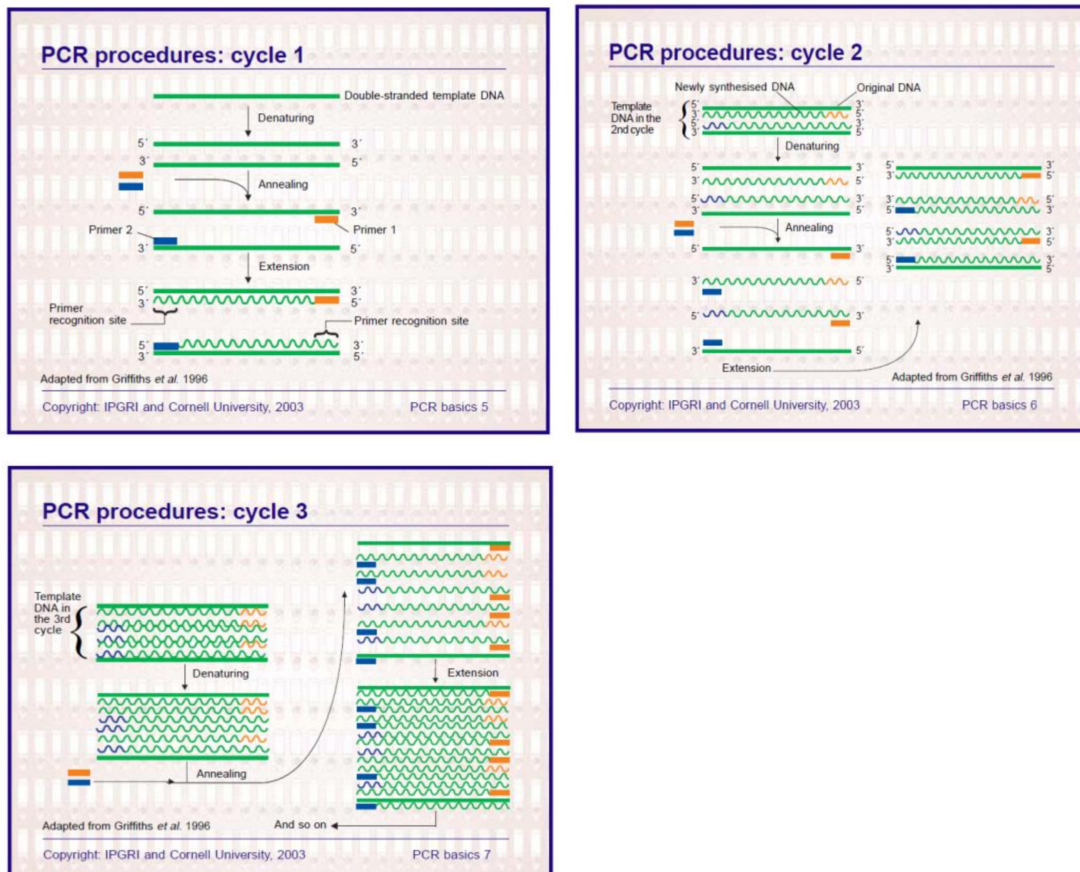


Figure 5: The characteristic cyles of a polymerase chain reaction (Griffiths, 1996**)**

Real time PCR

Real-time PCR is the technique of collecting data throughout the PCR process as it occurs, thus combining amplification and detection into a single step. This is achieved using a variety of different fluorescent chemistries that correlate PCR product concentration to fluorescence intensity. Reactions are characterized by the point in time (or PCR cycle) where the target amplification is first detected. This value is usually referred to as cycle threshold (C_t), the time at which fluorescence intensity is greater than background fluorescence. Consequently, the greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower C_t .

Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Especially the high sensitivity of the real-time PCR is a big advantage. (Medrano, 2005)

Real time PCR - procedure:

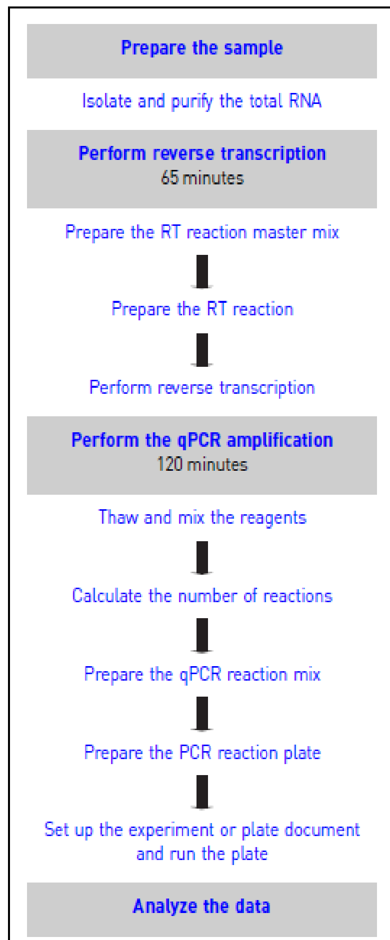


Figure 6: Procedural overview of TaqMan® Small RNA Assays (BIO-RAD)

1) Isolation/Purification of RNA from animal cells

For the whole RNA purification process from animal cells using spin technology, the QIAGEN RNeasy® kit is used.

This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane.

Efficient disruption and homogenization of the starting material is an absolute requirement for all total RNA purification procedures. Therefore, 700 µl of RLT-Lysis buffer and 7 µl of β-Mercaptoethanol are added to the tube, containing the transfected cell pellet. This lysate is placed into a QIAshredder spin column and spun for 2 minutes at maximum speed in a microcentrifuge. The lysate is homogenized as it passes through the spin column

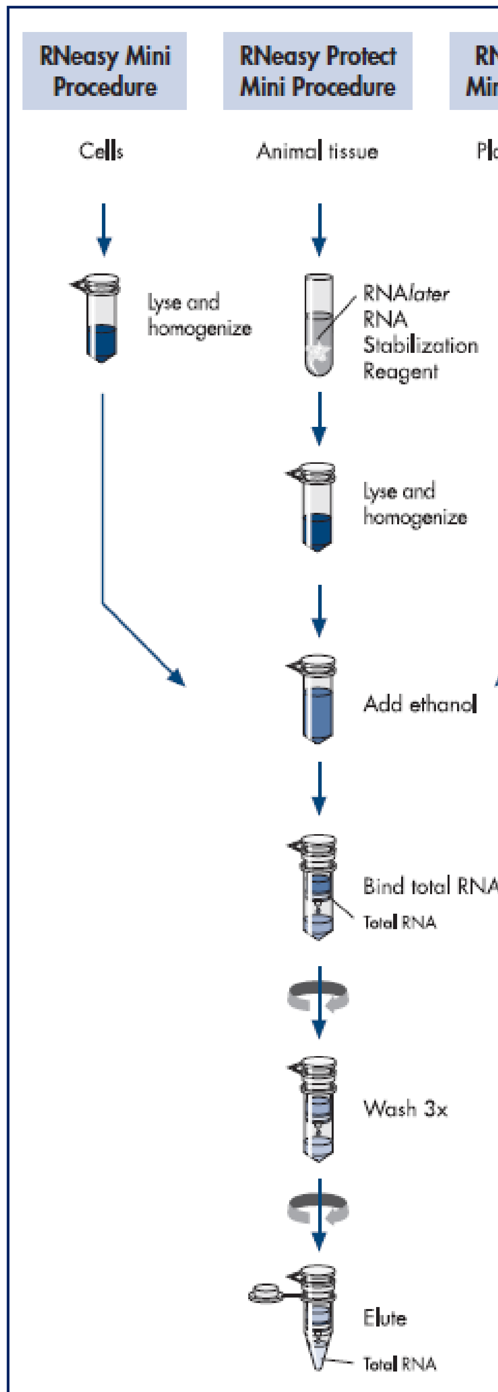


Figure 7: RNA purification using RNeasy technology (QIAGEN**)**

The RTL-Lysis Buffer is a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column and is centrifuged for one minute at 10.000 rmp

With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. As most RNAs are <200 nucleotides 100% ethanol instead of 70% is used for miRNA purification. All the other steps remain the same. (QIAGEN)
Total RNA bind to the membrane and contaminates are efficiently washed away using the washing buffer RW1 and RPE. High-quality RNA (miRNA) is eluted in RNase-free water. All bind, wash, and elution steps are performed by centrifugation in a microcentrifuge (6) .

2) Measuring the concentration of the RNA/miRNA.

After the purification the RNA/miRNA concentration is determined with a NanoDrop 2000 spectrophotometer with a sample retention technology which measures samples (0.5 µl) with the absorbance rate of A260 and the sample purity of 260/280 (Thermo Scientific, Rockford, IL).

The purified RNA is now ready for use in downstream applications such as RT-PCR and real-time RT-PCR or cDNA synthesis.

3) cDNA synthesis

DNA copies of mRNAs are called complementary DNAs or cDNAs. (Lodish H, 2000) It is DNA synthesized from a mRNA template in a reaction catalyzed by the enzyme reverse transcriptase and the enzyme DNA polymerase.

The key to forming *cDNA* is the enzyme reverse transcriptase. A retrovirus uses this enzyme to form a DNA-RNA hybrid in replicating its genomic RNA. Reverse transcriptase synthesizes a DNA strand complementary to an RNA template if it is provided with a DNA primer that is base-paired to the RNA. We can use a simple sequence of linked thymidine [oligo(T)] residues as the primer. This oligo(T) sequence pairs with the poly(A) sequence at the 3′ end of most eukaryotic mRNA molecules (Figure 8). The reverse transcriptase then synthesizes the rest of the cDNA strand in the presence of the four deoxyribonucleoside triphosphates. The single-stranded DNA is converted into double-stranded DNA by creating another primer site. The enzyme terminal transferase adds nucleotidesComplementary DNA for all mRNA that a cell contains can be made, inserted into vectors, and then inserted into bacteria. Such a collection is called a cDNA library. (Berg JM, 2002)

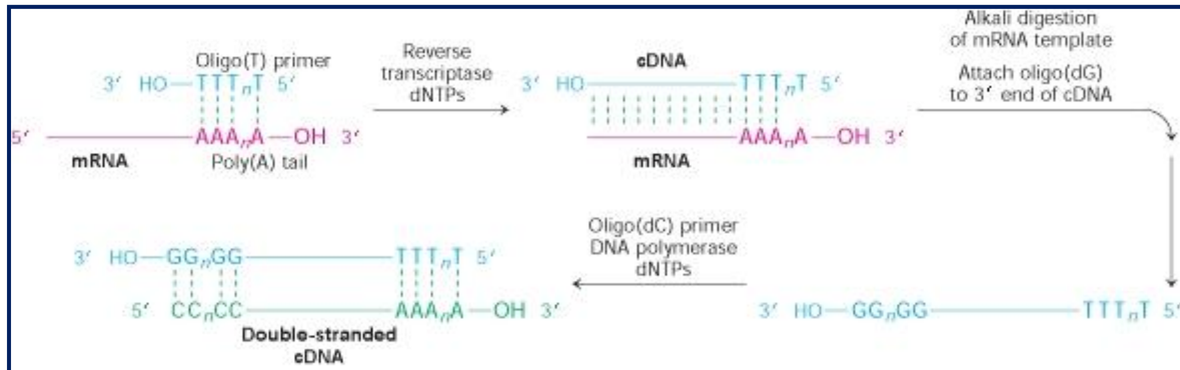


Figure 8: Formation of a cDNA Duplex (Berg JM, 2002)
(A cDNA duplex is created from mRNA by using reverse transcriptase to synthesize a cDNA strand, first along the mRNA template and then, after digestion of the mRNA, along that same newly synthesized cDNA strand.)

cDNA synthesis - procedure

To use a system for first-strand cDNA synthesis, the iScript cDNA synthesis kit from BIO-RAD is used. This kit contains the RNase H^+ iScript reverse transcriptase for sensitivity, a premixed RNase inhibitor to prevent indiscriminate degradation of RNA template, and a unique blend of oligo(dT) and random primers. (BIO-RAD)
The samples are mixed together according this protocol:

Components	Volume per Reaction
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease-free water	x µl
RNA template (100 fg to 1 µg total RNA)*	x µl
Total volume	20 µl

Figure 9: Preparation of cDNA synthesis (BIO-RAD)

Afterwards the samples are incubated according the following protocol (Figure 10) in a PCR machine.

