The effects of siRNA-mediated knockdown of MITF and AP-2a in melanoma

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Abstract

Melanoma is one of the most aggressive forms of skin cancer, and is very difficult to treat as resistance to existing treatments almost invariably develops. The disease is genetically heterogenous which further increases the challenges of developing new treatments against it. Exploring how differing levels of different proteins affect the tumor cells is the key in identifying new targets for future treatments.

MITF is the master transcription factor of melanocytes, and is shown to be able to control a large portion of the melanocyte genome. While it is only directly mutated in about 20% of melanomas, it is directly or indirectly deregulated in most patients. This, combined with the MITF-m-isoform of the protein having melanocyte-specific expression, makes the gene interesting as potential future drug target against melanoma. Increased knowledge about its targets and its effect on gene expression would provide valuable insights. Here, we demonstrate the effects MITF downregulation have on the tumor suppressor genes AP-2a and AP-2c, the membrane bound growth factor receptors ERBB2 and ERBB3, and the intracellular signaling proteins AKT and ERK. To compare samples, siRNA-mediated gene knockdown in SK-Mel28 melanoma cells was performed by using the transfection methods Photochemical Internalization and Lipofectamine 2000. Photochemical Internalization was shown to significantly affect MITF levels and was abandoned in favor of Lipofectamine 2000 transfection. Knockdown of MITF was shown to reduce levels of AP-2a significantly, while increasing levels of ERBB3. These effects were consistent at both a protein level and RNA level. Furthermore, reduced levels of MITF resulted in increased signaling in the PI3K/AKT-pathway, a pathway that also has been previously shown to be regulated by ERBB3. Lowered levels of AP-2a was found to reduce AKT-signaling. The MAPK/ERK-pathway was also examined, but no change was observed after either MITF or AP-2a siRNA knockdown. MTS assay experiments showed an increase in metabolic activity in cells transfected with MITF siRNA. These results indicate that MITF regulate genes capable of both assisting and preventing melanoma progression.

Abbreviations

Abbreviation	
PCI	Photochemical internalization
LF2000	Lipofectamine 2000
qPCR	Quantitative PCR (Real Time RT-PCR)
RT-PCR	Reverse transcriptase PCR
AP-2a	Activator protein 2 alpha
MITF	Microphthalamia-associated transcription factor
ERK	Extracellular signal-regulated kinase
PBS	Phosphate buffered saline
TBS-T	Tris-buffered saline w/ tween
ddH_2O	Double distilled water
BRAF	Mitogen activated protein kinase kinase kinase (MAPKKK)
SK-Mel28	Sloan-Kettering Institute Melanoma cell line 28
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
AKT	Protein Kinase B (PKB)
ERBB3	Receptor Tyrosine kinase erbB3
MAPK	Mitogen activated protein kinase (ERK)
RGP	Radial growth phase
VGP	Vertical growth phase
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
a-MSH	Alpha melanocyte-stimulating hormone
kDa	Kilodaltons
TFEB	Transcription factor E-box protein
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
b-HLH	Basic helix-loop-helix motif

c-Kit	Stem cell growth factor receptor (SCGF) / tyrosine-protein kinase kit
SOX10	Sry-related HMG-box 10
CRD-BP	Coding region determinant-binding protein
c-Myc	v-myc avian myelocytomatosis viral oncogene homolog
PP-2A	Protein phosphatase-2A
p53	Tumor protein 53
p21	Cyclin-dependent kinase inhibitor 1A (P21, WAF1, CIP1, CDKNI1A)
HER	Human epidermal growth factor receptor (ERBB, EGFR)
FBS	Fetal bovine serum
DMSO	Dimethyl sulphoxide
RPMI	Roswell Park Memorial Institute
mRNA	Messenger RNA
dsRNA	Double-stranded RNA
RISC	RNA-induced Silencing Complex
siRNA	Small Interfering RNA
PS	Photosensitizer
$TPPS_{2a}$	Disulfonated*meso*-tetraphenylporphine
dNTP	Deoxynucleotide triphosphate
cDNA	Complementary DNA
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
MOPS	3-(N-morpholino) propane sulfonic acid
BSA	Bovine serum albumin
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
pAKT	Phosphorylated AKT

pERK	Phosphorylated ERK
H3	Histone 3
AP-2C	Activator protein 2 gamma
LDS	Lithium dodecyl sulfate
FDA	US Food and Drug Administration
MEK1	MAPK extracellular kinase 1
RTK	Receptor tyrosine kinase
Taq	*Thermos Aquaticus*
NaCl	Sodium chloride
Ab	Antibody
TBP	Tubulin binding protein
RPLPO	Large Ribosomal Protein
ROS	Reactive Oxygen Species
DTIC	Dacarbazine
PEI	Polyethylenimine
^{o}C	Degrees Celcius
mTOR	Mechanistic Target of Rapamycin (serine/threonine Kinase)
IL-2	Interleukin-2
PD-1	Programmed Cell Death Protein 1
CDK	Cyclin-dependent kinase

1 - Introduction

1.1 Cancer

Cancer is a group of diseases caused by abnormalities in the cells of the body, causing cell growth to be deregulated, and later grants the cells the ability to spread to different parts of the body. Most types of cancers initially form a concentrated mass of cells, known as a tumor. The cells continue to grow uncontrollably and attain the ability to spread to other locations of the body, forming new tumors. If left untreated, the uncontrolled growth of cancer cells will cause displacement and damage of surrounding tissue and organs, eventually leading to the death of the diseased individual.

Carcinogenesis, the process in which a normal cell transforms into a cancer cell, is when a cell undergoes multiple genetic and/or epigenetic changes. These changes generally offer proliferative advantages to the cell. In 2000, Hanahan and Weinberg proposed six hallmarks that would define a cancer cell [1]: autonomy from growth signals, evasion of growth inhibitory signals, resistance to death by apoptosis, unlimited replicative potential, the ability to cause angiogenesis, and the ability to invade other tissues and metastasize. In 2011, they updated this list with four more hallmarks [2]: genome instability, tumor-promoting inflammation, the ability to reprogram metabolism, and evasion of destruction by the immune system. *(figure 1)*

Figure 1: The hallmarks of cancer, as presented by Hanahan and Weinberg in 2011. Mutations contributing to one of these attributes are essential for carcinogenesis [2].

There are two broad types of genes that are mutated in mutagenesis: *proto-oncogenes* and *tumor suppressor genes*. Proto-oncogenes are generally genes that drive cell proliferation, growth and division, and acquire mutations that *increase* their activity by either increasing the quantity of said gene, or through a functional change that makes the gene more active. Tumor suppressor genes, on the other hand, prevent cell proliferation, and acquire mutations that *decrease* their activity either partially or completely.

There are many physical and chemical agents that can potentially initiate the development of cancer. Most of these agents, commonly known as carcinogens, work by inducing DNA damage in cells. Examples of this include chemical compounds that bind to or react with DNA, and high-frequency electromagnetic radiation. Common for these carcinogens is that they induce breaks in the DNA strands, and damages the bases that make up the DNA code [3]. Ultraviolet radiation can cause damages by creating pyrimidine dimers, which causes the DNA to be unreadable by the proteins that attempt to access the genetic code [4, 5]. Chemicals that bind to DNA can make the process of replication more prone to errors [6, 7]. Certain viruses can also function as carcinogens,

by shutting down genes important for regulating cell division [8].

The cells in the human body do have mechanisms to repair the vast majority of the damages induced by carcinogens. However, over time DNA damages will still have a chance to accumulate and will generally pose an increased risk for cancer development later in life.

As cancer is a disease derived from the cells in the body rather than an external infectious agent, treatment of cancer can be challenging, and often induce severe side-effects. Common ways to treat diagnosed cancers involve surgery to remove tumors, use of chemical compounds that kill fast-growing cells (chemotherapy) and radiation therapy where tumors are irradiated by gamma radiation. Another strategy that is becoming more common is to use medication that primarily affect cells with specific mutations, known as personalized cancer therapy [9-12]. Much of the current research on cancer aimed at finding potential targets for such therapy, as well as developing medication against specific targets [8].

Cancer is one of the most common causes of death in the developed world. In Norway there were 29 907 new cases of cancer registered in 2011, as well as 10 970 deaths caused by cancer in the same year [13]. On a worldwide basis, it accounts for roughly one in eight of all deaths. As such, cancer is very much a relevant research topic; increased understanding of the molecular processes that underlies cancer has the potential to save many lives, as well as increasing our overall understanding of how living cells function.

1.2 - Melanoma

Melanoma is a form of cancer derived from the pigment-producing cells known as melanocytes. Melanocytes are found in the skin, but also in the eye and the colon. Melanoma can occur in any region where melanocytes are present, but the disease is primarily known as a skin cancer. The neoplasm usually takes the form of an irregular, growing mole on the skin. The neoplasm has three sequential growth phases: radial growth phase(RGP), vertical growth phase(VGP) and the metastatic phase. During the radial growth phase, the tumor expands without penetrating deeper into the skin layer; the vertical growth phase involves growth deeper into the skin layers, and during the metastatic phase the cancer spreads to other sites in the body [14].

Melanoma is a very aggressive form of cancer, and is considered the most lethal type of skin cancer, accounting for less than 2 % of total skin cancer cases. While being responsible for approximately 75% of all skin cancer related deaths. One of the risk factors most commonly associated with melanoma development is exposure to ultraviolet (UV) radiation. UV radiation can induce DNA damage [4], and cells in the skin are commonly exposed to UV radiation from the sun. Melanocytes function as a defense mechanism against this: they produce the pigment known as melanin, which absorbs portions of the damaging radiation before it can cause harm in the body. The incidence of melanoma is thereforehigher in fair-skinned individuals than dark-skinned individuals, as their lower levels of melanine makes them more susceptible to UV-induced DNA damage [15]. Individuals that have been frequently sunburned in the past are also at a higher risk than the general population.. Melanoma has become more common in Norway today compared to the 1950s, an increase that has been linked to changes in UV exposure and the use of tanning beds. In 2012 a total of 1755 new cases of melanoma were registered in Norway**[**13].

If identified before metastasis surgical removal of the tumor is an effective treatment that reduces the possibility of recurrence in most patients. However, if the melanoma has spread, the survival rate of patients greatly decreases. Melanoma has a high level of heterogeneity, which makes it more complicated to develop single treatments that can cover for all melanoma subtypes. [20] Treatments against metastatic melanoma include; chemotherapy, radiation therapy, and immunotherapy (Table 1). Traditionally, the alkylating agent Dacarbazine (DTIC) has been used to treat advanced melanoma, but the drug has a low response rate (7-13%) [17-19] and has severe side-effects. In recent years, multiple targeted therapies have been developed and approved for melanoma treatment. [21] Of particular note here is Vemurafenib, a kinase inhibitor specific for BRAF with a V600E-mutation. The drug has been approved by the FDA in the United States, and shows good initial response rate against advanced melanoma with a BRAF(V600E)-mutation. Unfortunately, the cancer almost invariably develops resistance towards the drug when administered as the sole treatment [4-6], but Vemurafenib shows great promise when administered together with other drugs as a combination therapy. [21]

Immunotherapy, a modality designed to direct the immune system towards the cancer cells, is also showing great potential for treatment of melanoma, and the effects therapeutic antibodies on

advanced melanoma is a promising field of research. Of particular note are Ipilimumab and Nivolumab: the former is an antibody that blocks the CTLA-4-receptor on the surface on T-cells, which initiates an immune response against tumor cells. [22, 23] Nivolumab functions by binding to the PD-1 receptor of the melanoma cells, directing the immune system to those cells. Both sets of treatments have individually been shown to increase survival rates amongst patients. [17, 23] However, a combination of the two drugs has shown far greater response rates than each drug administered alone, and a Ipilimumab-Nivolumab-combination therapy has been suggested as a potential treatment for the future. [24, 25] Table 1 outlines several drugs used against melanoma, as well as their targets.

Treatment	**Target of inhibition**	**Reference**
Imatinib	c-KIT	[26]
Vemurafenib	BRAF(V600E)	[21, 22, 27]
Interleukin-2 (IL-2)	IL-2 receptor	[28]
CDK Inhibitors	Cyclin Dependent Kinases	[27, 29]
Sorafenib	BRAF	[21]
Rapamycin	mTOR	[30]
Nivolumab	PD-1	[17, 21, 24, 25]
Ipilimumab	CTLA-4	[21-25, 31]
Dabrafenib	BRAF	[20, 21]

Table 1: Melanoma treatments and their targets

1.3 - Personalized cancer therapy

In recent years, changes have happened in the approach to cancer research and treatment. It is difficult to predict patient prognosis based on the anatomical origin of the tumor. Also, while traditional treatments like chemotherapy and radiotherapy have proven effective, they also come with serious side effects. The doctrine of "personalized cancer therapy" has therefore gained significant popularity. The idea that identifying the phenotypes of individual patients, and picking a treatment specifically for the mutations the tumors in the patient has acquired, is potentially more effective method than attempting to use a catch-all treatment. As genetic sequencing is becoming

cheaper and faster, and more drugs targeting specific proteins are being developed, this method is becoming more and more feasible, as mutations can be identified and treatments can be tailored specifically for the patient. The advances in sequencing and expression assay technology allow individual tumors to be analyzed for specific mutations, providing valuable information that can help inform on the effectiveness of different treatments. This ability to identify mutations greatly increases the value of treatments targeting genes that may not always be mutated.