Incubate complete reaction mix:
5 minutes at 25ºC
30 minutes at 42ºC
5 minutes at 85ºC
Hold at 4ºC (optional)

Figure 10: following parameters are set on the PCR machine (BIO-RAD)

4) Micro cDNA

To create microRNA Assay, the Applied Biosystems® TaqMan® MicroRNA Reverse Transcription Kit is used, which has the necessary components for optimal performance in TaqMan® MicroRNA Assays. Components of this kit are used with the RT primer provided with the TaqMan® MicroRNA Assay to convert miRNA to cDNA.

The master mix is prepared as seen in the following table (figure 11).

Component	Master mix volume per 15-µL reaction*
100mM dNTPs (with dTTP)	0.15 µL
MultiScribe™ Reverse Transcriptase, 50 U/µL	1.00 µL
10X Reverse Transcription Buffer	1.50 µL
RNase Inhibitor, 20 U/µL	0.19 µL
Nuclease-free water	4.16 µL
Total volume	**7.00 µL**

* Each 15-µL RT reaction consists of 7 µL master mix, 3 µL of 5X RT primer, and 5 µL RNA sample.

Figure 11: Preparation of the RT master mix1 (Invitro, Life Technologies**)**

Afterwards, the RT reaction, including no-template controls and RT minus controls, is added to the tubes, containing the master mix. Also, the denatured RNA is added. (1 to 10 ng of RNA per reaction).

Reverse Transcription:

In a PCR System machine (Applied Biosystems), the samples are incubated first for 30 minutes at 16 °C, then keep for 30 minutes at 42 °C and the finally they are hold 5 minutes at 85 °C.

5) TaqMan® Small RNA Assays

This kit contains preformulated primer and probe sets designed to detect and quantify mature microRNAs using real-time PCR instruments (Applied Biosystems). The assays can detect and quantify small RNA in 1 to 10 ng of total RNA.

Each TaqMan® Assay includes:

• One tube containing small RNA-specific RT primer

• One tube containing a mix of small RNA-specific forward PCR primer, specific reverse PCR primer and small RNA-specific TaqMan® MGB probe

After the cDNA samples are done, the TaqMan® Assay (20X) is thawed and the number of reactions that are need for each assay is calculated. The reaction mix is prepared as shown in figure 12:

Component	Volume per 20-µL Reaction	
	Single reaction	Three replicates§
TaqMan® Small RNA Assay (20X)	1.00 µL	3.60 µL
Product from RT reaction*	1.33 µL	4.80 µL
TaqMan® Universal PCR Master Mix II (2X), no UNG‡	10.00 µL	36.00 µL
Nuclease-free water	7.67 µL	27.61 µL
Total volume	**20.00 µL**	**72.01 µL**

Figure 12: Preparation of the qPCR reaction mix (Invitro, Life Technologies)

To prepare the PCR reaction plate, 33 µl of the complete qPCR mix (assay and RT product including non-template controls) are pipetted in triplicates (11µl/well) into a 96-well-plate and are run according the following protocol. (Figure 13)

Step	Optional AmpErase® UNG activity*	Enzyme Activation	PCR	
	HOLD	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 minutes	10 minutes	15 seconds	60 seconds

Figure 13: Thermal cycling conditions (Invitro, Life Technologies)

The gene expression levels are quantified using 7500 Fast Real-time Sequence detection system Software. The relative expression was calculated using the comparative Ct method.

6) Ct value

The qPCR data can be calculated by the ΔΔCt (delta-delta-Ct) method. (see fig. xx)
Due to pipetting errors, sample-to-sample differences, sample quality, differences in the starting material and other problems. Samples are normalized to RNU48 or HPRT. These house-keeping genes are expressed at a constant level and they are unaffected by investigated experimental treatment.
For this normalization step the CR value of the reference gene is subtracted from the Ct value of the gene of interest (ΔCt). To get the ΔΔCt value, the ΔCt of the control sample is substracted from the ΔCt of the treated sample. The last step is to calculate the ratio. (Figure 14)

ΔCt = Ct of analyzed gene – Ct of reference gene (e.g. RNU)

ΔΔCt = ΔCt of treated gene - ΔCt of control gene

Ratio : $2^{-\Delta\Delta Ct}$

Figure 14: Formula to calculate the ΔΔCt value

In reference to the different methods of knockdown verifications shown in figure 4, the western blot analysis also belongs in the group of important techniques used to understand if a special gene is expressed or suppressed.

5e Western Blot Analysis

To detect small quantities of a particular protein in the presence of many other proteins, an immunoassay technique called Western blotting is used.(Figure 4). A (protein) sample is subjected to electrophoresis on an SDS-polyacrylamide gel. Blotting transfers the resolved proteins on the gel to the surface of a polymer sheet to make them more accessible for reaction. An antibody that is specific for the protein of interest is added to the sheet and reacts with the antigen. The antibody-antigen complex on the sheet then can be detected by rinsing the sheet with a second antibody specific for the first (e.g., goat antibody that recognizes mouse antibody). A radioactive label on the second antibody produces a dark band on x-ray film (an autoradiogram). Alternatively, an enzyme on the second antibody generates a colored product, as in the ELISA method. Western blotting makes it possible to find a protein in a complex mixture. (Berg JM, 2002)

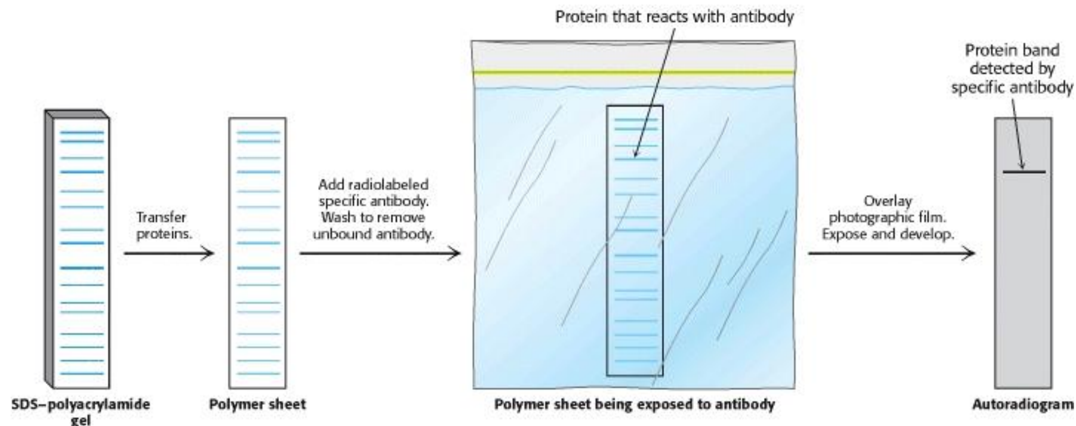


Figure 15:Western Blotting
Proteins on an SDS-polyacrylamide gel are transferred to a polymer sheet and stained with radioactive antibody. A band corresponding to the protein to which the antibody binds appears in the autoradiogram. **(Berg JM, 2002)**

1) Protein extraction

Protein purification is a series of processes necessary to isolate a single type of protein from a complex mixture.

Proteins from various cell lines samples need to be extracted efficiently and without degradation to make the best use of a limited resource.

The most common method in biomedicine to solubilize proteins for identity and quantity determination is by solubilization with the ionic detergent sodium dodecyl sulfate (SDS).

In our case, we use RIPA lysis buffer, containing 0.1% SDS. (Christer Ericsson)

RIPA buffer is one of the most reliable buffers used to lyse cultured mammalian cells from both plated cells and cells pelleted from suspension cultures. This buffer enables protein extraction from cytoplasmic, membrane and nuclear proteins and is compatible with many applications, including reporter assays, protein assays, immunoassays and protein purification. (Pierce)

As this buffer does not contain protease or phosphatase inhibitors, additionally a protease/phosphatase inhibitor is added to prevent proteolysis and phosphorylation of proteins. (OpenWetWare contributors)

Proteine Extraction - Procedure:

After the growth medium is removed from the adherent cells, a two time's wash of the cell pellet with PBS takes place. Afterwards cold RIPA buffer is added to the cells. (1ml of buffer per 75 cm2 flask containing 5x×106 melanoma cells.) The whole lysate is kept on ice for 20 minutes. (OpenWetWare contributors) Next the mixture is applied to a sonificator. This machine ultrasonicaly disrupt cells. The tip of the machine vibrates in a longitudinal direction and transmits this motion to the solution. Cavitation results, in which microscopic vapor bubbles are formed momentarily and implode, causing powerful infinitesimal shock waves to radiate throughout the solution in proximity to the radiating face of the tip. (Biologics, Inc.)

After the cell disruption, the lysate is centrifugated at 13.000 rpm for 8 minutes at 4°C in order to pellet the cell debris.

The last step is to transfer the supernatant to a new tube for further analysis. (OpenWetWare contributors)

2) Yield of extracted protein – Bradford Assay

In order to determine the total yield of extracted proteins, a standard curve has to be set up. (Christer Ericsson)

In our case we use the Bio Rad Protein Assay. It is based on the method of Bradford and is a simple and accurate procedure for determining concentration of solubilized protein. The Bio-Rad Protein Assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. It involves the addition of an acidic dye to protein solution, Coomassie® Brilliant Blue G-250 dye, which absorbance is shifting from 465 nm - 595 nm when binding to protein occurs. The subsequent measurement at 595 nm with a spectrophotometer or microplate reader The Coomassie blue dye binds to primarily basic and aromatic amino acid residues (arginine)

Comparison to a standard curve provides a relative measurement of protein concentration.

Bradford Assay - Procedure

To 5µl cell lysate (varies according to protein concentration) add 995µl (1:5 diluted Bio-Rad reagent (dilution factor 1:200)) is added and the whole solution is incubate at room temperature for 5 minutes. Afterwards the absorbance at 595nm is measured.