One of the most common examples of a success story in personalized cancer therapy is Vemurafenib. Developed by Plexxicon and approved by the FDA in 2011, it is a kinase inhibitor specific for BRAF with a valine-glutamine substitution at the amino acid side chain valine-600 [21, 22, 27]. This specific mutation is common in melanoma and results in constant proliferative signaling, and for that reason vemurafenib is commonly used to treat melanoma patients with tumors positive for BRAF(V600E). Initial response rates are high, and as the inhibitor is specific for the mutated BRAF, the side-effects are mild in comparison to chemotherapeutic agents like DTIC [11]. However, resistance to the drug invariably develops after a few months, and disease relapse is very common [11, 21, 27]. It has been shown that combining Vemurafenib with another target-specific drug greatly reduces the risk of tumor cells developing drug resistance [27]. The goal is to discover new drug that targets will give more tools in the fight against cancer and be less straining on the patients compared to current therapies. For this reason, an important focus of cancer research is examining the potential of new genes as cancer drug targets.

An ideal drug target is a gene that is important for cancer development, can be easily targeted, is commonly found in the cells, and causes less severe side effects in the patient compared to other treatments. BRAF(V600E) is a prime example of a gene exhibiting all of these attributes [101]. Determining genes with similarly suitable properties could improve chances of finding future treatment methods.

1.4 - Signaling pathways in melanoma

Alteration of the signaling pathways in a cell is an essential part of carcinogenesis. For a cell to become cancerous, it is necessary to deregulate pathways that control cell growth, metabolism, apoptosis and gene repair, in ways that favor proliferation and cell division [32]. In melanoma, the pathways that are the most relevant for disease progression include the MAPK-pathway, the PI3K/AKT-pathway, the Wnt/B-catenin-pathway and A-MSH-pathway [33]. Table 2 outlines some of the most common genetic changes found in melanoma.

Mutation	Incidence	Reference
BRAF(V600E)	~50%	[34]
NRAS	15-25%	[35]
MITF amplification	21%	[36]
p53	13%	[33]
p21	30-40%	[33]

Table 2: Common mutations in melanoma

One of the greatest challenges with melanoma treatment is how resistance towards drugs often arise. Identifying mechanisms behind how resistance develops is therefore important in improving patient survivability. Increased signaling in the PI3K/Akt-pathway and the MAPK/ERK-pathway are often found in cells that have been treated with BRAF(V600E)-inhibitors and developed resistance to them [37-39]. This highlights the importance of these pathways for cancer cell survival. The microphthalamia-associated transcription factor (MITF) is one of the most important transcription factors in melanocytes [36, 40], and has shown to be linked to BRAF(V600E)-resistance [77], as well as being able to influence both the PI3K/AKT- and MAPK/ERK-pathways [62, 77, 78]. Increased insight in how MITF is linked to these pathways could provide valuable knowledge in how to combat melanoma.

1.5 - Microphthalmia-Associated Transcription Factor (MITF)

The Microphthalmia-Associated Transcription Factor is a 52kDa basic Helix-loop-helix transcription factor that acts as the master regulator of melanocyte development. [36, 40] It belongs to the MiT-family of transcription factor proteins, along with Transcription Factor E Box-proteins TFEB, TFEB3 and TFEC [16, 41]. These proteins are capable of forming homodimers and heterodimers involving other proteins from the MiT-family, but not other bHLH-proteins [42]. There are nine isoforms of the protein. The isoform MITF-M has melanocyte-specific expression *(figure 2)* [16, 36]. In a hetero- or homodimeric form, MITF is capable of binding to a 10 base-pair motif known as the M-box, with the sequence of 5'-GTC ATG TGC T-3', and the E-box, with the

consensus sequence of 5'-CANNTG-3' [79].

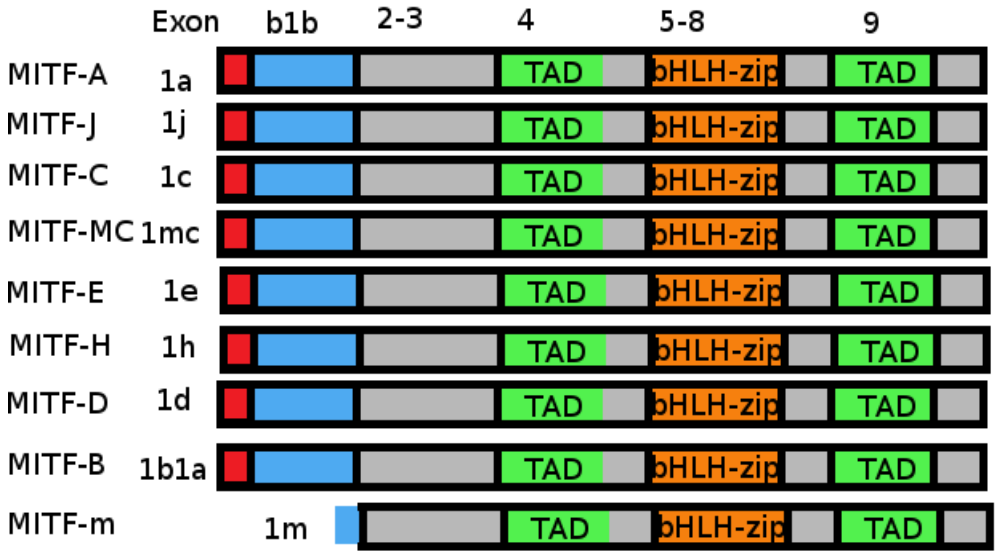


Figure 2: the nine different isoforms of MITF. The melanocyte-specific MITF-M is the only splice variant that lacks the b1b-part of exon 1, and instead has the 1m exon. All other isoforms have b1b plus an unique exon. Schematic diagram based on Levy et al [36].

MITF levels are commonly altered in melanoma cells compared to healthy melanocytes. However, in comparison to many other genes relevant to cancer development, the MITF gene itself is relatively rarely mutated [43]. Several studies have found amplification of the MITF gene in about 20% of melanoma tumors, as well as a relevant point mutation (E318K). Additionally, these studies have concluded that upstream regulators and the tumor microenvironment are more important factors in terms of regulating MITF levels than direct mutations are [43, 44]. Certain phenotypes of cancer cells are indicated to be correlated with different levels of MITF activity: low activity is associated with stemness; medium levels are associated with proliferation and invasion; high levels are associated with differentiation [45, 46].

MITF transcription is regulated by a multitude of pathways, some of which are shown in figure 3. Of particular note are the transcriptional promoting effects of Wnt, c-KIT, BRAF(V600E) and SOX10, all of which are commonly deregulated in melanoma [43, 47]. BRAF(V600E) is capable of influencing multiple pathways that modulate MITF transcription, both as activators and suppressors. BRAF(V600E)-positive melanoma cells are associated with low levels of MITF, however [48].

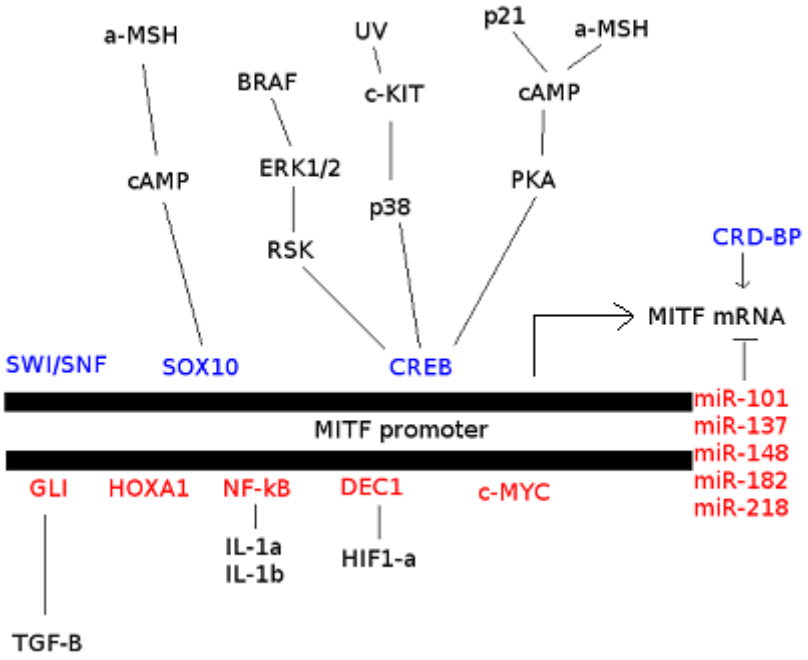


Figure 3: the effects of different pathways on MITF expression. Proteins shown in green function as enhancers of expression, while proteins shown in red function as repressors. Adapted from a figure from Hartman *et al [45].*

Post-transcriptional regulation of MITF activity is achieved through regulation of transcript stability. The protein complex coding region determinant-binding protein (CRD-BP) helps stabilizing the mRNA. Several micro-RNAs are capable of binding to MITF mRNA, which promotes enzymatic breakdown of the transcript. The protein can also be phosphorylated at Ser-73 and Ser-409, which both makes the protein transcriptionally active. Phospho-Ser-73 also flags MITF for degradation by the ubiquitin-proteasome pathway [43, 49]. Interestingly, c-KIT and BRAF(V600E) are indicated to take part in this phosphorylation, in addition to their transcription-promoting effects on MITF, though they exert their effects indirectly rather than actively phosphorylating MITF themselves [48, 49].

MITF-m is capable of affecting genes involved in a vast array of functions, including differentiation, proliferation, senesence and migration [45]. The gene is important in melanocyte development, and is commonly altered directly or indirectly in melanoma. For these reasons, efforts are being made to investigate the downstream targets of MITF, as well as its potential as a future therapeutic target. MITF-m having melanocyte-specific expression further increases the interest of

the gene, as it could make it possible to specifically target cells expressing this isoform. However, this is complicated by the complex expression model of MITF, as different melanoma cell phenotypes express different levels of MITF [45]. Thus, it is hard to predict the effects of targeting MITF, and certain papers have concluded that MITF itself may not be a druggable target, and suggests focusing on the targets of MITF, and its regulators [45, 102].

In-house data indicates that members the AP-2 family of transcription factors could possibly be affected by MITF levels, as well as members of the HER / ERBB-family. Members of the AP-2 family have been noted as tumor suppressors [51-53], while ERBB-proteins function as extracellular receptors that relay extracellular growth signals to the cells [64, 66]. To attain further knowledge of the effects of MITF in melanoma cells, this project will explore the effect MITF has on members of these families.

1.6 - Activator Protein 2 (AP-2)

The activator protein 2(AP2)-family of 52 kDa basic helix-loop-helix transcription factors. The proteins in this family contain a highly conserved b-HLH-motif that binds GC-rich areas in DNA (figure 4) [50], and are capable of forming both homo- and heterodimers. Humans have 5 genes in the AP-2-family: AP-2a, AP-2b, AP-2c, AP-2d and AP-2e.

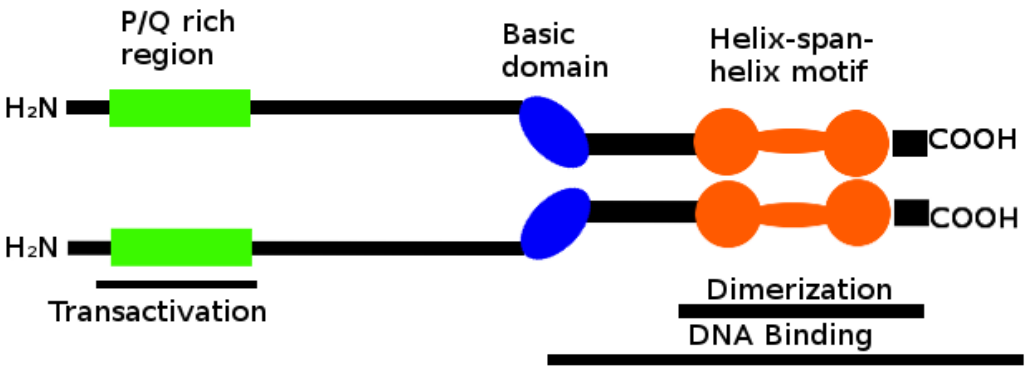


Figure 4: a representation of an AP-2a homodimer. The bHLH-domain is responsible for DNA binding and dimerization. Schematic diagram adapted from The AP-2 family of transcription factors (*Eckert et al 2005)* [50].

The structure of an AP-2 alpha monomer consists of the N-terminal transactivation domain [50] and the C-terminal DNA binding domain which is also responsible for dimerization. The homodimer binds to the consensus sequence of 5'-GCCNNNGGC-'3 [51]. AP-2a is reported to have four isoforms (1a, 1b, 1c and 1d) , with 1a being the most expressed isoform, though the 1b and 1c isoforms are commonly upregulated in breast cancer cells [85].

In recent years, the importance of AP-2 alpha as a tumor suppressor has been increasingly well-documented. The gene has been found to be widely downregulated and/or disrupted in all stages of melanoma, and a high ratio of cytoplasmic to nuclear localization of AP-2 alpha has been found to correlate with poor patient prognosis [52]. Additionally, AP-2 alpha knockdown has been demonstrated to reduce levels of other tumor suppressor genes [51, 53], and the up-regulation of genes associated with cell proliferation and disease progression [54, 55], The gene is also shown to cooperate with p53 in order to induce p21 expression [56, 92], an anti-proliferative kinase that regulates the activity of several key proteins in cell cycle progression [57]. Furthermore, AP-2a has been shown to be a positive regulator of the serine-threonine-phosphatase PP-2A A Alpha, a major serine-threonine phosphatase responsible for as much as 50% of the phosphatase activity in

eukaryotes [80]. Its importance in melanocytes is also highlighted by its reported co-localization with MITF at promoters for genes involved in pigment cell differentiation [83]. AP-2C is not as prominently reported on as a tumor suppressor gene in melanoma compared to AP-2a, but studies have shown that reduced levels of AP-2C contribute to melanoma tumor progression [81]. The gene is also a regulator of extracellular matrix 1 (ECM1) which is over-expressed in melanoma cells [82].

Increased knowledge of how AP-2 transcription factors affects melanoma cells could therefore provide key insights in how to combat melanoma.

1.7 - ERBB3

ERBB3 (HER3) is a member of the human epidermal receptor family, a family of receptor tyrosine kinases, which also encompasses HER2 / ERRB2, Epidermal Growth Factor Receptor (EGFR) and HER4 / ERBB4 [58]. These cell surface receptors relay growth factor signals to the cell by activating several important pathways for cell proliferation, including the PI3K / AKT-pathway and the MAPK / ERK-pathway [59, 60]. The ERBB-family proteins have a similar structure consisting of three main domains: an extracellular ligand binding domain, a single hydrophobic transmembrane region, and an intracellular region containing a conserved tyrosine kinase domain. The receptors are activated by assembling into homo- or heterodimers of other members of the family upon binding of extracellular ligands, with the notable exception of ERBB2, which is unable to bind any known ligand [61].

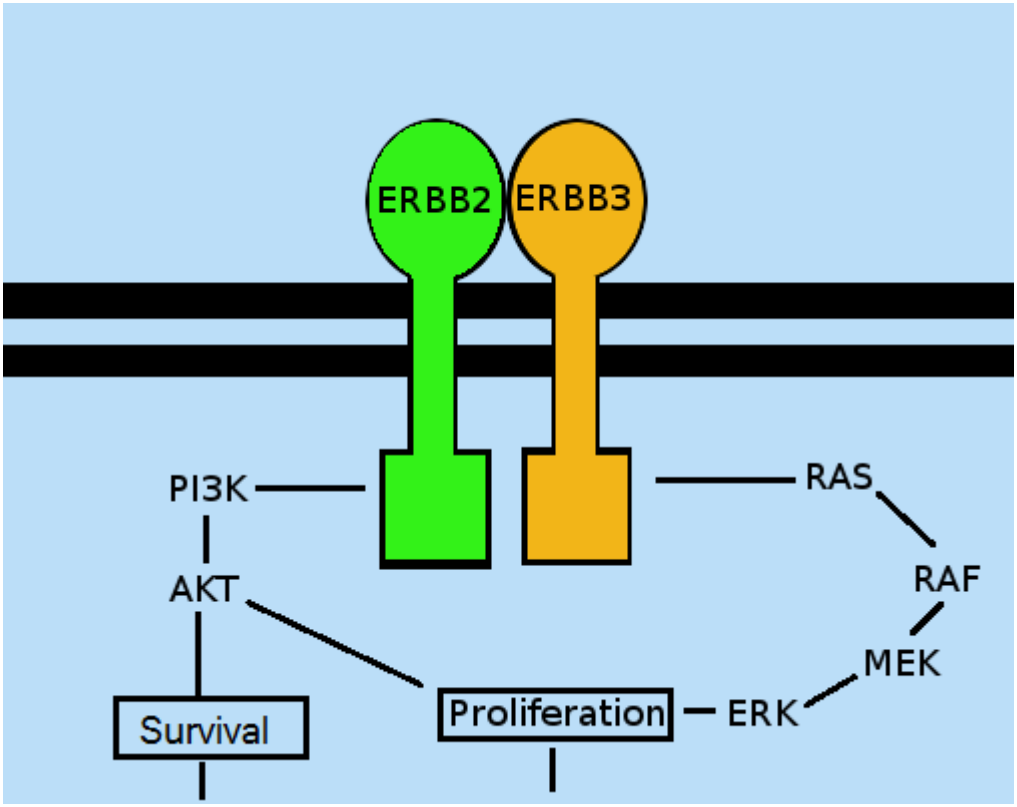


Figure 5: Representation of ERBB2/ERBB3 heterodimer signaling. Based on a figure by Gala et al [62].

ERBB3 is unique in the ERBB-family: until recently, it was thought that this receptor was enzymatically inactive. Although research has shown that the protein has an ability to bind ATP and autophosphorylate [63], it is unable to phosphorylate other proteins. It requires heterodimerization with another ERBB-protein in order to relay extracellular signals, though it has also been shown to dimerize with non-ERBB-family proteins [64, 65]. For this reason, the protein has been neglected somewhat by researchers as a potential therapeutic target in comparison to ERBB2 and EGFR: both of these have targeted therapies developed against them [61]. It has been shown that ERBB3-ERBB2-heterodimers are the most active of the ERBB-dimers [66].

ERBB3 is commonly found to be upregulated in several cancers. The ERBB2/ERBB3-dimer is shown to be essential for tumor formation in breast cancer [67]. Blocking the dimerization of ERBB3 has thus been explored as a way to use it as a therapeutic target [68]. ERBB3 has been demonstrated to be upregulated in cells resistant to ERBB2- and EGFR-treatments [65, 69, 70]. and increased ERBB-activity is also implicated to be one of the mechanisms behind resistance towards

treatments with BRAF(V600E)-inhibitors [60]. All of these discoveries highlight the importance of attaining further knowledge about ERBB3.

1.8 - Aims of the project

- Obtain efficient gene silencing of MITF and AP-2a in a cultured melanoma cell line
- Investigate a possible correlation between MITF and AP-2 alpha expression
- Investigate the effects of MITF and AP-2 alpha gene silencing on AP-2 gamma, ERBB2 and ERBB3 expression levels
- Investigate the effects of MITF and AP-2 alpha gene silencing on signaling in the PI3K/AKT-pathway and MAPK/ERK-pathway

2 - Methods and materials

2. 1 - Cell culturing

The cell line used for the project was the metastatic melanoma cell line SK-Mel28. In-house data shows that this cell line has an amplification of the MITF gene, and high expression levels of MITF protein and mRNA. The cells were grown in Nunc EASYFlask 75 cm^2 (Thermo Scientific) and were kept at 37 ^oC with 5% CO_2, using RPMI-1640 medium (Sigma) supplemented with glutamax and fetal bovine serum (FBS).

Cells are stored in liquid nitrogen when not in use, to prevent genetic changes from accumulating in the cells. While frozen, the cells were kept in growth medium supplemented with FBS and the cryoprotective agent dimethyl sulphoxide (DMSO), the latter to prevent ice crystal formation which might damage the cells. When the cells were to be cultured, they were removed from the freezer and thawed in a water bath at 37 ^oC. When thawed, the cells were added to the cell culturing flasks, and 20 ml of RPMI-1640 growth medium was added to the flask. After 24 hours, when the cells had adhered to the surface of the flask bottom, the medium in the flask was changed to remove any traces of DMSO, which is otherwise toxic to the cells.

The cell flasks were inspected by microscope daily, and when the confluence in the flask was high enough, the cells were split by the use of trypsin (usually 3-5 days after the last splitting), and samples were transferred to 6-well cell growth plates. When the cell confluence in the wells had reached at least 60%, the plates were ready for transfection.

2.2 - Transfection

Transfection methods are used to introduce foreign oligonucleotides into cells. The types of oligonucleotides introduced are commonly used to affect the expression levels of a gene of interest. Introducing the mRNA of a particular gene into a cell can lead to transiently increased levels of the translated protein in the cell. Similarly, introducing oligonucleotides that interact with and/or disrupt

the existing mRNAs in the cells can lead to transiently downregulated levels of the gene in question. Disruption of mRNA happens when the interfering RNA bind to the mRNA, forming double-stranded RNA molecules. The dsRNA-molecules are recognized and divided into short fragments by the enzyme Dicer. These fragments are degraded by the RISC-complex [71].

For a transfection technique to be effective, it needs to effectively facilitate the uptake of the oligonucleotides into the cytoplasm of the cells. This can be challenging, as the negatively charged lipid surface of the cell membrane will repel negatively charged oligonucleotides, and the hydrophobic inner of the lipid bilayer also acts as an effective barrier that prevents uptake of the hydrophilic lipids. The method used should as far as possible not indirectly affect gene expression by itself, and have low toxicity.

In this project, two transfection methods were used to introduce siRNAs targeting MITF-m and AP-2a in an attempt to downregulate them.

The sequences of the siRNAs used were:

AP-2a sense: GCA-GUA-GCU-GAA-UUU-CUC-A55

AP-2a antisense: UGA-GAA-AUU-CAG-CUA-CUG-C55

and

MITF sense: GCA-GUA-CCU-UUC-UAC-CAC-U55

MITF antisense: AGU-GGU-AGA-AAG-GUA-CUG-C55

For each transfection done in this project, there were made at least three control samples in addition to the samples transfected with AP-2a siRNA and MITF-siRNA, shown in figure 6. The controls in the LF2000-transfected cells involved one set sample with LF2000 added, one sample with LF2000 and negative control siRNA added, and one untreated sample. The PCI-transfected cells had one control with PEI, $TPPS_{2a}$ and 435 nm light treatment, one control with PEI, $TPPS_{2a}$ negative control siRNA and 435 nm light treatment, one control with only 435 nm light treatment, and one untreated control.

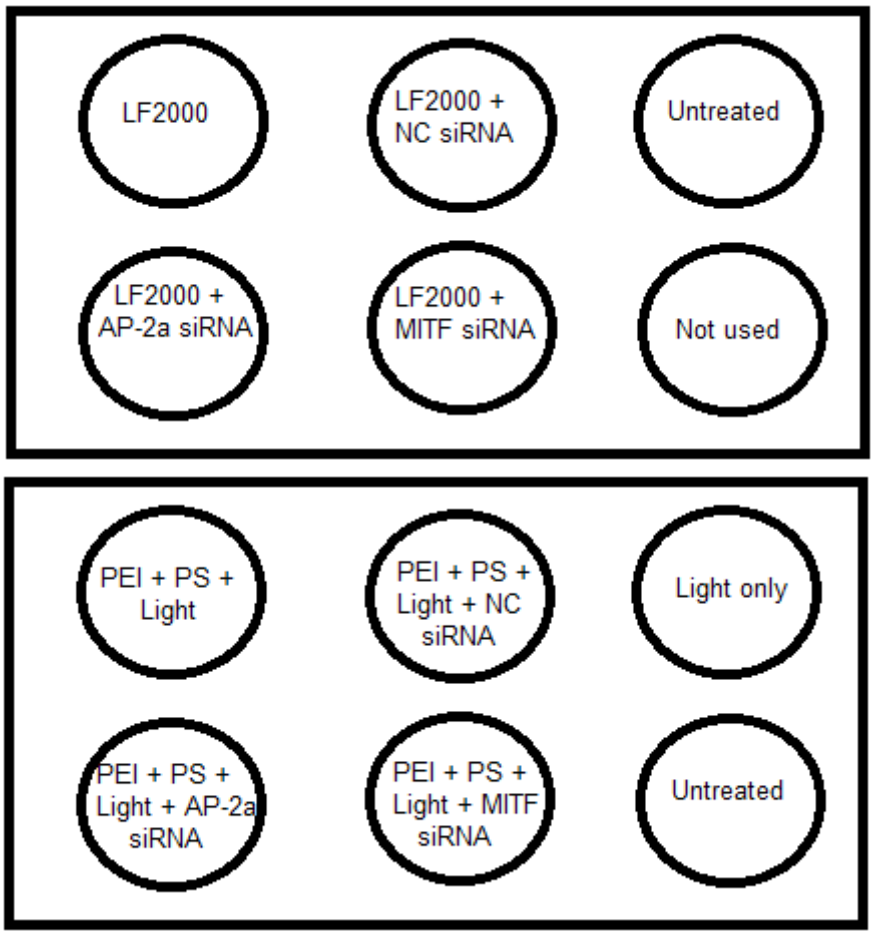


Figure 6: Representation of the wells with plated cells in LF2000-transfected cells and PCI-transfected cells, respectively.

2.2.1 - Transfection: Photochemical Internalization (PCI)

Photochemical internalization is a transfection method where macromolecules are delivered into cells through disruption of the endocytic pathway. Macromolecules are taken up by the cells through endocytosis, and the endosomes are destroyed by use of photosensitizers: molecules that are excited by certain wavelength of electromagnetic radiation [72, 73]. These sensitizers are hydrophobic, and will therefore be prone to integrate themselves into the membranes of the cells. The RNA that is to be taken up by the cells is mixed with a positively charged complexing agent, which facilitates attraction to outer cell membranes. The outer cell membrane will then fold inwards and form vesicles called endosomes that will contain the RNA and complexing agent. This process is called endocytosis. When the cells are exposed to electromagnetic radiation of the wavelength that excites the photosensitizers, the excitation will facilitate the generation of free radicals and

reactive oxygen species that will react with and damage local molecules. As the sensitizers are inserted into cell membranes, and thereby endosomes, the endosomes will be destroyed, and the contents of the vesicles will be released [74, 75].

The light-source used in the PCI is a LumiSource lamp (PCI Biotech), which delivers blue light at 435 nm with a fluence rate of 5.1 mW/cm^2. The photosensitizer used was disulfonated*meso*-tetraphenylporphine($TPPS_{2a}$) and the transfection reagent was polyethylenimine (PEI).

The transfection procedure was done as follows:

1) Add 200 µl of RPMI-1640 medium to an eppendorf tube, and add 50 nMol of siRNA with 8 µl of PEI. Mix gently and incubate at room temperature for 30 minutes.

2) After incubation, add 800 µl of medium mixed with photosensitizer(PS) to the tubes. The concentration of PS post mixing should be 0,5 µg/µl.