Next, a standard curve (see table 1) using a serial dilution series (0 – 25 ng/ml) of a know protein sample concentration is prepared. Therefore, bovine serum albumin (BSA) (1mg/ml) is dissolved with water or 0.15M NaCl.

Table 1: Set-up of a standard curve for estimating the protein concentration

BSA(µl)	Water(µl)	Bio-Rad reagent 1:5 dilute in water(µl)
0	25	975
5	20	975
10	15	975
15	10	975
20	5	975
25	0	975

The standard curve is performed in duplicates and after 5 minutes waiting time the reading at 595 nm is done. (BIO-RAD Life Science Research)

Electrophoresis

Electrophoresis is a technique for separating, or *resolving,* molecules in a mixture under the influence of an applied electric field. Dissolved molecules in an electric field move, or migrate, at a speed determined by their charge mass ratio. For example, if two molecules have the same mass and shape, the one with the greater net charge will move faster toward an electrode. (Lodish H, 2000)

3) SDS-Polyacrylamide Gel Electrophoresis

Because many proteins or nucleic acids that differ in size and shape have nearly identical charge/mass ratios, electrophoresis of these macromolecules in solution results in little or no separation of molecules of different lengths. However, successful

separation of proteins and nucleic acids can be accomplished by electrophoresis in various *gels* (semisolid suspensions in water) rather than in a liquid solution. Electrophoretic separation of proteins is most commonly performed in *polyacrylamide gels.* These gels are cast between a pair of glass plates by polymerizing a solution of acrylamide monomers into polyacrylamide chains and simultaneously cross-linking the chains into a semisolid matrix. The *pore size* of a gel can be varied by adjusting the concentrations of polyacrylamide and the cross-linking reagent. (Lodish H, 2000)

When a mixture of proteins is applied to a gel and an electric current applied, smaller proteins migrate faster than larger proteins through the gel. The rate of movement is influenced by the gel's pore size and the strength of the electric field. The pores in a highly cross-linked polyacrylamide gel are quite small. Such a gel could resolve small proteins and peptides, but large proteins would not be able to move through it.

In what is probably the most powerful technique for resolving protein mixtures, proteins are exposed to the ionic detergent SDS (sodium dodecylsulfate) before and during gel electrophoresis (figure 15). SDS denatures proteins, causing multimeric proteins to dissociate into their subunits, and all polypeptide chains are forced into extended conformations with similar charge mass ratios. SDS treatment thus eliminates the effect of differences in shape, so that chain length, which reflects mass, is the sole determinant of the migration rate of proteins in SDS-polyacrylamide electrophoresis. Even chains that differ in molecular weight by less than 10 percent can be separated by this technique. Moreover, the molecular weight of a protein can be estimated by comparing the distance it migrates through a gel with the distances that proteins of known molecular weight migrate.

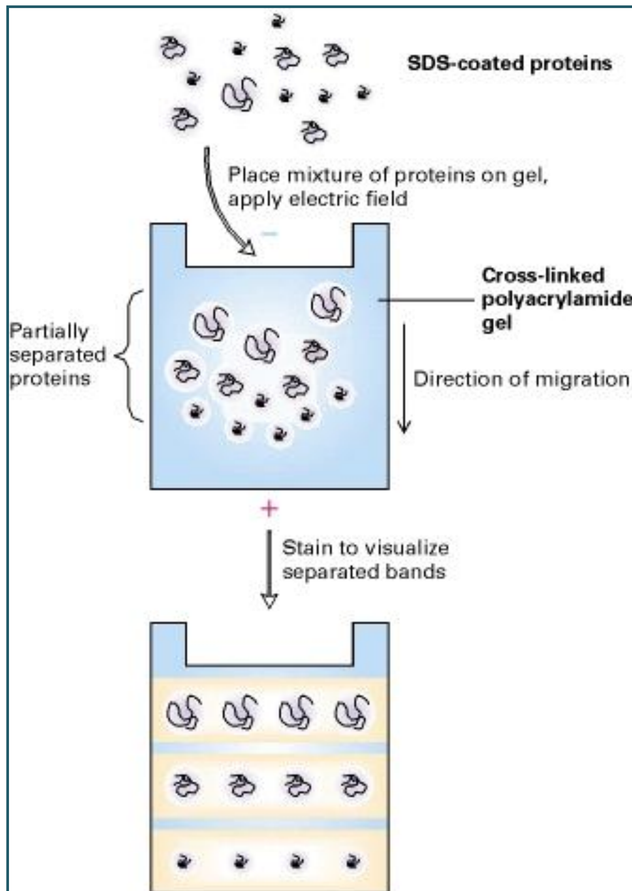


Figure 16: SDS-polyacrylamide gel electrophoresis (technique for separating proteins at good resolution) (Lodish H, 2000)
First the protein mixture is treated with SDS, a negatively charged detergent that binds to proteins. This binding dissociates multimeric proteins and forces all polypeptide chains into denatured conformations with nearly identical charge mass ratios. During electrophoresis, the SDS-protein complexes migrate through the polyacrylamide gel. Small proteins are able to move through the pores more easily, and faster, than larger proteins. Thus the proteins separate into bands according to their size as they migrate through the gel. The separated protein bands are visualized by staining with a dye

SDS-PAGE - Procedure

To pour the gel, the gel apparatus has to be set up. According to the size of the particular protein, an appropriate percentage of the gel has to be chosen.

Table 2: *Information regarding to the percentage of gel to be used for the protein of particular molecular mass*

Gel %	Protein Size (kDa)
15	12-43
10	16-68
7	36-94
5	57-212

Next all reagents (figure 17) are mixed gently and are filled into the chamber of the apparatus. Afterwards, approximately 1ml of distilled H2O is put on the top of the layer in order to remove air bubbles.
After the polymerization of the separating gel, the overlay is decanted and the stacking monomer is prepared (see figure 18). The comb is inserted and before running the electrophoresis, the gel has to be completely polymerized. (www.dartmouth.edu, 2011)

Separating gel

Solutions / Gel %	7%	10%	12%	15%
Distilled H_2O	5.1 ml	4.1 ml	3.4 ml	2.4 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	2.5 ml	2.5 ml
20% (w/v) SDS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	2.3 ml	3.3 ml	4.0 ml	5.0 ml
10% (w/v) ammonium persulfate	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml
Total Volume	**10.005 ml**	**10.005 ml**	**10.005 ml**	**10.005 ml**

Figure 17: *Reagents involved in the preparation of the separating gel*

Stacking gel

Stacking Gels, 4.0% gel, 0.125 M Tris, pH 6.8	
distilled H_2O	3.075 ml
0.5 M Tris-HCl, pH 6.8	1.25 ml
20% (w/v) SDS	0.025 ml
Acrylamide/Bis-acrylamide (30%/0.8% w/v)	0.67 ml
10% (w/v) ammonium persulfate	0.025 ml
TEMED	0.005 ml
Total Stack monomer	**5.05 ml**

Figure 18: *Reagents involved in the preparation of the stacking gel*

After the protein samples (according to their concentration) are mixed with standard 2x sample Buffer (0.5M Tris-HCl, pH 6.8,4.4% SDS, 300mM Mercaptoethanol, 10mg/ml Bromphenol Blue) and protein loading buffer 2x, they get a heat treatment (95°C) for five minutes.
Afterwards, the samples are loaded into the wells on the gel and voltage is applied in order to separate the proteins.

The gel electrophoresis is running with a 5X SDS-PAGE Running Buffer.

4) Transfer proteins to membrane

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. There are a variety of methods that have been used for this process, including diffusion transfer, capillary transfer, heat-accelerated convectional transfer, vacuum blotting transfer and electroelution. (Asai, 1993)

In our case, we use the most common method, semi-dry blot technique, which is based on the electrophoretic transfer giving advantages in speed and transfer efficiency. (Westermeier, 2005) This method uses the electrophoretic mobility of proteins to transfer them from the gel to the membrane. Electrophoretic transfer of proteins involves placing a protein-containing polyacrylamide gel in direct contact with a piece of nitrocellulose (protein-binding support) and "sandwiching" this between two electrodes submerged in a conducting solution. When an electric field is applied, the proteins move out of the polyacrylamide gel and onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel. (Thermo Scientific)

It is important to exclude excess moisture and air bubbles trapped in the filter papers and membrane when setting up the transfer.

(Polvino, 1983)

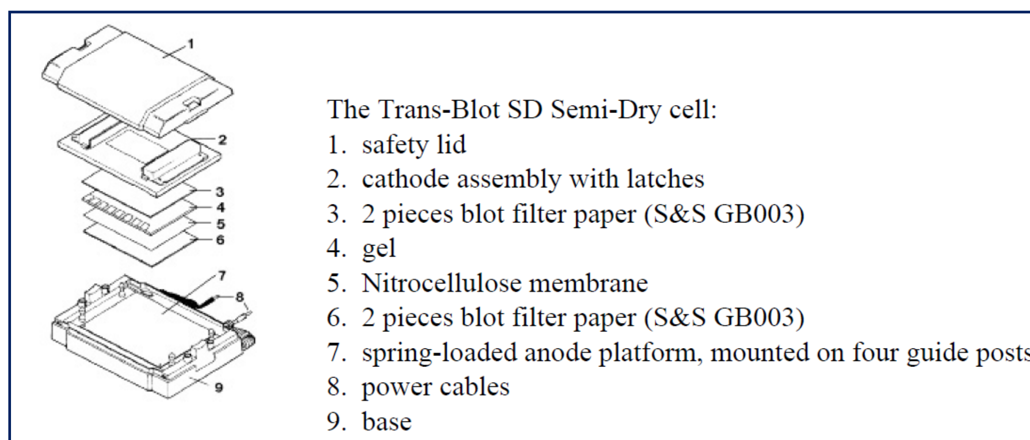


Figure 19: Assembling of a Semi-dry blot (Asai, 1993**)**

5) Blocking non-specific sites

The membrane supports used in Western blotting have a high affinity for proteins. Therefore, after the transfer of the proteins from the gel, it is important to block the remaining surface of the membrane to prevent nonspecific binding of the detection antibodies during subsequent steps. (Thermo Scientific)

First, the blot is washed a total of three times with TBST, then incubated (60 minutes) in a protein solution (5% non-fat dried milk in TBST), which will block all remaining hydrophobic binding sites on the nitrocellulose sheet. To remove nonspecifically bound antibodies, the nitrocellulose must be washed three times for five minutes with TBST. (Asai, 1993)

6) Primary and secondary antibodies

Once transferred on to nitrocellulose, the separated proteins can be examined further. This involves probing the blot, usually using an antibody to detect a specific protein. The blocked membrane is incubated for 60 minutes in a dilution of 1 µl in 20 ml of blocking buffer with an anti-serum or primary antibody directed against the protein of interest. This IgG molecule will bind to the blot if it detects its antigen, thus identifying the protein of interest. (Keith Wilson, 2000)

In general, the primary antibody which recognizes the target protein in a Western blot is not directly detectable. (Thermo Scientific). In order to visualize this interaction, the blot is incubated further in a solution of tagged secondary antibody, which is directed against the IgG of the species that provided the primary antibody. For example, if the primary antibody was raised in a rabbit then the 2nd antibody would be anti-rabbit IgG.)