3) Remove the growth medium in the cells that are to be transfected, and add the medium with siRNA and PS to the wells containing the cells

4) Wrap the cells in tinfoil to protect them from light, and incubate them overnight at 37 degrees C at 5% CO_2

5) After incubation, wash the cells three times with RPMI-1640 medium, and add 3 ml of RPMI-1640 medium with FBS and GlutaMax. Incubate for 4 hours at 37 ^oC at 5% CO_2

6) Expose the cells to monochromatic light from LumiSource lamp for 45 seconds

7) Incubate the cells for 24 or 72 hrs prior to harvesting for protein/and or total RNA

2.2.2 - Transfection: Lipofectamine 2000

Lipofection is a transfection method that uses positively charged lipids as a method to deliver nucleic acids into target cells. As mentioned earlier, one of the issues with delivering nucleic acids into cells is that the negatively charged nucleic acids will be repelled by the negatively charged outer lipid layer of the cell membrane, as well as being unable to pass through the hydrophobic lipid bilayer. By complexing the nucleic acids with positively charged lipids, the complexes are capable of passing through the cell membrane and into the cells. The cationic lipid formations used in our experiments were Lipofectamine 2000 (Invitrogen).

50 nMol of siRNA was complexed with 7,5 µl of Lipofectamine 2000 in 1 ml of pure RPMI-1640 medium, and were incubated for 30 minutes in order for complexing to finish. This was afterwards added to the target cells, and left for 24, 48 or 72 hours before the cells were taken for harvesting.

2.3 - Cell harvesting and RNA extraction

In order to examine the effects of the transfection protocols on the cells, the contents of the cells have to be isolated for total RNA. RNA was extracted from lysed cells by use of GenElute RNA kit (Sigma Aldrich). The procedure was done as follows:

1) The growth medium was removed from the cells, and the cells were washed three times with PBS

2) 650 ml lysis buffer with 0,1% mercaptoethanol was added to each well

3) The cell lysate was added to a filtration column

4) Centrifuge the columns for 2 minutes at 14.000 x g

5) Discard the column and measure the amount of filtrate. Add a volume of 70% ethanol to the filtrate. Mix by vortexing.

6) Up to 700 µl of filtrate-ethanol-mix was transferred to a binding column, and centrifuged at 14.000 x g for 15 seconds. The filtrate was discarded. This step was repeated until all the lysate had been run through the column.

7) 500 µl of wash solution #1 was added to the column, and centrifuged at 14.000 x g for 15 seconds. The column was moved to a new 2 ml collection tube, the filtrate was discarded.

8) 500 µl of wash solution #2 was added to the column, and centrifuged at 14.000 x g for 15 seconds. The filtrate was discarded.

9) 500 µl of wash solution #2 was added to the column, and centrifuged at 14.000 x g for 2 minutes. The filtrate was discarded.

10) The column was centrifuged an additional time at 14.000 x g to dry the column.

11) The column was moved to a new 2 ml collection tube. 50 µl of elution solution was added to the

column, and the column was centrifuged for 1 minute at 14.000 x g. The filtrate was reapplied to the column with a pipette, and centrifuged for 1 minute.

12) The samples were measured for RNA concentration and purity by NanoDrop 2000 Photospectrometer (Thermo Scientific) by measuring absorption of electromagnetical wavelengths of 260, 280 and 230 nm.

2.4 - RNA precipitation

If the RNA samples were not pure enough for further experimental usage, they were precipitated and resuspended in elution buffer to remove any impurities. This would decrease the total RNA yield in the samples, and was only done if the absorption ratio between 260 nm and 280 nm, as well as 260 nm and 230 nm, was unsatisfactory. A higher ratio than 1.4 was considered good for both 260/280 and 260/230.

The procedure was done as follows:

1) Move the elution buffer containing extracted RNA to an eppendorf tube, and add an amount of isopropanol equal to the volume of the elution buffer, and an amount of 3M Sodium Acetate (pH = 5,2) equal to 1/10th of the original volume, to the tube. Incubate overnight at -20 degrees C

2) Spin the samples down at 13,000g/min at 4 ºC for 40 minutes

3) Check if a pellet has formed in the tubes, then remove the supernatant, wash the samples with 75% EtOH

4) Spin the samples down at 13,000g/min at 4 ºC for 15 minutes

5) Remove the EtOH, leave the tubes open for 1-2 minutes for the remaining of the EtOH to evaporate

6) Resuspend the pellet in elution buffer, examine the RNA amount and purity as described in the RNA extraction paragraph

2.5 - iScript cDNA synthesis

In order to measure and compare the concentration of mRNA in the samples, the samples would be subjected to reverse transcriptase cDNA synthesis. This process uses the enzyme reverse transcriptase to create double-stranded complementary DNA from single-stranded mRNA, making the samples more stable and, more importantly, sets the samples up for real-time PCR.

For cDNA synthesis, the iScript cDNA Synthesis kit provided by Qanta was used. The synthesis was run on a GeneAmp PCR System 9700 provided by PE Applied Biosystems.

Tubes were set up containing the following:

-1 μg of sample RNA

- 4 μl of 5x iScript Mix

- 1 μl of iScript Reverse Transcriptase

- RNase-free water to make the total volume of the tube 20 μl

The tubes were then run for synthesis with a program of 5 minutes at 22 ºC, 30 minutes at 42 ºC, 5 minutes at 85 ºC before cooling down to 6 ºC. The cDNA samples were diluted to a concentration of 10 ng/μl and were stored at 4 ºC until further use.

2.6 - Real-Time Polymerase Chain Reaction (qPCR)

Real-time polymerase chain reaction is a procedure used for quantitative comparison of gene expression in multiple samples. The process works by making a solution of DNA, primers for the gene one wishes to examine, a heat-stable DNA polymerase, deoxynucleotides (dNTP) and a dye that binds to double-stranded nucleotide strands, and follows the following steps:

- heating up the samples for the DNA to denature

- cooling the samples down enough for primers to anneal to the sample, and for the heat-stable DNA polymerase to create new double-stranded DNA

These steps are repeated many times, doubling the amount of double-stranded DNA in the solution for every run. When a dye that binds double-stranded DNA is present in the sample, it is possible to measure the absorption of electromagnetic radiation at a certain wavelength in real time, and by comparing the growth of the absorption rate between wells, it is possible to see what samples have higher or lower levels of the genes of interest.

Two types of genes are examined in parallel here: the genes of interest for the experiments, and (at least) two housekeeping genes, which are genes that are required for basic cellular function. As the expression of these genes should be unaltered in all the samples, having similar levels of housekeeping genes between the samples serve to ensure that equal amounts of total cDNA have been added to each sample [76].

For Real-Time PCR, PerfeCta SYBR Green Supermix provided by Qanta was used for the sample solutions. The samples were put on 96 well plates and the plates were covered by optical tape, both provided by Bio-Rad. The runs were conducted on a CFX Connect Real Time System, provided by Bio-Rad.

1) make a master mix for each gene of interest containing 30 µl SYBR green supermix, 16,4 µl ddH2O, 1,8 µl of forward primer and 1,8 µl of reverse primer for each sample

2) transfer 50 µl of the master mix to tubes

3) add 10 µl of cDNA to the tubes, mix

4) add 25 µl of this mix to adjacent wells in a 96-well Bio-Rad optical plate, creating two parallels

5) cover the plate with optical tape

6) measure the difference in changes in absorbance over time as the PCR process runs

Run setup:
1 - 95,0 ºC for 3:00
2 - 95,0 ºC for 0:15
3 - 60,0 ºC for 0:35
4 - return to step 2, repeat 39 times

5 - 95,0 ºC for 0:10

6 - 60,0 ºC for 0:30

7 - Establishing Melt Curve: 60,0 ºC for 0:08, increments of 0,5 ºC for 0:08, up to 95,0 ºC

8 - 4,0 ºC for 0:20

END

2.7 - Protein harvesting

In order to examine the changes the transfections would cause at protein levels in the cell, it was necessary to extract proteins from the cells. The procedure for this was done as follows:

1) Cells are washed three times with cold PBS

2) 1 ml of cold PBS is added to the cells. The cells are then suspended into the PBS by use of a scrape.

3) The contents of the well is transferred to a eppendorf tube and put on ice.

4) The eppendorf tubes are spun at 4 ºC at 13,000 g x min for 15 minutes

5) The supernatant is removed, and the pelleted cells are put at -80 ºC overnight or longer

6) When the cells are removed from the freezer, they are put on ice, and lysis buffer with a protease inhibitor (Complete Mini) and a phosphatase inhibitor (pSTOP) are added to the pellet immediately

7) The tubes are vortexed, put on ice for 15 minutes, and vortexed again afterwards

8) The content of the tubes are sonicated three times, for three seconds each

9) The tubes are centrifuged at 13.000 g x min for 15 minutes at 4 ºC

10) The supernatant containing the protein is transferred to a new set of tubes. The pellet is discarded.

2.8 - Protein concentration measurement by BioRad assay

To measure the protein concentration in the samples containing the extracted protein, the BIO-RAD Protein Assay was used. This assay uses a solution containing the dye Coomassie Brilliant Blue G-250, which changes color when it binds to amino acid side chains in proteins. The samples are examined by WALLAC 1420 MULTILABEL COUNTER, which measures the sample absorption

at 595 nm. This absorption rate is compared to standard samples of a known concentration.

A total of 10 µl of ddH2O with a protein concentration of 0, 1, 2, 3, 4 and 5 µl is mixed with 100 µl of dye solution in the wells of a 96 well cell culturing plate. Three parallels of each protein concentration are made, and the average absorption of each of these is used to create the standard curve. Afterwards, two parallels of 10 µl of each unknown protein sample is applied to the plate and mixed with 100 µl of dye solution. The absorption is subsequently measured.

2.9 - Western blot

Western blot is a very powerful technique used to compare the levels of protein in samples. It is done by denaturing the proteins in the samples of interest through reduction and heating, as well as adding sodium dodecyl sulfate (SDS) to the samples. SDS is a negatively charged detergent that helps denature the cells, as well as giving each protein in the sample approximately the same charge/size-ratio. The samples can then be separated by applying them to a polyacrylamide-gel and then using an electrical field to separate them. Since the size/charge-ratio of the proteins in the samples is approximately equal, the proteins will be separated primarily based on size. A protein standard with visible bands of known concentrations is also added to the gel, which makes it possible to locate where in the gel proteins of a certain size will be found. After gel electrophoresis, the proteins on the gel are transferred from the gel to a nylon membrane by the use of an electrical current (blotting). These proteins can then be visualized by exposing them to antibodies specific for the gene in question.

The polyacrylamide gels used in this project was NuPAGE Bis-Tris 4-12% gels (Life Technologies). The blotting apparatus used is an iBlot (Invitrogen), and the blotting stack used is iBlot (Life Technologies).

The procedure is done as follows:

1) 20-30 µg of protein are added to the tubes, along with 1 µl of reduction agent and 2,5 µl of a loading buffer. Total volume sample is adjusted to 10 µl by adding double-distilled water.

2) Samples are put on a 70 ºC heating block for 10 minutes to denature the proteins

3) Samples are spun down and put on ice

4) A polyacrylamide-gel mounted in a gel electrophoresis apparatus, and the apparatus is filled with 1x MOPS, up to the point where it covers the wells in the gel. The wells are washed by the use of syringe filled with 1x MOPS.

5) ~10 µl of the samples are added to the individual wells of the gel, as well as 2,5 µl of a ladder solution in one of the wells.

6) Electrophoresis runs at 150V for 90 minutes, or as long as is necessary to achieve a good separation of the proteins

7) The membrane is removed from its container, the gel is put in a blotting device along with the nylon membrane, and the desired blotting program is allowed to run

The process of visualizing the proteins is as follows:

1) The membrane is put in a blocking solution (5% dry milk or 5% bovine serum albumin) for 60 minutes with agitation, and is then washed briefly with 0,1% TBST.

2) The membrane is incubated with a primary antibody overnight at 4 ºC with agitation. Specifics for the solutions of the antibody varies depending on the antibody

3)The next day, the membrane is washed with 0,1% TBST 3 times for 10 minutes each

4) After washing, the membrane is incubated with a secondary antibody for 60 minutes with agitation. Afterwards it is washed with TBST 3 times for 10 minutes.

5) The membrane is put in a G:BOX visualizing machine (Syngene) and is added a visualization solution(Thermo Scientific). The machine then visualizes the strength of the protein bands in the gel.

The specifications and condition for each primary and secondary antibody used for visualization is shown in the appendix.

2.10 - MTS assay

To investigate whether knockdown of MITF and AP-2a had an effecton metabolism, the MTS assay was used. The basis for the assay is the dye MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). It is yellow in its base state, but in the presence of NADP(H)-dependent oxidoreductases it is reduced to formazan, which has a purple color and an absorbance peak at 490 nm. This assay can be used to measure the metabolism of the cells: a higher absorbance at 490 nm indicates a higher level of viable cells.

400 µl of MTS solution (20% of the volume of growth medium) was added to each well. The cells were packed in aluminium foil and incubated at 37 ºC. They were later inspected for visible changes in medium color. If changes were observed, the plates were analyzed in a WALLAC 1420 MULTILABEL COUNTER for absorption at 490 nm.