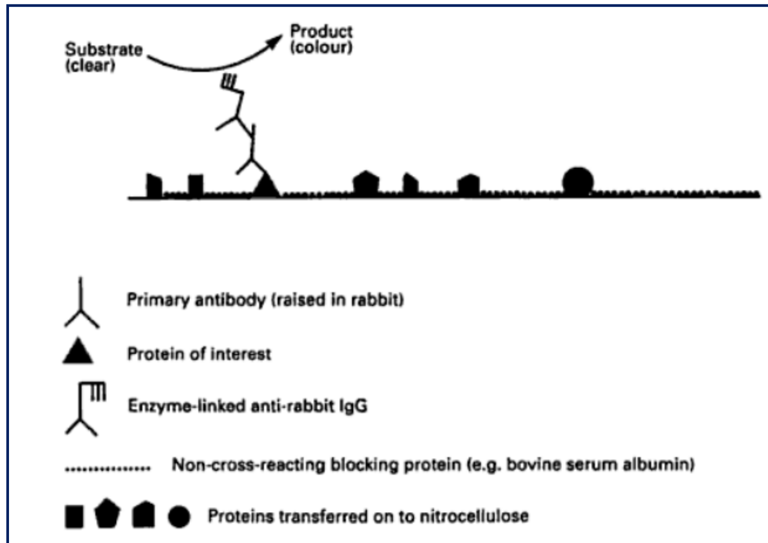


Figure 20: The use of enzyme-linked second antibodies in immunodetection of protein blots
1) The primary antibody (e.g. raised in a rabbit) detects the protein of interest on the blot. 2) Enzyme-linked anti-rabbit IgG detects the primary antibody. 3) Addition of enzyme substrate results in coloured product deposited at the site of protein of interest on the blot (Keith Wilson, 2000)

7) Detection method

One of the most common detection methods is to use an enzyme-linked secondary antibody. In our case, we use the Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc.), which enhances chemiluminescence detection of Western blots). (Keith Wilson, 2000)
After a 60 minute incubation time of the secondary antibody in a TBST dilution, the nitrocellulose membrane is covered with the western blotting luminal reagent. After the membrane is covered with this reagent, it is put into a developing cassette and the blot is exposed to an X-ray film.

In the presence of hydrogen peroxide and the chemiluminescent substrate luminal, HPO oxidizes the luminal with concomitant production of light. – The intensity is increased 100-fold by the presence of this chemical enhancer. The light emission can be detected by exposing the blot to a photographic film. (Thermo Scientific)

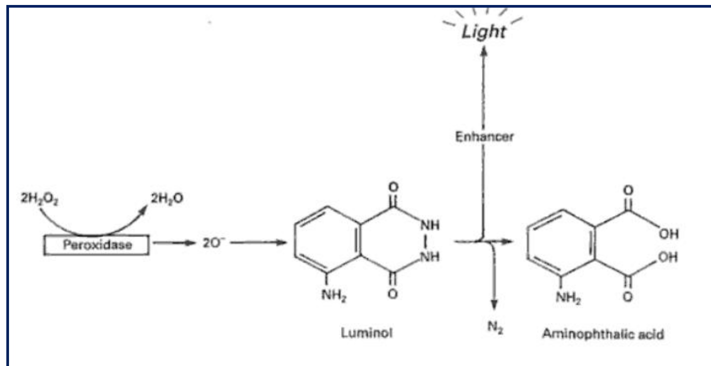


Figure 21: The use of enhanced chemiluminescence to detect horseradish peroxidase: Chemiluminescence is a property of chemical reactions which emit light as a byproduct. Luminol is one of the most widely used chemiluminescent reagents. The Oxidation of luminol by peroxide results in creation of an excited state product called 3-aminophthalate*. This product decays to a lower energy state by releasing photons of light. *(Keith Wilson, 2000) (Thermo Scientific)*

8) Outcome Western

For analyzing and assessing the transfer efficiency and for estimating the molecular weight of the proteins of interest, a Protein Kaleidoscope standard is used. (Precision Plus Protein™ Kaleidoscope Standards, BIO-RAD) (Bio-Rad Laboratories, Inc.)

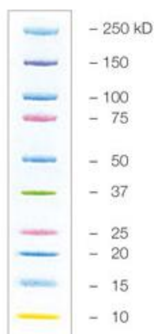


Figure 22: Precision Plus Protein™ Kaleidoscope Standards #161-0375, BIO-RAD

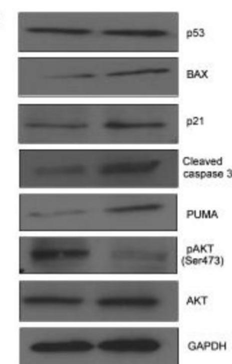
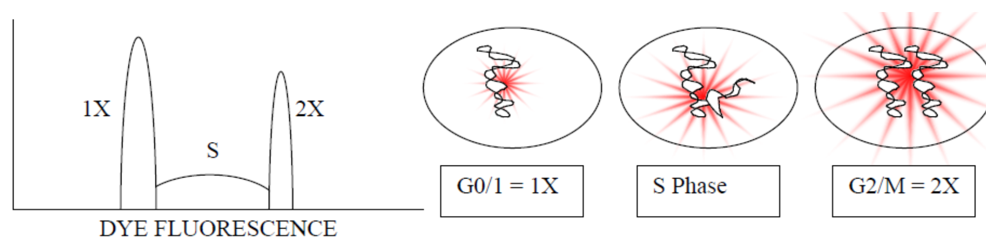


Figure 23: Example of outcome of western blotting on a x-ray film (Dar AA, 2010)

5f Cell Cycle Assay

Analysis of a population of cells' replication state can be achieved by fluorescence labeling of the nuclei of cells in suspension and then analyzing the fluorescence properties of each cell in the population. Quiescent and G1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity. Cells in G2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity. Since the cells in S phase are synthesizing DNA they will have fluorescence values between the 1X and 2X populations.



The resulting histrogram consists of three populations: two Gaussian curves (1X and 2X peaks) and the Sphase population. Adjacent populations overlap each other. Because of this, a modeling program is required to de-convolute the populations and assign percentage values to each population. Expert and subjective review of the modeling software's cell cycle phase percentage assignment is the final stage of cell cycle analysis prior to reporting the results. (UCL - University College London)

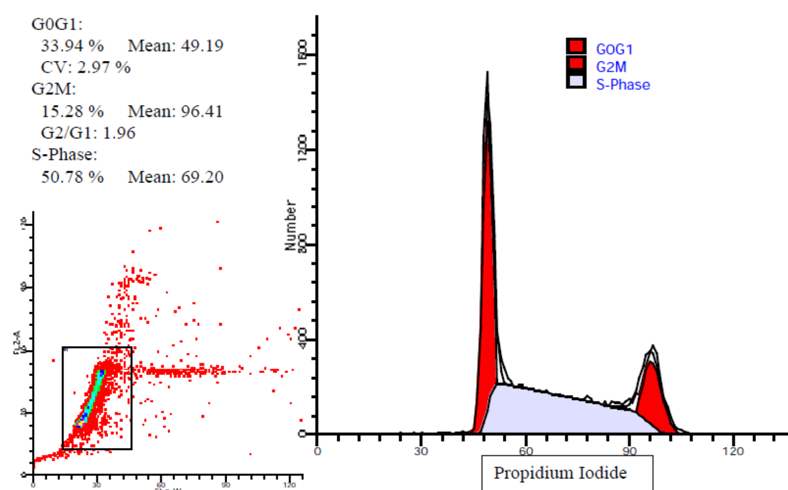


Figure 224: fluorescence-activated cell sorter histogram showing each phase oft he cell´s cell cycle

Cell reproduction occurs by an elaborate series of events called the cell cycle, whereby chromosomes and other components are duplicated and then distributed into two daughter cells. A complex network of regulatory proteins governs progression through the steps of the cell cycle.

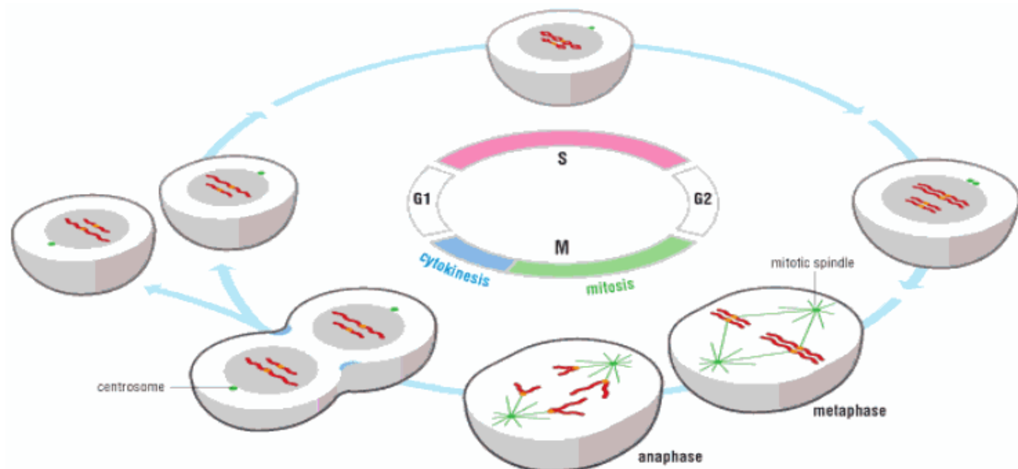


Figure 25: The events of the eukaryotic cell cycle

S phase: the cell-cycle phase during which DNA replication and chromosome duplication occurs.

G2: the cell-cycle gap phase between S phase and M phase

M phase: the cell-cycle phase during which the duplicated chromosomes are segregated and packaged into daughter nuclei (mitosis) and distributed into daughter cells (cytokinesis)

G1: the cell-cycle gap phase between M phase and S phase

G0 : a prolonged non-dividing stat that is reached from G1 when cells are exposed to extracellular conditions that arrest cell proliferation

The central events of cell reproduction are chromosome duplication, which takes place in the S phase, followed by chromosome segregation and nuclear division (mitosis) and cell division (cytokinesis), which are collectively called M phase. G1 is the gap phase between M and S phases; G2 is the gap phase between S and M phases. Metaphase is the stage in mitosis when sister chromatids are aligned on the mitotic spindle and anaphase is the stage when sister chromatids are separated and pulled to opposite spindle poles. In most cells, the discrete events of the cell cycle occur against a background of continuous cell growth. (Morgan, 2007)

6. RESULTS

The expression of miR-18b in different human melanoma cells (C8161.9, Lox, D04, 1205-Lu and WM3211) is mainly analyzed by quantitative real time-PCR (qRT-PCR) and western blot analyses. This expression is compared with a normal melanocyte cell line (HEM).