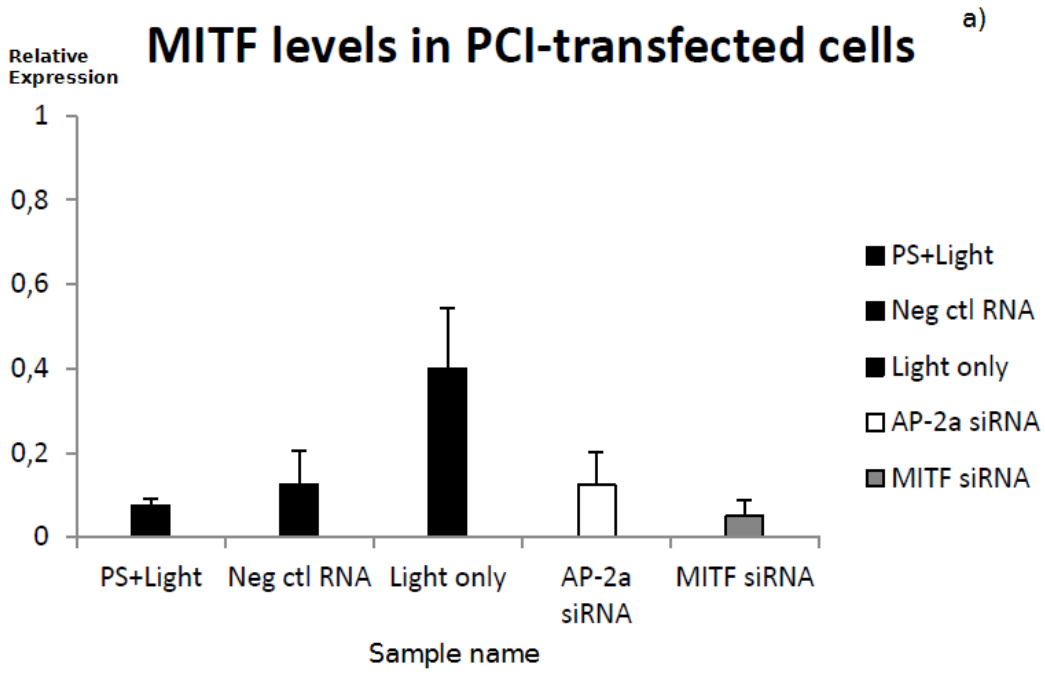
3 - Results

3.1 - PCI greatly affects MITF and AP-2a mRNA levels

Several transfection protocols have been established for delivery of siRNA-molecules into human cancer cell lines. Here, we investigated if transfection by PCI would provide an efficient method of siRNA-delivery in the melanoma cell line SK-Mel28. The protocol described in the materials and methods section has been optimized for the SK-Mel28 cell line by in-house personell, and was used for this reason. The delivered siRNA was targeting MITF and AP-2a, as well as a scrambled sequence used as negative control, and MEK1 as a positive control (data for MEK1 not shown). Investigation of the effects of the PCI-transfection on the SK-Mel28 cells were done by qPCR. The transfected cells were lysed and their RNA was purified and the RNA-levels were measured before cDNA was made from the samples. The cDNA was then used for qPCR analysis. Tubulin binding protein (TBP) and Large Ribosomal Protein (RPLPO) were used as control samples, and are not shown in the result. MEK1 was used as positive control in the earliest transfection attempts to ensure the efficiency of the protocol (results not shown).

Results are shown in figure 7. Transfecting cells with MITF siRNA was shown to reduce the levels of MITF mRNA in the cells comparison to the control samples (figure 7a), and the same effect was observed for AP-2a in cells transfected with AP-2a siRNA (figure 7b). In addition, the cells transfected with MITF siRNA would lead to reduced levels of AP-2a mRNA in comparison to the controls (figure 7b). This supports the hypothesis that MITF directly influences the levels of AP-2a in the cells.

Additionally, it was also clear that PCI as a transfection method had a significant effect on the levels of MITF in the cells. Compared to the untreated samples, applying light to the cells would reduce MITF levels by ~50%, and treating the cells with both light and photosensitizer would further lower MITF-levels by 60% as compared to the light-treated control samples. As such, it was decided to abandon PCI as the transfection method of choice for the remaining of the project.



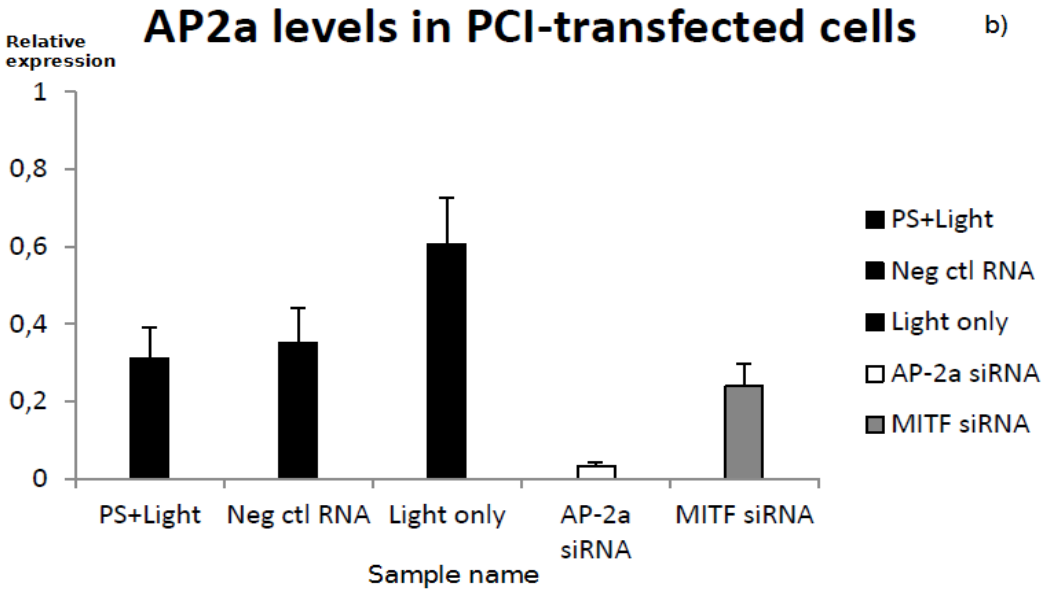


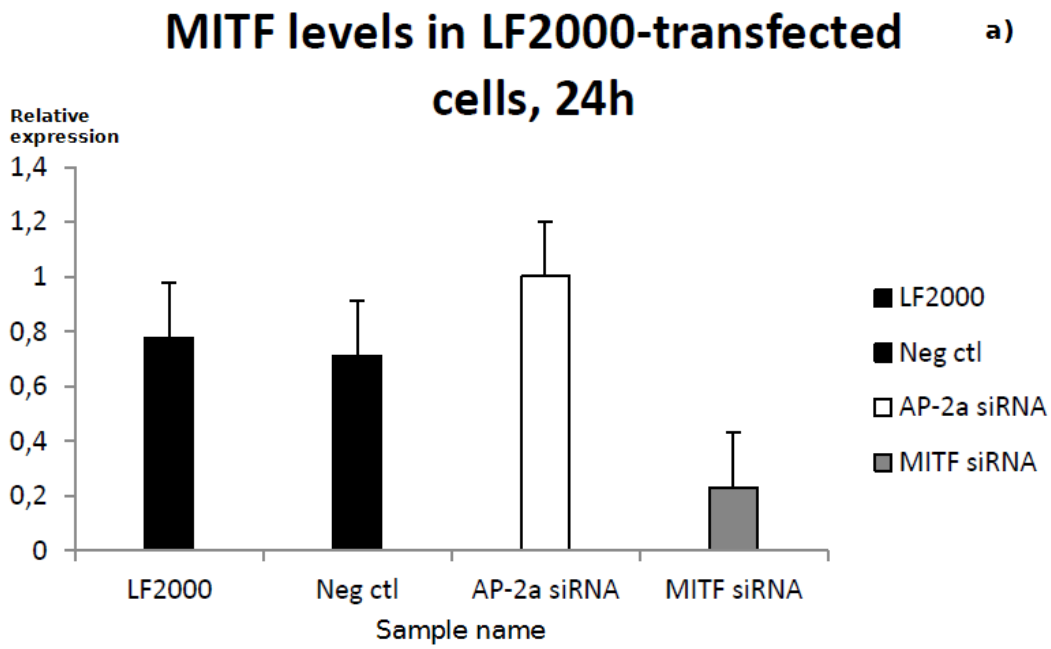
Figure 7: PCI-induced siRNA transfection after 24 h by Real-Time PCR. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Figure 7a) shows the MITF levels after various treatments, 7b) show the levels of AP-2a mRNA. The untreated control sample was set as baseline control. Bars are presented as the average of three individual experiments and error bars show standard deviation between the samples.

3.2 - Successful siRNA knockdown with LF2000 after 24 h

After deciding to abandon PCI as the transfection method of choice, Lipofectamine 2000 was elected to be tested and compared with PCI as a transfection method. The protocol listed in the Materials and Methods-section has been optimized for transfection of SK-Mel28 by in-house personell. As with PCI, the siRNAs used were targeting MITF and AP-2a, as well as a scrambled negative control RNA sequence.

The samples transfected with LF2000 were examined by qPCR. As with the PCI-transfected samples, the RNA from the transfected samples was collected, measured, and used for cDNA synthesis. Samples were collected after either 24 hours or 72 hours, to compare the efficiency of the protocol at different intervals. TBP and RPLPO were used as controls and their are not shown.

Figure 7 and 8 show the results from samples that were harvested after 24 hours. The samples transfected with MITF siRNA were shown to have lowered levels of MITF and AP-2a (figure 7a), while cells transfected with AP-2a siRNA showed lowered levels of AP-2a (figure 7b). Compared to the samples prepared with PCI, the control samples were much closer to the untreated baseline control for both MITF and AP-2a.



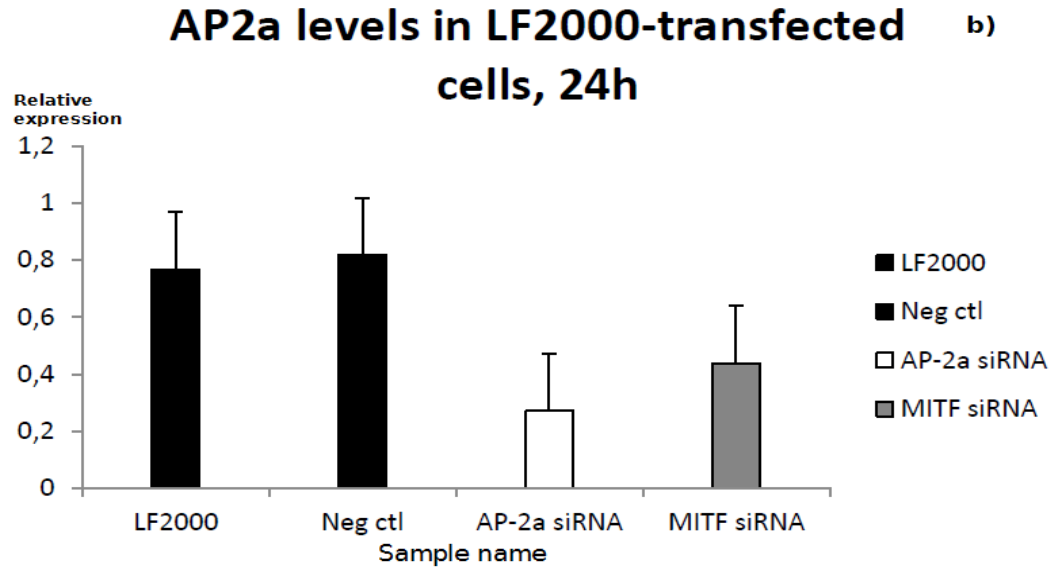
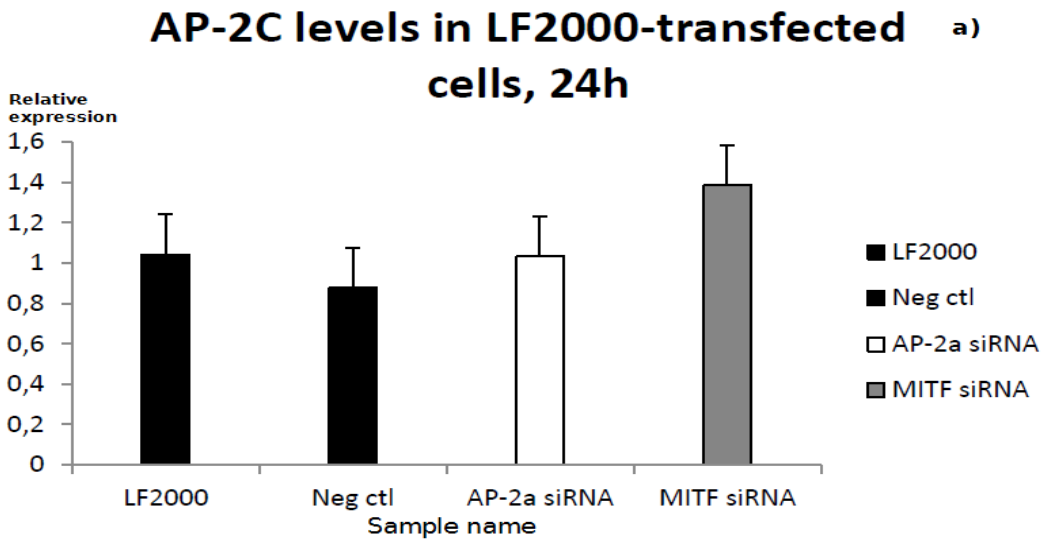
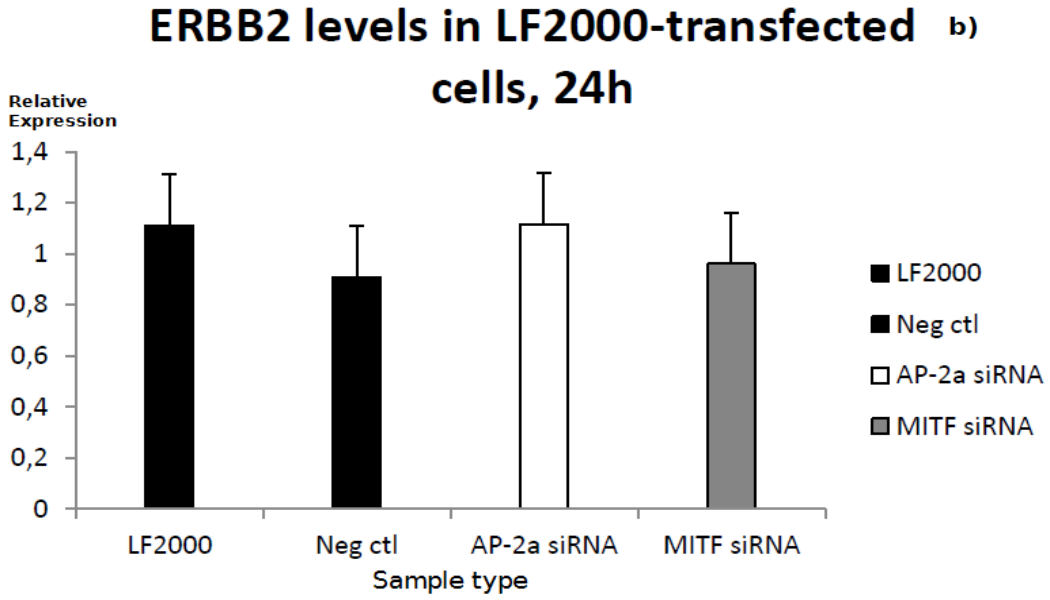


Figure 8: LF2000-induced siRNA transfection after 24 h by Real-Time PCR. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Figure 8a) shows the MITF levels after various treatments, 8b) show the levels of AP-2a mRNA. The untreated control sample was set as baseline control. Bars are presented as the average of three individual experiments and error bars show standard deviation between the samples.