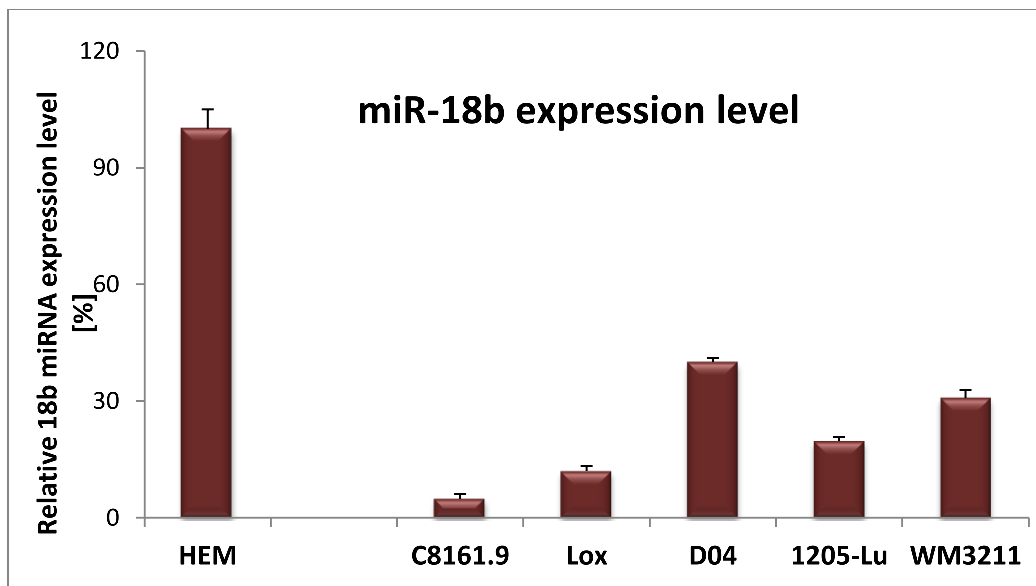


Figure 26: *miR-18 expression level in percentage in a normal human melanocyte (HEM) and five melanoma cell lines as determined by qRT-PCR.*

The expression level of miR-18b in a panel of human melanoma cell lines and normal melanocytes is shown in figure 26. The results indicate a significant down-regulation in expression of miR-18b in melanoma cells as compared with normal melanocytes. This data suggests that miR-18b has a potential tumor suppressing role in melanoma. However, there was a difference in the miR-18b expression level among the different melanoma cell lines.
The cell line C8161.9 shows the most miR-18b downregulation, followed by the other melanoma cell lines Lox, 1205 Lu, WM3211 and DO4.

To analyze the 18b-expression in tissue samples, quantitative RT-PCR (miR qRT-PCR) analysis on an independent cohort of nevus and melanoma tissues was performed. miR qRT-PCR of nevus (n=30) and metastatic (n=35) samples indicate

that miR-18b expression is significantly downregulated in metastatic samples when compared with nevi. (figure 27).

Comparing fig. 27 with fig.28 shows that the relative expression level for the nevus samples is in a range between the value 2.3 and 96.6. In comparison, the miR-18b expression in melanoma samples is only varying between a value of 0.3 and 12.9. This result demonstrates that miR-18b act as a tumor progression marker in melanoma.

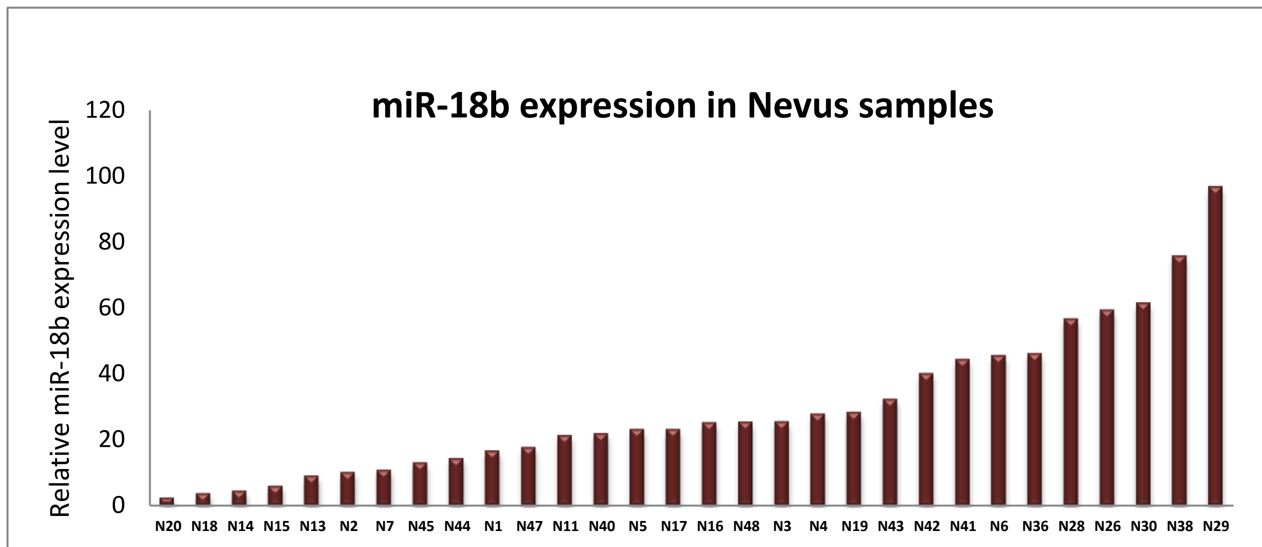


Figure 27: qRT-PCR analysis for miR-18b expression in 30 nevus samples. miR-18b expression in nevus samples is high.

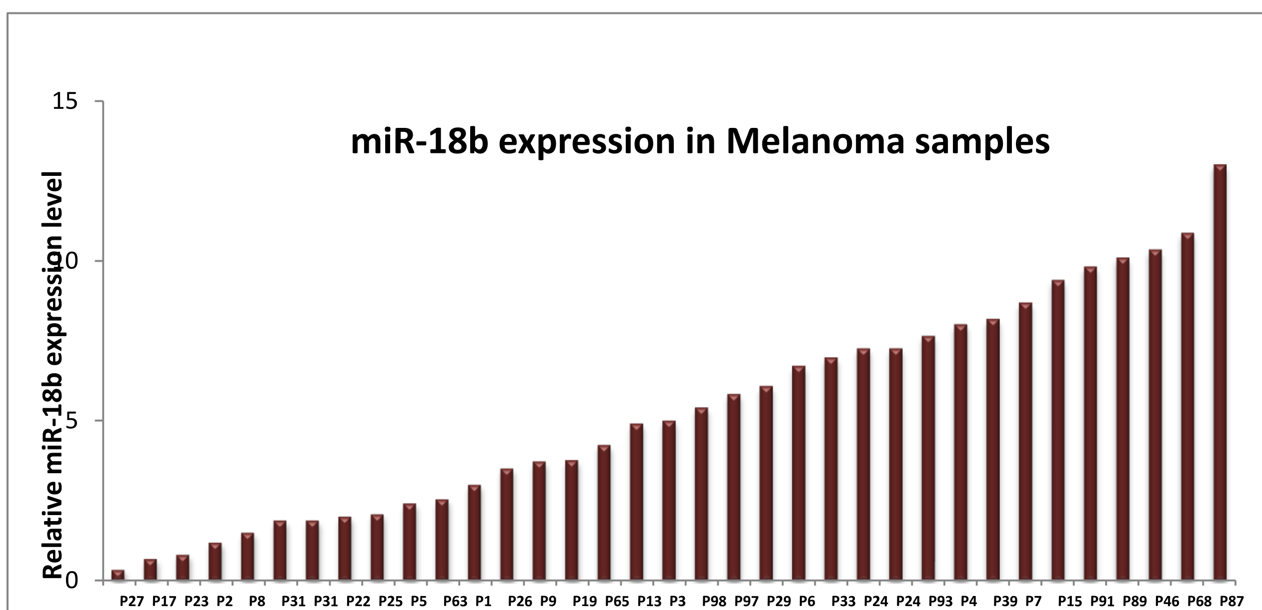


Figure 28: qRT-PCR analysis for miR-18b expression in 35 melanoma samples. miR-18b expression in maelanoma samples is low.

In order to demonstrate whether the overexpression of miR-18b in melanoma cell lines can regulate protein levels and alter downstream signaling events, 1205-Lu cells were transfected with miR-18b. This results in miR-18b over expression as determined by miR qRT-PCR analysis. Transient transfection was carried out by Lipfectamine.

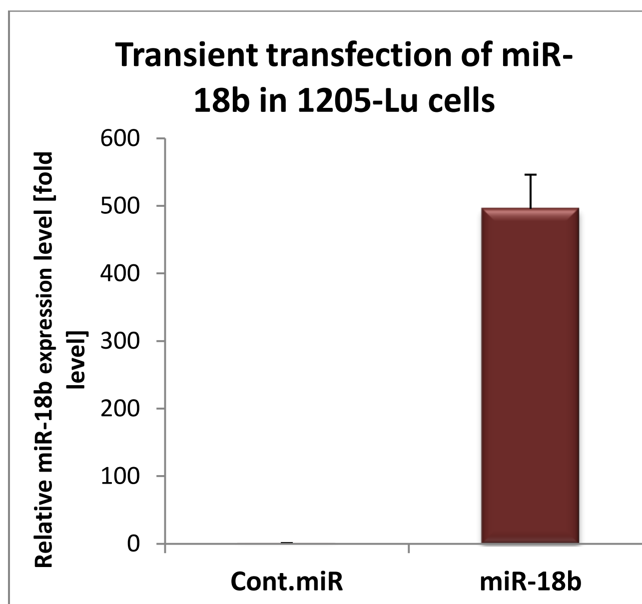


Figure 29: Relative miR-205 expression level in 1205-Lu cells after transiently transfection with miR-18b as determined by miR qRT-PCR; Neg control pre-miR is taken as a control.

Figure 29 shows the relative expression level of miR-18b in 1205 Lu cells after transient transfection. As a control and for comparison, cells expressing negative control pre-miR are taken.

1205-Lu Cells Transiently Transfected With miR-18b Inhibits Melanoma Cell Proliferation, Colony Formation and Induce Apoptosis

To determine if miR-18b overexpression affects cell proliferation, melanoma cells were transfected transiently with miR-18b. Therefore a cell survival assay of 1205-Lu cells was performed, plating cells in a 96-well plate at a density of 3 x 10^3 cells per well. The cell viability assay was performed at 24, 48 and 72 hours after transfection using the cell counting kit 8. A significant decrease in cell proliferation was observed

over time in 1205-Lu cells expressing miR-18b compared with cells expressing negative control pre-miR. (Fig. 30).

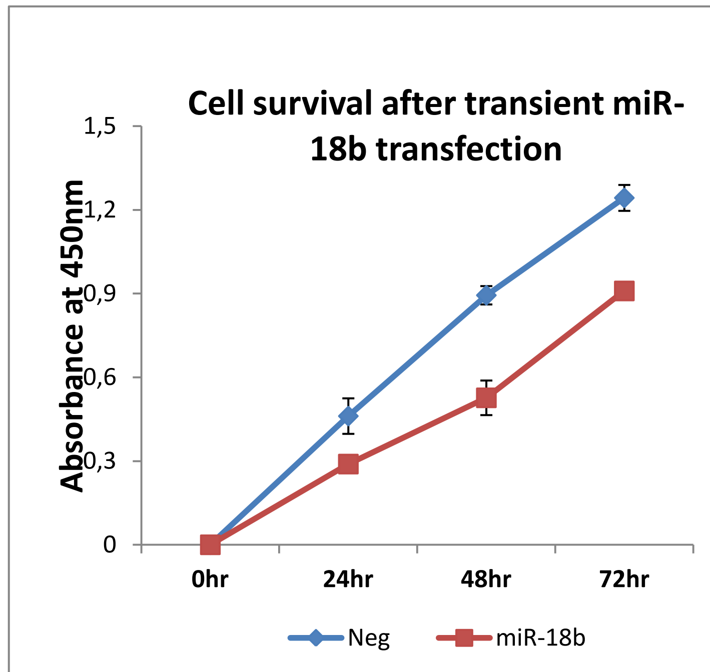
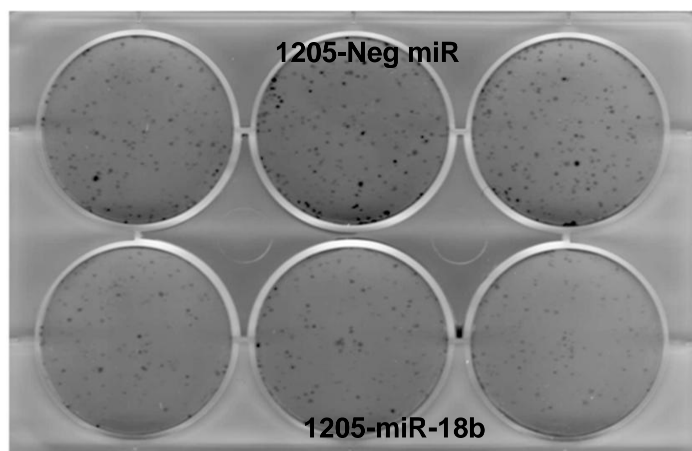


Figure 230: Outcome of a cell survival assay; the proliferation ability of 1205-Lu cells after miR-18b transfection is reduced significantly when compared with negative control pre-miR (Neg)



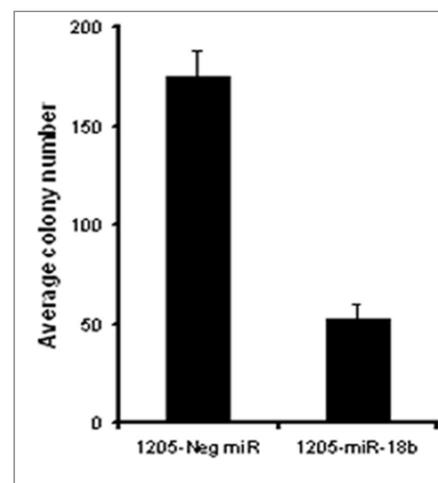


Figure 31: Transient overexpression of miR-18b suppresses colony formation, inducing apoptosis.
Cell survival analysis of 1205-Lu cells overexpressing miR-18b as compared with control miR expressing 1205 Lu cell.

We further examined the effects of miR-18b on melanoma cell viability using a colony formation assay. Figure 31 is showing a 6-well plate, where 500 cells/well were plated and were allowed to grow until visible colonies appeared. Colonies were stained with Giemsa and counted. The first row contains cells expressing negative control pre-miR acting as the control cell line. The second row shows 1205-Lu cells, transfected with miR-18b.

The miR-18b transfected 1205-Lu cells showed low colony formation ability, as both the size and number of foci in miR-18b expressing cells were suppressed when compared with negative control pre-miR expressing cells.

The diagram (figure 31) shows the comparison of the average colony number within the two cell lines, 1205-Neg miR and 1205-Lu miR-18b. The control cell line shows an average colony number of 170. In contrast the 1205-miR-18b cells have poor colony formation ability with an average number of 53. This indicates that the microRNA 18b is blocking colony formation and thus cell survival.

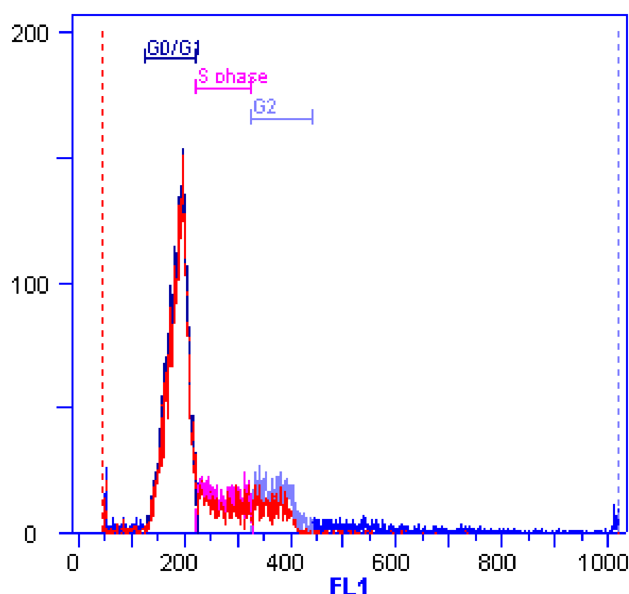


Figure 32: cell cycle assay (fluorescence-activated cell sorter) of 1205-Lu Neg-miR

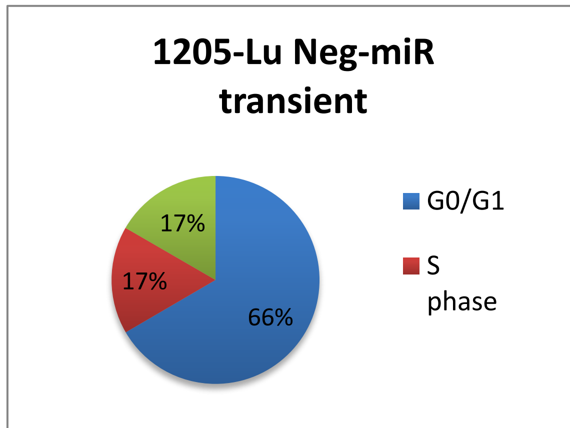


Figure 33: percentage of cells being in different phases of the cell cycle

Table 3: Counted cells and total percentage of 1205-Lu Neg-miR cells being in different phases of the cell cycle

Region	Count	Pct % Total
G0/G1	6.025	60,25%
S phase	**1.516**	**15,16%**
G2	1.507	15,07%

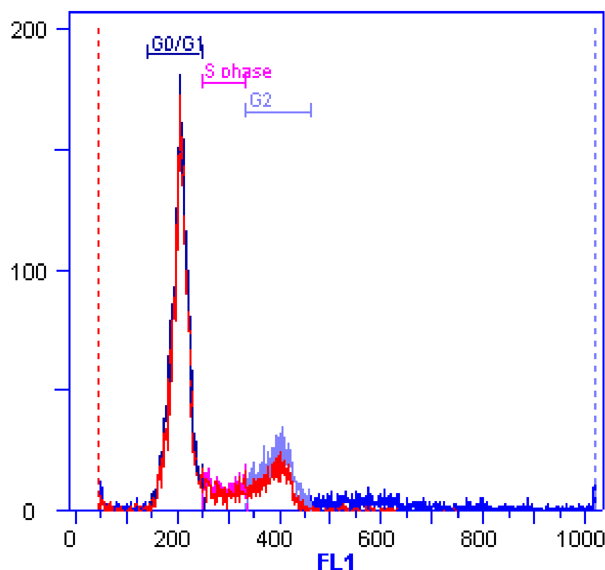


Figure 34: cell cycle assay (fluorescence-activated cell sorter) of 1205-Lu transient transfected with miR-18b

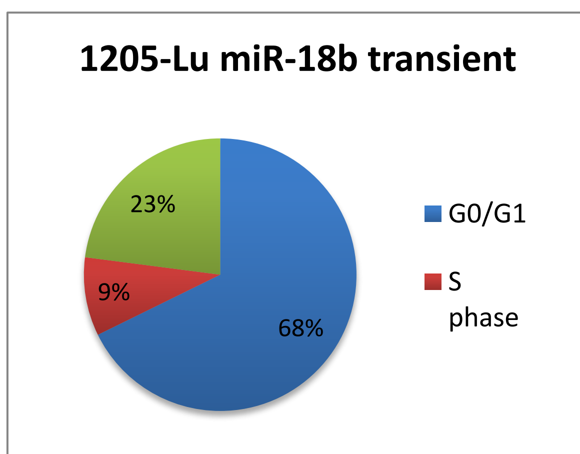


Table 4: Counted cells and total percentage of 1205-Lu Neg-miR cells being in different phases of the cell cycle

Region	Count	Pct % Total
G0/G1	5.872	58,72%
Sphase	**808**	**8,08%**
G2	1.990	19,90%

Figure 35: percentage of cells being in different phases of the cell cycle

Figure 32 and 34 show the outcome of the cell cycle assay. The different stages of the eukaryotic cell cycle are indicated. For this experiment, the S phase is the focus because this specific phase is where DNA replication and chromosome duplication occurs.

Figure 32 displays each stage of the cell cycle of 1205-Lu Neg-miR transiently transfected cells acting as a control. The cells shows a G0/G1 phase fraction of 60.3%, which is in between a normal range. Cells in the G2 phase of the cell cycle consist of 15.1% of the total cells. The percentage of 1205-Lu Neg-miR transient transfected cells in the S phase is 15.16% (figure 33 and table 3). If this value is compared to the DNA synthesis (S phase) fraction of 1205-Lu miR-18b transient cells, the value is significantly high. (Figure 34)

Only 8.08% of the miR-18b expressing 1205-Lu cells are in the DNA replication phase of the cell cycle. Comparing the cell cycle arrest at the G2-M phase in miR-18b transfected cells with the Neg miR transient cells, the fraction is higher in miR-18b. miR-18b has a fraction of 16.65% and in comparison only 11.33% of 1205-Lu Neg-miR cells are in the G2 phase of the cell cycle (table 4 and figure 35). This indicates that the time to undergo cytokinesis takes longer in 1205 miR-18b expressing cells. This experiment proves the role of miR-18 as a tumor suppressor because it blocks cell cycle.