As successful MITF- and AP-2a knockdown had been demonstrated, the levels of AP-2C, ERBB2 and ERBB3 in the same samples were examined. Figure 9 shows mRNA levels of AP-2C, ERBB2 and ERBB3 in samples harvested 24 hours after transfection. Results show an upregulation of AP-2C mRNA levels by approximately 40% in the samples transfected with MITF siRNA (figure 9a). Furthermore, ERBB3 mRNA levels were more than 2-fold upregulated in the samples transfected with MITF siRNA (figure 9c), while the ERBB2 levels were unchanged (figure 9b).





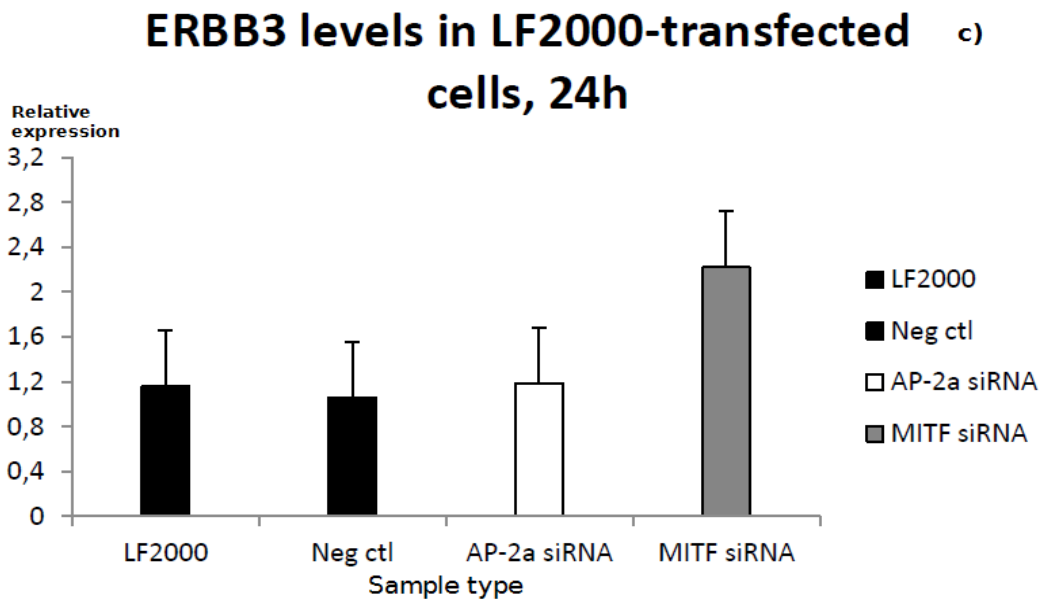


Figure 9: LF2000-induced siRNA transfection after 24 h by Real-Time PCR. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Figure 9a) shows the AP-2C levels after various treatments, 9b) show the levels of ERBB2 mRNA, 9c) show the levels of ERBB3 mRNA. The untreated control sample was set as baseline control. Bars are presented as the average of three individual experiments and error bars show standard deviation between the samples.

3.3 - MITF and AP-2a knockdown affects pAKT protein levels after 24 h

While effects on the RNA levels of the transfected cells showed clear effects on RNA-levels, these differences may not be as pronounced at the protein level. A slow rate of protein turnover for the examined proteins would mean any changes in mRNA levels would not immediately translate to reduced protein levels. Thus, it was deemed necessary to investigate how MITF- and AP-2a knockdown affected protein expression. Protein samples harvested from cells transfected with Lipofectamine 2000 were harvested after 24 hours and analyzed by western blot. The levels of AP-2a, MITF and ERBB3 in transfected cells were compared to the control samples, and the loading was evaluated by using Histone 3 as a loading control.

The results of the western blot analysis is shown in figure 10. These results mirrored the results of the qPCR-results. Samples transfected with MITF siRNA had its levels of MITF and AP-2a greatly reduced in comparison to the control samples, and significantly increased levels of ERBB3. Samples transfected with AP-2a siRNA had very low levels of AP-2a in comparison to the controls, with ERBB3 and MITF unaffected by the AP-2a siRNA treatment.

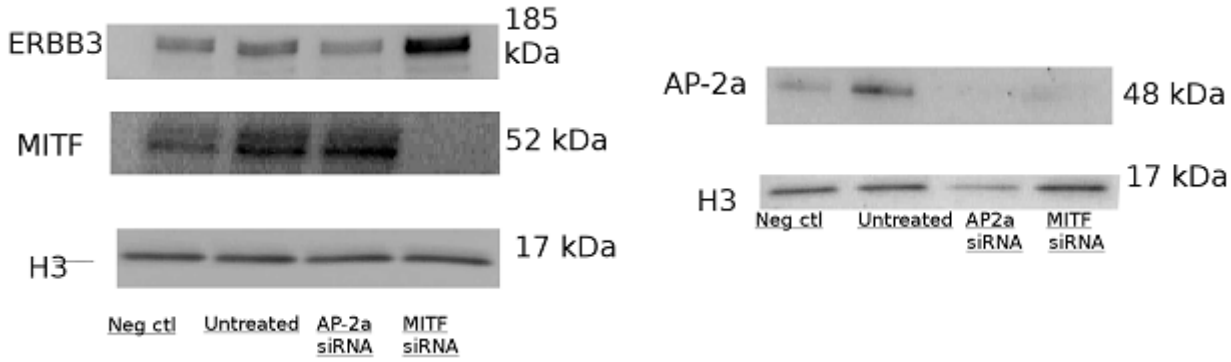


Figure 10: LF2000-induced siRNA transfection after 24 h by western blot. H3 was used as loading control.

One of the goals of this project was to examine the effects MITF has on proliferative signaling and survival signaling in melanoma cells. For this reason, the levels of AKT, ERK, pAKT and pERK in the transfected samples were also examined. AKT, ERK and pERK levels were unchanged in all samples. pAKT levels were elevated in MITF-transfected samples, and reduced in AP-2a transfected samples (figure 11).

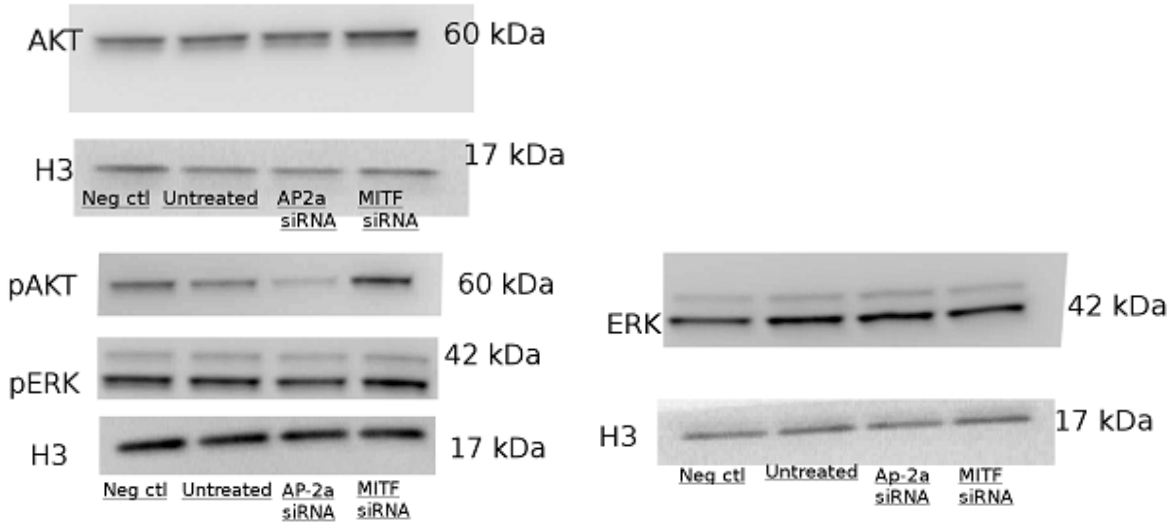


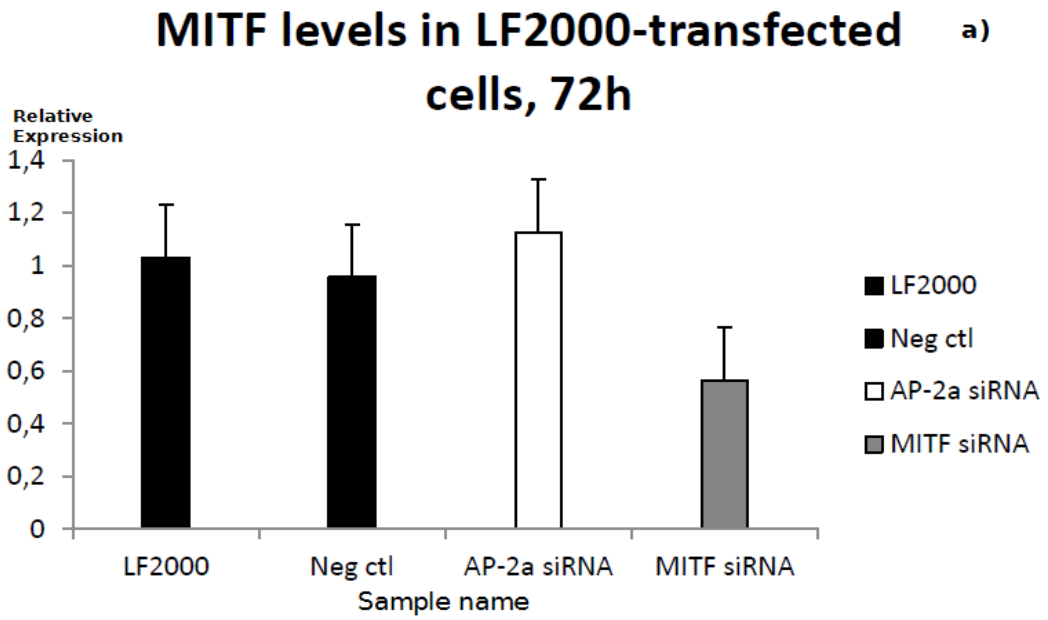
Figure 11: LF2000-induced siRNA transfection after 24 h by western blot. H3 was used as loading control.

3.4 - qPCR data from LF2000-transfected samples after 72 h

In the 24 h transfections, several interesting effects were observed after MITF and AP-2a siRNA knockdown, in particular the upregulation of pAKT and ERBB3. To investigate whether these effects were short lived, 72 h transfections were carried out and analysed for both protein and mRNA. The procedure used in the 72 h transfections was, aside from the transfection time,

otherwise equal to the 24 h transfections.

Figure 12 and 13 show the results from samples that were harvested after 72 hours. The results were consistent with the samples that were harvested after 24 hours. Transfection with AP-2a siRNA resulted in reduced levels of AP-2a (figure 12b), transfection with MITF resulted in reduced levels of MITF (figure 12a), as well as reduced levels of AP-2a (figure 12b), and elevated levels of AP-2C and ERBB3 (figure 13). The effects of the siRNA on the target genes were less pronounced compared to the cells harvested 24 hours after transfection.