Stably Expressed miR-18b Cells Block Cell Survival, Cell Proliferation, Colony Formation and Cell Cycle

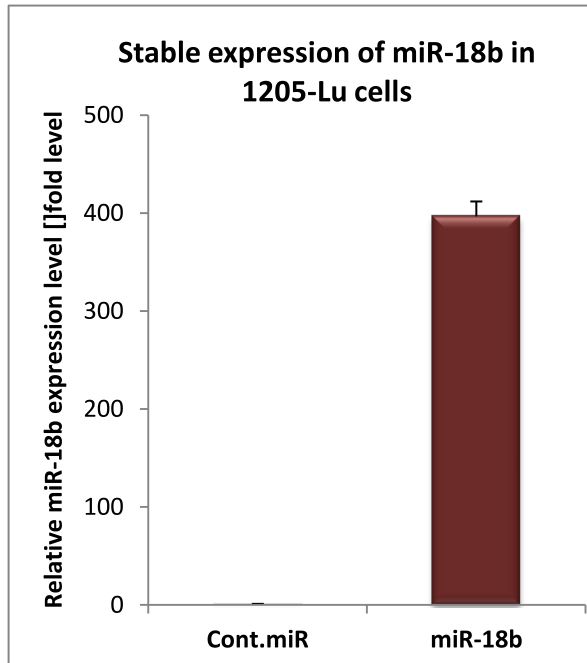


Figure 36: Relative miR-205 expression level in 1205-Lu cells after stably transfection with miR-18b as determined by miR qRT-PCR;
Neg control pre-miR is taken as a control

In order to confirm these findings, we generated stable expressing 1205-Lu miR-18b cells. These cells are generated by transfecting miR-18b expressing vector having puromycin resistance gene. After 48 hours of transfection, 1µl/ml of puromycine was added to cells till visible resistant colonies formed. These cells stably express 18b microRNA under puromycin selection. (Figure 36)

The overexpression of miR-18b was confirmed by miR qRT-PCR analysis.

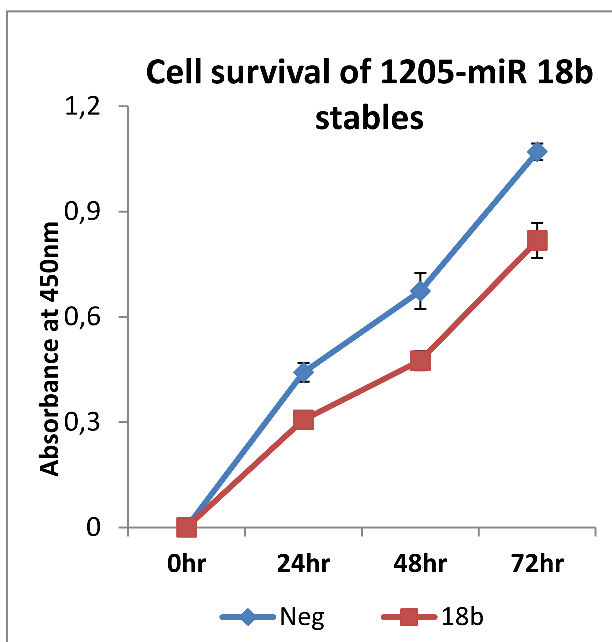


Figure 37 Stable over expression of miR-18b in melanoma cell lines significantly suppresses cell proliferation, which is reduced in miR-18b transfected cells compared with negative control pre-miR (Neg.).

1205-Lu cells expressing miR-18b exhibited a significant suppression in cell proliferation compared with the control vector-expressing cells. This means cell proliferation is significantly suppressed.

The cell viability assay was performed at 24, 48 and 72 hours. The diagram shows a significant decrease in cell proliferation over time in 1205-Lu cells expressing miR-18b compared with cells expressing negative control pre-miR. (Figure 37).

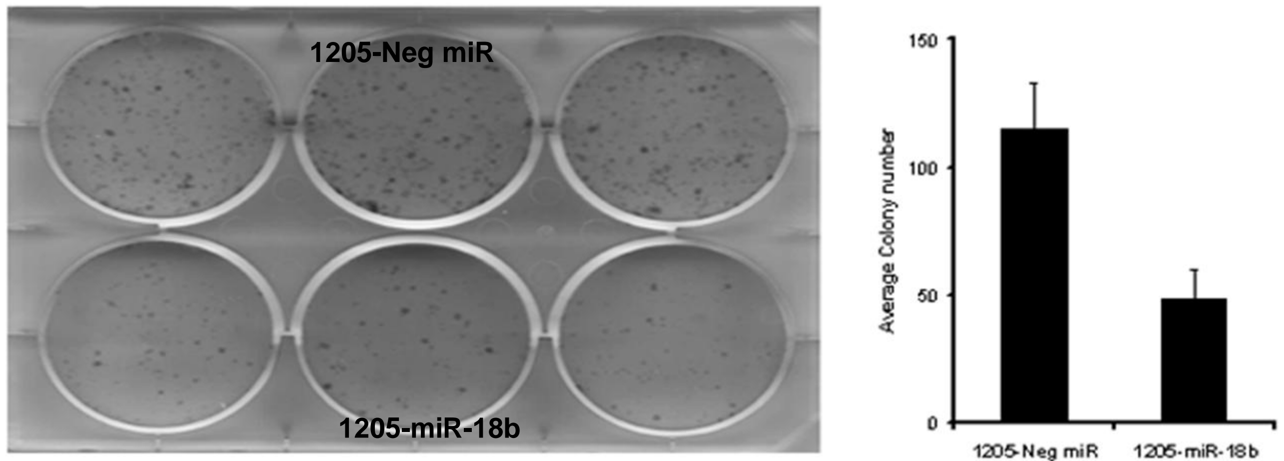


Figure 38: miR-18b overexpression significantly inhibits the colony formation of melanoma cells

Figure 38 shows the colony formation capability of miR-18b stably transfected cells. A highly significant decrease in the over expressing cells and the colony formation capability of miR-18b compared to the control cells is observed. The bar graph shows the mean number of colonies. The average colony number is 115 for the 1205 Neg-miR, and, in comparison, the average number of the formed colonies for 1205 miR-18b is 52, which is significantly lower. In conclusion, miR-18b suppresses colony formation and proliferation.

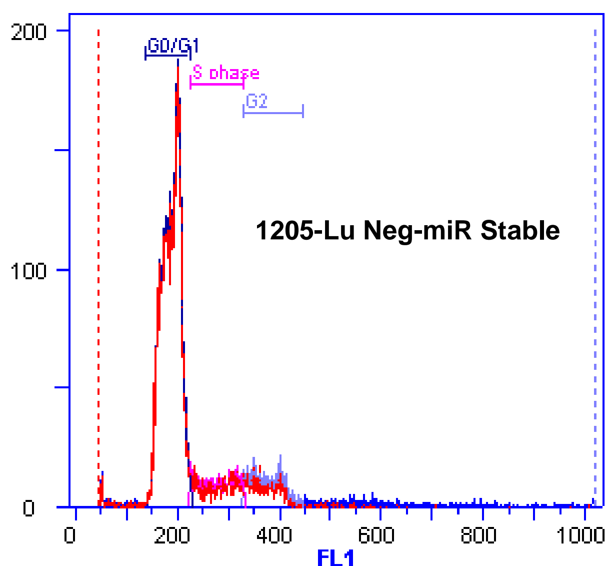


Figure 39: cell cycle assay (fluorescence-activated cell sorter) of 1205-Lu Neg-miR

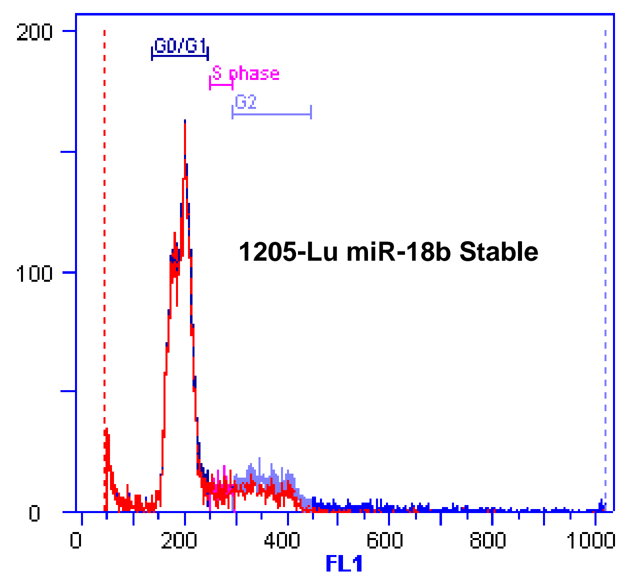


Figure 40: cell cycle assay (fluorescence-activated cell sorter) of 1205-Lu transient transfected with miR-18b

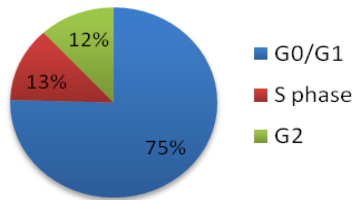


Figure 41: percentage of 1205-Lu Neg-miR cells being in different phases of the cell cycle

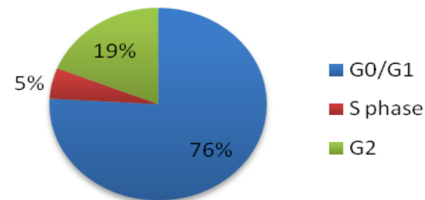


Figure 42: percentage of 1205-Lu miR-18b cells being in different phases of the cell cycle

Table 5: total percentage of 1205-Lu Neg-miR cells being in different phases of the cell cycle

Region	Pct % Total
G0/G1	71.89%
Sphase	12,08%
G2	11.33%

Table 6: total percentage of 1205-Lu-miR-18b cells being in different phases of the cell cycle

Region	Pct % Total
G0/G1	66.80%
Sphase	4.55%
G2	16.65%

To acquire these findings, a cell cycle analysis using a fluorescence-activated cell sorter is used to determine the percentage of cells in different phases of the cell cycle. (Figure 39 and 40). Fig. 39 displays the outcome of the cell cycle assay of 1205-Lu stably expressing Neg-miR cells, and Fig 40 gives the results of 1205-Lu miR-18b for the same experiment.

Table 5 shows that 71.89% of the 1205-Lu Neg-miR cells are in the G0/G1 stage with 11.33% of cells in the G2 phase and 12.08% of cells in the DNA synthesize phase. In comparison, the result of the cell cycle assay performed on 1205-Lu stably expressing miR-18b show the similar results as in transient transfected mir-18b cells. A fraction of 66.80% of cells are in G0/G1 phase with 16.65% of cells undergoing cytokinesis and only 4.55% of the cells in the S-phase (Table 6). Subsequently, stably expressed mir-18b cells block cell survival, cell proliferation, colony formation and cell cycle.

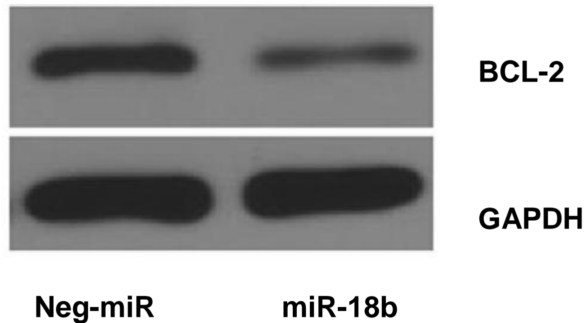


Figure 43: *Stable overexpression of miR-18b inhibits cell proliferation. Western blot analysis showing suppression in the pro-proliferation protein BCL-2*

Also, a significant suppression of BCl-2 was observed when performed in western blot analysis. When comparing the 1205-Lu transfected miR-18b cells with the control (Neg-miR), this shows that the microRNA 18b acts as a tumor suppressor in melanoma by blocking cell cycle, cell survival, and proliferation (Figure 43).

7. Discussion

As miRNAs modulate gene expression, it is not surprising that they have been implicated in regulating a wide variety of biological processes. Various studies have characterized the modulation of miRNA expression in cancers and identified a number of miRNAs that are up- or downregulated in tumors (7-8). If miRNAs are significantly suppressed in melanoma and other types of cancer, they are acting as tumor suppressors. miRNAs are called oncogenes if they are overexpressed (5).

In order to find out the role of microRNA-18b in melanoma, the melanoma cell line 1205-Lu was transfected with miR-18b to investigate its role as a tumor suppressor or oncogene.

In the present study, we examined the expression pattern and functional significance of miR-18b in melanoma progression. The expression of miR-18b in cancer is controversial because it has been found to be either upregulated (9) or downregulated for example in brain tumor.

miR-18b is downregulated in melanoma (tissue and cell lines)

It was found that *miR-18b is significantly downregulated in metastatic melanoma* when compared with nevi. The reduced levels of miR-18b in metastatic samples in an independent tissue set confirmed its role as a tumor progression marker in melanoma. The downregulation of miR-18b expression was also shown in melanoma cell lines when compared with normal melanocytes. We analyzed the cell lines C8161.9, Lox, D04, 1205-Lu, and WM3211. All of these melanoma cell lines showed a low miR-18b expression level compared to the melanocyte cell line (HEM). With 1205-Lu, we achieved the best outcomes. Therefore, more experiments were done working with this 1205-Lu melanoma cell line. The significant suppression of miR-18b in melanoma tumors and melanoma cell lines suggests a tumor suppressor role in melanoma.

miR-18b Inhibits Melanoma Cell Proliferation and Colony Formation

To determine if miR-18b overexpression affects cell proliferation, melanoma cells were transfected transiently with miR-18b. Transient transfection was carried out by Lipofectamine® 2000" (Invitrogen) (see "transfection – methods"). As seen in the results (Fig. 30 – 35), the various experiments determined that miR-18b blocks cell survival, colony formation, and reduces tumor cell growth.
The cell survival assay, which was performed according the Luciferase assay from Promega, shows that miR-18b transfected cells induces apoptosis earlier than the control Neg miR (Fig. 30). In conclusion, the miR-18b blocks cell survival.

Also, the proliferation assay (Fig. 31) shows that the reduced ability to proliferate for cells in transient transfected with miR-18b. In a 6-well plate, 500 cells/well were plated and allowed to grow until visible colonies appeared. Colonies were stained with Giemsa and counted. One can clearly observe that the number of formed colonies is significantly more for the control Neg-miR than for miR-18b transfected cells. This is another indication that microRNA-18b is blocking suppressing colony formation.

With a fluorescence-activated cell sorter, a cell cycle analysis were performed with 1205-Lu cells transfected with miR18-b and with Neg-miR 1205-Lu cells. The central events of cell reproduction are chromosome duplication, which takes place in the S phase, followed by chromosome segregation, nuclear division (mitosis), and cell division (cytokinesis) which are collectively called M phase. G1 is the gap phase between M and S phases; G2 is the gap phase between S and M phases
This means that we are especially interested in the S-phase where the DNA synthesis takes place.

The percentage of overexpressing 1205-Lu Neg-miR transient transfected cells being in the S phase is significantly more if this value is compared to the DNA synthesis (S phase) fraction of 1205-Lu miR-18b transient cells (Figure 32-35).
In addition, we observed that cell cycle arrest at the G2-M phase in miR-18b transfected cells is higher than in the control cells. This means that cells in this phase need more time to undergo cytokinesis.
Hence, miR-18b expression suppress the cell cycle of 1205-Lu cells.

Stable overexpression of miR-18b suppresses colony formation, tumor growth and induces apoptosis

To further examine the effects of miR-18b overexpression, stably expressing miR-18b 1205-Lu cells were generated. These cells are transfected by miR-18b expressing vector having puromycin resistance gene. After 48 hours of transfection, 1µl/ml of puromycin was added to cells till visible resistant colonies are formed. These cells are stably expressing 18b microRNA under puromycin selection. (Figure 34)

Transient cells are mostly for rapid analysis because foreign DNA is only delivered into the nucleus but is not integrated into the genome. Gene expression is studied shortly after the transfection procedure usually within the 24 to 72 hours
To get convincing results, all these previous experiments are done with stably generated cells too.
Stably transfection is used for stable induction of expression. The gene not only introduced into the cell but also integrated into the host DNA and carried along with it during cycles of cell division.

As expected, the results for the 1205-Lu cells stably tranfected miR-18b are similar to the past experiments done with miR-18b transient transfected cells. Thus, miR-18b stably transfected cells suppress colony formation, tumor growth, and induce apoptosis. (Figure 36– 42)

Western blot confirms our theory that miR-18b is a tumor suppressor marker (Figure 43). BCL-2 is a human proto-oncogene located on chromosome 18. High level of the Bcl-2 protein protects the cells from early death by apoptosis. BCL-2 helps in proliferation but is downregulated in this experiment with miR-18b. This means that microRNA-18b blocks the ability of cell proliferation.

In order to make improvements to this study, in vivo tumor growth of subcutaneous injection into nude mice could be performed. As well as tumor cell invasion into matrigel of 1205-Lu cells overexpressing miR-18b versus expressing Neg-miR (control) could be done.

As melanoma increases faster than other cancers, it is vitally important to uncover and use genetic and epigenetic regulatory mechanisms during the development and progression of melanoma for a better prevention and diagnosis (4). Therefore further experiments with regard to the epigenetic regulator mechanisms, such as DNA methylation could be done.

8. CONCLUSION

This study demonstrates that microRNA 18b (miR-18b) has the role of a tumor suppressor in melanoma, which means it is downregulated in this type of cancer. The expression of miR-18b is significantly suppressed in melanoma specimens when compared with nevi and is correlated inversely with melanoma progression. miR-18b in 1205 Lu cells after transient transfection inhibit cell proliferation, colony formation and induces apoptosis. Also stable overexpression of miR-18b inhibits cell survival, cell cycle, cell invasion and reduces tumor cell growth.
These findings confirmed the role of miR-18b as a tumor progression marker in melanoma.

9. ACKNOWLEDGEMENTS

I want to thank my supervisor Altaf A. Dar for supporting me in my work and for his feedback and inputs for this thesis.

10. REFERENCES

1. Dar AA, Majid S, de Semir D, Nosrati M, Bezrookove V, Kashani-Sabet M. miRNA-205 suppresses melanoma cell proliferation and induces senescence via regulation of E2F1 protein. J Biol Chem 2011; 286: 16606-14.
2. Hacinliyan A, Skarlatos Y, Sahin G, Atak K, Aybar OO. Possible stretched exponential parametrization for humidity absorption in polymers. Eur Phys J E Soft Matter 2009; 28: 369-76.
3. Mueller DW, Bosserhoff AK. Role of miRNAs in the progression of malignant melanoma. Br J Cancer 2009; 101: 551-6.
4. Howell PM, Jr., Li X, Riker AI, Xi Y. MicroRNA in Melanoma. Ochsner J 2010; 10: 83-92.
5. Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev 2009; 28: 369-78.
6. Dar AA, Majid S, Nosrati M, de Semir D, Federman S, Kashani-Sabet M. Functional modulation of IGF-binding protein-3 expression in melanoma. J Invest Dermatol 2010; 130: 2071-9.
7. Jiang J, Lee EJ, Gusev Y, Schmittgen TD. Real-time expression profiling of microRNA precursors in human cancer cell lines. Nucleic Acids Res 2005; 33: 5394-403.
8. Gaur A, Jewell DA, Liang Y, et al. Characterization of microRNA expression levels and their biological correlates in human cancer cell lines. Cancer Res 2007; 67: 2456-68.
9. Gottardo F, Liu CG, Ferracin M, et al. Micro-RNA profiling in kidney and bladder cancers. Urol Oncol 2007; 25: 387-92.

- Asai, D. D. (1993). Methods in Cell Biology - Antibodies in Cell Biology. Academic Press Inc.
- Berg JM, T. J. (2002). Biochemistry. 5th edition. New York: W H Freeman.
- Biochemica. (2004). Gene Knockdown.
- Biologics, Inc.. Manassas, Virginia, USA.
- BIO-RAD, Hercules, CA, USA.
- Bio-Rad Laboratories, Inc. Precision Plus Precision Plus Instruction Manual. Hercules, CA, USA.
- BIO-RAD Life Science Research.Bio-Rad Protein Assay. Hercules, CA.
- Bruce Alberts, A. J. (2002). Molecular Biology of the Cell, 4th edition. New York: Garland Science.
- Chesnoy, S. a. (2000). Annual Review of Biophysical Biomolecular Structure .

- Christer Ericsson, M. N. Protein Extraction From Frozen Brain Samples. Cancer Center Karolinska, Stockholm, Schweden.
- Freshney, I. Culture of animal cell, 6th edition. 2007 . Wiley-Blackwell.
- Griffiths, A. J. (1996). An. New York: W.H. Freeman and Co.
- Herlyn Meenhard. Philadelphie, PH. (2008)
- Invitro, L. T. Carlsbad, CA.
- Invitro, Life Technologies. Carlsbad, CA.
- Keith Wilson, J. W. (2000). Principles and Techniques of Practical Biochemistry. Fifth Edition . Cambridge University Press.
- Kim Tae Kyung, J. E. (2010). Mamalian cell transfection. Springer.
- Life Technologies Corporation. (2011).
- Lodish H, B. A. (2000). Molecular Cell Biology. 4th edition. New York: W.H. Freeman.
- Markus R. Wenk, A. Z. (2007). A manual for biochemistry protocols. Jahn-Thorsten-Schantz.
- Medrano, M. L. (2005). Real-time PCR for mRNA quantitation.
- Morgan, D. O. (2007). The cell cycle principles of control.
- OpenWetWare contributors. (2008). Total Protein Isolation Using RIPA Lysis Buffer.
- Pierce. Rockford, Illinois, IL.
- Polvino, S. (1983). BioRad Trans-Blot SD Semi-Dry Electrophorectic Cell Instruction Manual. Electrophoresis .
- QIAGEN, RNeasy® Mini Handbook. Düsseldorf.
- Ryan, J. A. (2000). Introduction into animal cell culture.
- Thermo Scientific Western Blotting. Rockford, IL, USA.
- Thermo Scientific. SuperSignal West Pico Chemiluminescent Substrate Kit. Rockford, Illinois.
- CL - University College London. (2009) Cell Cycle Basics.
- Westermeier, R. (2005). Electrophoresis in practice: a guide to methods and applications of DNA and protein separation. WILEY-VCH.
- Wiesner, C. P. (2010). Cell Culture Techniques_University of Applied Sciences IMC Krems.
- Winkler, A. (2008). Bachelor Thesis I.
- www.dartmouth.edu. (2011).