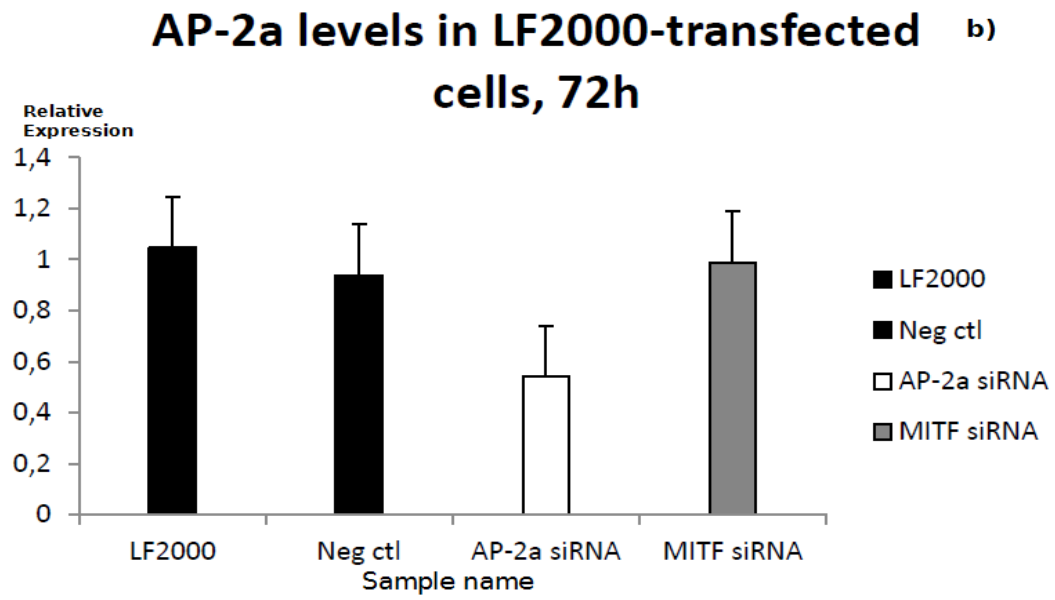


Figure 12: LF2000-induced siRNA transfection after 72 h by Real-Time PCR. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Figure 12a) shows the MITF levels after various treatments, 12b) show the levels of AP-2a mRNA. The untreated control sample was set as baseline control. Bars are presented as the average of three individual experiments and error bars show standard deviation between the samples.

As with the samples harvested after 24 h, the samples harvested after 72 h were examined for AP-2C and ERBB3. The levels of ERBB3 saw a twofold increase in the MITF-transfected samples in comparison to the control samples. Similarly, the AP-2C levels in the MITF-transfected samples were increased by around 40%. No significant change was observed in neither ERBB3 nor AP-2C in the samples treated with AP-2a siRNA.

In the 24 h samples, ERBB2 mRNA levels had also been investigated by qPCR. In those samples, transfection did not have any observable effect on ERBB2-expression. This, combined with time constraints on the project, led us to abandon qPCR analysis of ERBB2 on the 72 h samples.

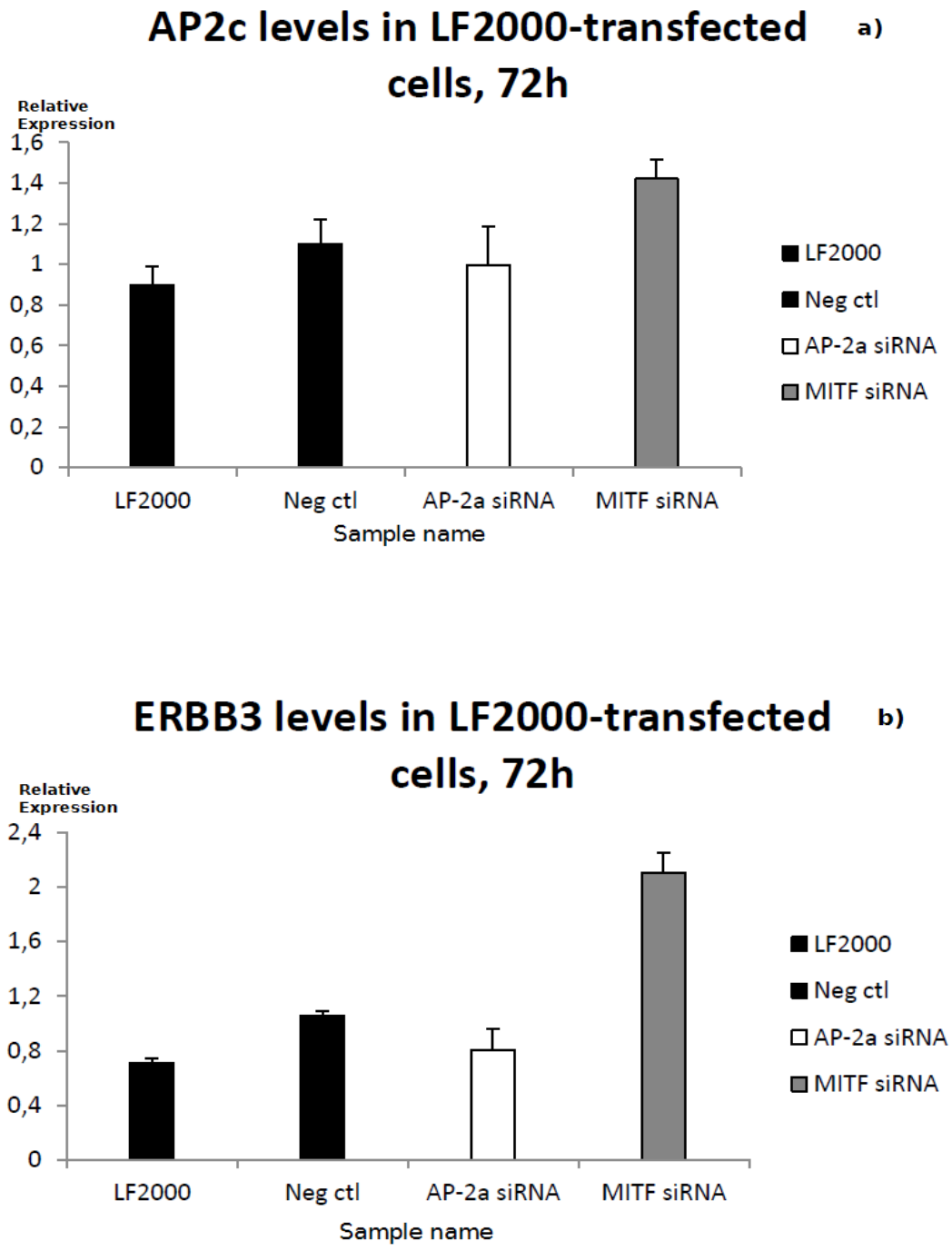


Figure 13: LF2000-induced siRNA transfection after 72 h by Real-Time PCR. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Figure 13a) shows the AP-2C levels after various treatments, 13b) show the levels of ERBB3 mRNA. The untreated control sample was set as baseline control. Bars are presented as the average of three individual experiments and error bars show standard deviation between the samples.

3.5 - Western blot analysis after 72 h

As with the qPCR-samples, the effects seen at a protein level after 24 h of LF2000-transfection showed several interesting effects, and it was decided to investigate whether the same effects would be observed after 72 h of transfection.

The results for the 72 h samples were consistent with the data obtained from the 24 h samples, and are shown in figure 14. MITF knockdown caused a very significant downregulation of MITF protein, caused a downregulation of AP-2a, and an upregulation of ERBB3 and pAKT, while AP-2a knockdown caused lowered levels of pAKT. The effect of the knock-down was not as pronounced in the 72 h samples as in the 24 h samples.

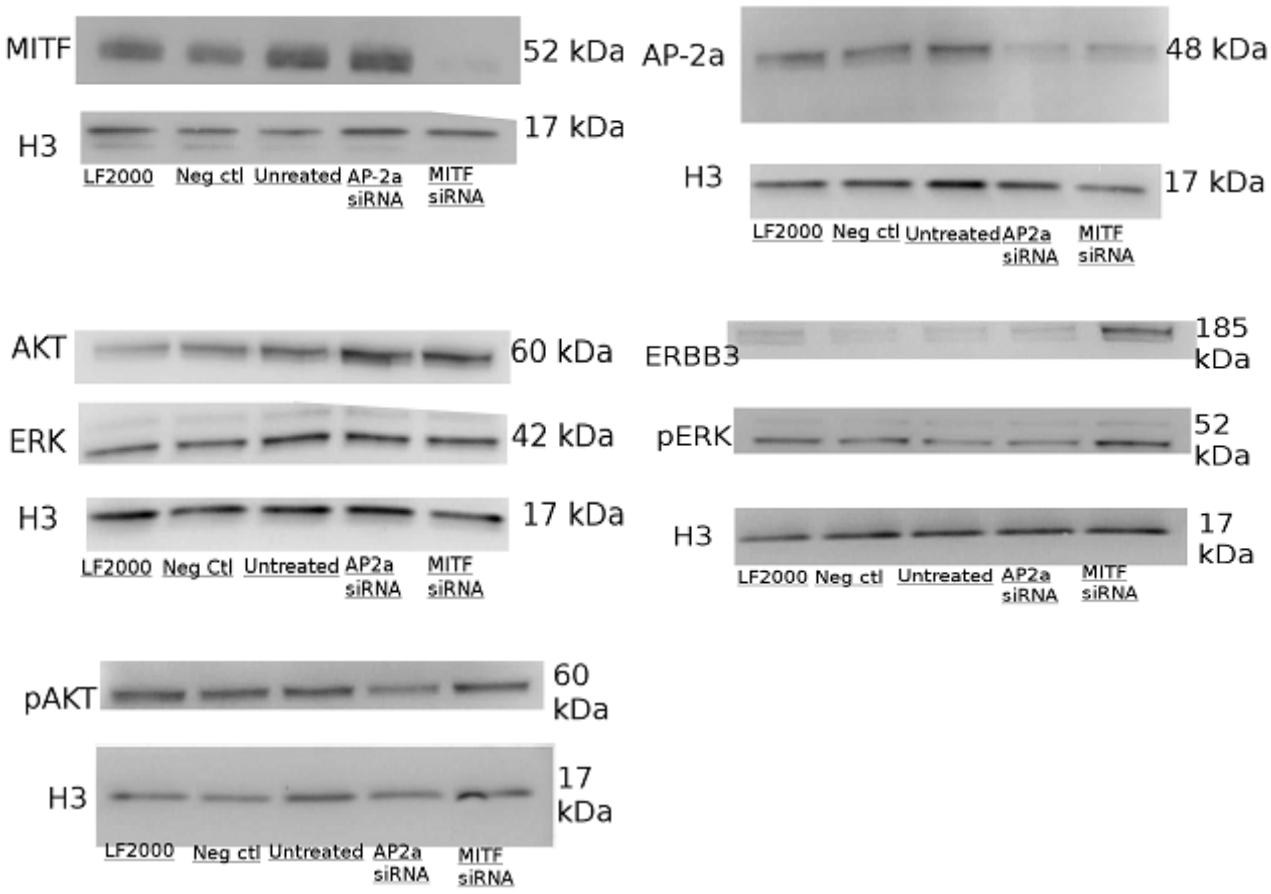


Figure 14: Results of the western blot run for samples transfected with MITF and AP-2a siRNA. H3 was used as loading control.

3.5 - MITF knockdown increases metabolic activity in SK-Mel28

The analysis of protein and mRNA levels in the transfected cells provide insights on how MITF and AP-2a knockdown affect specific proteins and signaling pathways, but not on how it affects the cell as a whole. To gain insight on this, the MTS assay was used. As described in the Materials and Methods-section, the dye MTS was added to the wells of the plated cells, and analyzed for 490 nm absorption after 5 minutes. The dye changes color as the cells metabolize, thus it gives a method for measuring metabolic activity: higher abs indicates increased activity.

Results of the MTS assay are shown in figure 15, with supplementary figures in Appendix D. The untreated cells had the consistently lowest abs. The MITF transfected cells had a higher abs. rate than the treated controls and the AP-2a transfected cells.

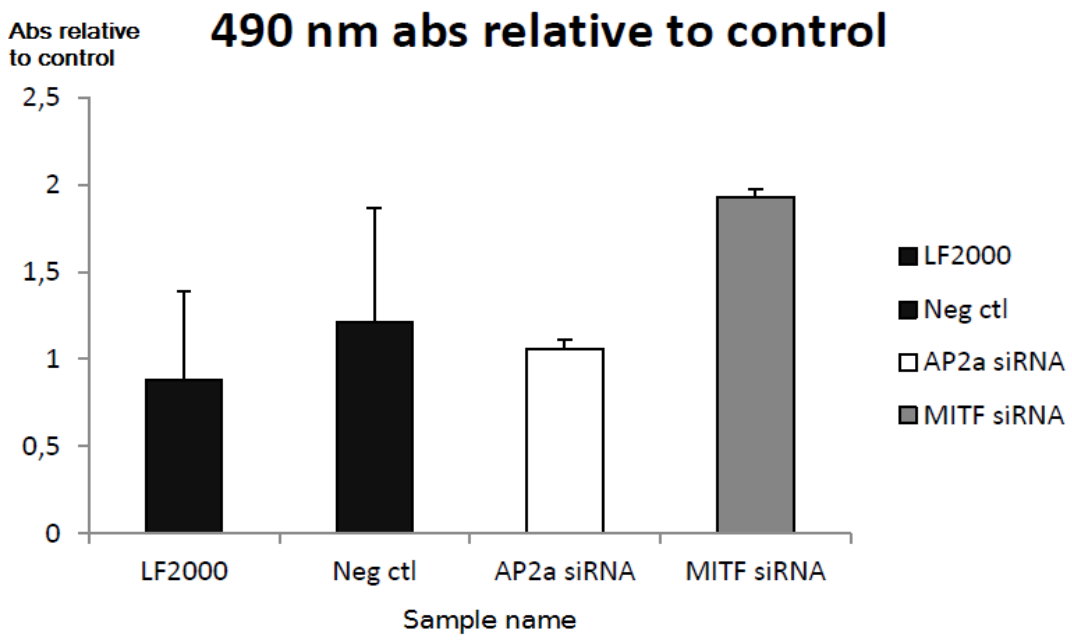


Figure 15: Relative absorption rate between transfected samples. Controls are shown in black bars, samples transfected with AP-2a siRNA in white and samples transfected with MITF siRNA in gray. Control Untreated control samples are set as baseline control. MITF transfected cells had the consistently highest abs. rates. Error bars show standard deviation between the samples. Absolute numbers for the abs. rates are found Appendix D.

4 - Discussion

4.1 - LF2000 is preferred over PCI as a transfection method for SK-Mel28

As stated in the introduction section, one of the goals of this project was to attain effective silencing of MITF and AP-2a. To this end, two different transfection methods were employed at the beginning of the project: PCI and Lipofectamine 2000. When comparing qPCR-data from cells transfected with the two transfection methods, it is clear that LF2000 is preferred to PCI for transfection of SK-Mel28, at least when the goal is to study MITF and AP-2a. Both methods were efficient for siRNA knockdown. Interestingly, MITF knockdown also resulted in reduced levels of AP-2a compared to the control samples, indicating that MITF is a regulating modulator of AP-2a expression. However, when comparing mRNA levels of MITF and AP-2a in the control cells of the PCI-transfected cells (figure 7) and in the LF2000-transfected cells (figure 8), treating the cells with 435 nm light caused a significant drop in MITF mRNA levels, while LF2000 did affect MITF expression far less severely. MITF is considered one of the most important genes in melanocyte differentiation and function, and is also responsible for melanin production in melanocytes [40]. As MITF levels are affected by UV light [84], it is less surprising that treating melanoma cells with 435 nm light will have an effect on the expression levels of MITF present in the cell. The differences in AP-2a mRNA levels between the treated controls and the untreated control can be explained by the differences in MITF levels, as knock-down of MITF resulted in reduced levels of AP-2a.

As mentioned above, it has been documented that exposing melanocytes to ultraviolet (UV) radiation can affect MITF levels [84]. However, in other studies, UV-treatment has resulted in *upregulation* of MITF levels, rather than the effect observed in this project. UV-radiation has a shorter wavelength (400 nm - 100 nm) than the light used for transfection in this project (435), so the data from this project does not directly contradict previous studies. The observed change in MITF indicates that different wavelengths of electromagnetic radiation could have opposing effects on MITF levels. Further experiments could verify this, and whether this is true for light of shorter wavelengths than what was used in this project as well.

The reagents used for transfection in PCI, PEI and $TPPS_{2a}$, caused a greater change in MITF levels

compared to LF2000. MITF- and AP-2a levels were reduced by 15-30% in samples where LF2000 was added when compared to untreated controls. Samples treated with light and photosensitizer were reduced by 70-80% in comparison to the light-treated controls, and 85-90% in comparison to the untreated controls. The functional mechanism of photosensitizers used in PCI is to become excited when exposed to light of the corresponding wavelength. This causes the formation of reactive oxygen species (ROS) [72], which causes the destruction of the cellular material close to the photosensitizers. The stress induced by the photosensitizers could activate signaling that affects MITF transcription. Shi *et al* showed recently that melanocytes respond to cellular stress by upregulating miR-25, a micro-RNA capable of negatively regulating MITF translation, supporting the observations in this project and providing a possible mechanism for these results [87].

LF2000 is a well described and widely used transfection reagent [88, 89]. It has been used in numerous scientific projects to facilitate the uptake of foreign substance into cells. From the results shown in this project, it is clear that use of LF2000 is preferable to PCI when transfecting SK-Mel28, as it is less disruptive towards one of the key melanocyte genes. Of note, there are also other available options for lipofection that have not been covered in this project that may be very useful for transfection of melanoma cell lines. Invitrogen, the producer of LF2000, is also offering Lipofectamine 3000, which their webpage describe as being less toxic than LF2000 while being more effective in numerous cell lines previously considered "hard to transfect". Another product described by Invitrogen is LF2000 RNAiMAX, which is a transfection agent optimized for siRNA delivery [90]. These agents could possibly provide even better siRNA knockdown than LF2000, but this remains to be investigated.

During the MTS assay experiments, it was observed an increase in metabolic activity in cells transfected with LF2000 in comparison to the untreated controls. As LF2000 is toxic [99], the increase in 490 nm absorption could be due to the cells reacting to the possible cell stress/damage induced by LF2000.

4.2 - Knock-down of MITF in SK-Mel28

siRNA-mediated knockdown of MITF and AP-2a were successfully carried out in the SK-Mel28 cell line. In samples where MITF were knocked down showed, relative to controls, reduced levels of AP-2a mRNA and protein, increased levels of AP-2C mRNA, and increased levels of ERBB3 mRNA and protein. In contrast, levels of ERBB2 mRNA, AKT protein and ERK protein were unaffected. However, levels of pAKT were found to be increased, while pERK levels were unaffected. These effects were consistent at both 24 and 72 h after transfection. A reduction in pAKT was observed in the samples transfected with AP-2a siRNA, but otherwise no other examined genes were found to be affected by AP-2a knockdown at either protein or mRNA level.

Cells with MITF-knockdown showed increased levels of pAKT, but unchanged levels of total AKT, indicating higher AKT activity in cells transfected with MITF. It has been reported previously that ERBB3 is capable of affecting signaling in the PI3K/AKT-pathway [58, 68]. As we observed an increase in phosphorylation levels of AKT, rather than on the amounts of expressed AKT protein in the cells, these findings support existing studies where ERBB3 was found to be a modulator of the PI3K/AKT-pathway [58, 68]. Before any conclusions can be made it is necessary to validate that the observed effect on PI3K/AKT-signaling is modulated by ERBB3 rather than an upstream gene targeted by MITF.

No changes in ERK and pERK levels were found in any western blot samples, even though increased activity in the ERBB3 has previously been shown to be able to affect signaling in the ERK/MAPK-pathway [64, 96]. According to in-house data, SK-Mel28 is homozygous for BRAF(V600E), which makes the ERK pathway constitutive activated [101]. This is a possible explanation for why no changes in the ERK or pERK levels were observed in samples where ERBB3 was affected. Further studies on the influence of ERBB3 and MITF on the ERK/MAPK-signaling in a cell line without a BRAF(V600E)-mutation is therefore recommended.

The implication of these results is that MITF is a negative regulator of ERBB3-transcription, which gives it indirect control over proliferative and survival signal pathways. To further examine the role of MITF in pAKT regulation, it would be necessary to ensure that it is the altered ERBB3-levels,

rather than another protein regulated by MITF, that is responsible for the increased AKT activity. This could be done by examining cells after simultaneously knocking down MITF and ERBB3, alone and in combination.

Downregulation of MITF resulted in an increase in AP-2C mRNA expression. As described in the introduction chapter, AP-2C functions as a tumor suppressor [81], and increased levels of AP-2C should therefore inhibit tumor formation. At the same time, MITF silencing caused reduced levels of the tumor suppressor AP-2a and elevated levels of the proliferative signaling receptor ERBB3. Both of these effects promote tumor growth. The fact that MITF knockdown caused both the up- and down-regulation of different tumor suppressor genes, suggests that MITF a very complex gene and is capable of functioning as both an oncogene and tumor suppressor.

The results from the MTS assay showed an increase in metabolic activity in the MITF siRNA transfected samples compared to the controls. It is possible that the observed changes in metabolic activity in the MITF-transfected samples were due to increased AKT-signaling, which in turn could be mediated by the measured change in ERBB3-levels. However, ERBB3 is one out of many genes under the transcriptional control of MITF. It is therefore difficult to determine if the observed effect was due to elevated ERBB3-signaling without further experiments. Furthermore, the standard deviation between the parallels in the control samples were larger than that of the samples transfected with siRNA. This makes it difficult to determine if the observed increase in metabolic activity in the MITF transfected samples was significant.

As MTS assays are used for measuring metabolic activity in cells, it may give an indication of the cell proliferation rate, as wells with higher cell counts are likely to have higher levels of metabolic activity. However, this does not directly confirm whether proliferation is actually affected, or if any changes in metabolic activity are for other reasons. The results will therefore have to be verified further. This can be done by cell density measurements or by use of a survivability assay, both alternative methods suited for of measuring cell proliferation. One such technique, IncuCyte, involves analyzing and comparing differences in cell confluency [91], which may offer a better measurement for proliferation and survival than the MTS assay.

MITF is a complex gene, which exerts influence on a large variety of genes, and where different expression levels of the gene are associated with different phenotypes in melanocytes [45]. It's difficult to predict the effects of targeting MITF therapeutically; in fact, some studies have

previously concluded that MITF is not a druggable target [102] and that it is likely more productive to examine its upstream modulators as therapeutic targets [45]. The results obtained in this study are consistent with these claims, as MITF silencing resulted in increased metabolic activity and AKT-signaling in SK-Mel28. Due to the limited scope of the project, however, no conclusions can be drawn on the viability of MITF as a therapeutic target from these data alone. On the other hand, the results of the study do show a correlation between MITF expression and AP-2a, AP-2C and ERBB3.

Knocking down MITF in this study resulted in increased pAKT, and an upregulation of metabolic activity in the SKmel-28 cell line. SKmel-28 is a metastatic cell line that has an amplification of the MITF gene and high levels of MITF expression, in addition to having a non-functional p53-gene and being homozygous for BRAF(V600E). (in-house data) It would be very interesting to investigate the response of MITF transfections attempted in this study in other cell lines. Considering how different levels of MITF expression is reported to be associated with different melanoma cell phenotypes, [45] the results of MITF silencing in melanoma cell lines with different levels of MITF expression could yield different results than those obtained here. Melanoma cell lines in a different disease stage could also react differently to MITF knockdown compared to metastatic cell lines. MITF silencing in SK-Mel28 did not affect the protein levels of ERK or pERK in this study. Investigating the effects of MITF silencing in a cell line that is not homozygous for BRAF(V600E) is recommended.

4.3 - Knock-down of AP-2a in SK-Mel28

AP-2a knockdown was not observed to affect the metabolic activity, as measured by the MTS assays. However, MITF knockdown which caused AP-2a downregulation was observed to increase the metabolic activity. AP-2a functions as a tumor suppressor gene and low levels of the gene is associated with disease progression in cancer cells [54]. One of AP-2a's functions is to cooperate with p53 to induce p21 expression in the cells, which again binds to the cyclin-dependent kinase 2 (CDK2). The CDK2-p21-complex prevents cell cycle progression past the G1-phase [92]. The SK-Mel28 cell line has a nonfunctional p53 gene, (in-house data) shutting down p53-p21-signaling independent of AP-2a. This could be a possible explanation for why reduced levels of AP-2a did not cause any changes in metabolic activity. Previous studies have also shown a correlation between ERBB2-overexpression and high levels of AP-2a in breast cancer [100]: ERBB2 has been found to affect the MAPK/ERK-pathway, and may give AP-2a indirect influence over ERK-signaling in cell lines that contain wild type BRAF. However, in this project, we did not detect any effect upon

ERBB2 mRNA when knocking down AP-2a.

Knock-down of AP-2a resulted in reduced pAKT-levels compared to the control samples. This was less apparent in the 72 h samples compared to the 24 h samples. As AP-2a is thought to function as a tumor suppressor, it was surprising to detect that lower AP-2a levels lead to a decrease in pAKT levels. It was also interesting to see MITF knockdown leading to AP-2a downregulation, yet MITF knockdown caused increased AKT-signaling rather than reduced. Sumigama *et al* [86] increased AP-2a levels in the SKOV3 ovarian cancer cell line and observed reduced levels of pAKT and pERBB2 as a consequence. Other studies have hinted that AP-2 proteins can function both as an oncogene as well as a tumor suppressor [97], and it is possible that AP-2a has a different function in melanoma compared to ovarian cancer. Sumigama *et al* also specifies that the SKOV3 cell line has a low initial level of AP-2a. The AP-2a levels in SK-Mel28 in comparison to other cell lines is at the present moment not known. As since the cell line has a high expression level of MITF, a gene that upregulates AP-2a, it is a possibility that this would cause an increase in AP-2a expression. This has to be verified, however. It is also known that cancer cells can have different expression profiles in regards to which AP-2a isoforms that are expressed in SK-Mel28. While the 1a isoform is the most highly expressed, it has been observed that breast cancer cell lines can have increased levels of the 1b and 1c isoforms [85]. Berlato *et al* shows the 1a isoform is reported as the only subunit capable of acting as a direct repressor, while the 1b and 1c isoforms are both capable of transactivating the ERBB2-promoter [85]. As ERBB2 is capable of affecting the PI3K/AKT-pathway, knocking down AP-2a could influence PI3K/AKT-signaling indirectly by upregulating ERBB2 through the 1b and 1c. However, no changes in ERBB2 mRNA-levels were detected in cells transfected with AP-2a siRNA (figure 9b). Further investigation on the role of AP-2a in melanoma cells is recommended to verify the validity of the results obtained in this project.

4.4 - Future perspectives

The results obtained in this study could indicate that ERBB3 is capable of affecting the AKT-pathway. Other studies have found similar results [96], and efforts to target ERBB3 therapeutically has been made [93-95]. As a surface receptor, a likely method for targeting ERBB3 would be antibodies. It has been attempted to create therapeutic antibodies that block ERBB2-ERBB3-dimerization, which is one possible method of action against ERBB3 [98]. MITF, however, may not be suited as a drug target, and needs further examination. Furthermore, the results obtained in this study will have to be verified to ensure their relevance. One way to accomplish this is to carry out MITF- and AP-2a silencing in other cell lines, particularly ones that are not BRAF(V600E)-

homozygous, or in a metastatic phase. The results of the MTS assay are also not enough to draw any conclusions, and it is recommended to supplement these results with data obtained with other techniques. Furthermore, the effects ERBB3 has on cell proliferation and survival needs to be further verified: while MITF-transfected cells had increased levels of both ERBB3 and pAKT, it is necessary to look at ERBB3 alone. This project has not examined how MITF levels affect the invasiveness of melanoma cell lines, which could be done through matrigel analysis [103]. Examining how MITF silencing affects melanocytes *in vivo* is also a way to provide useful research data on MITF. The results obtained on AP-2a also need to be further verified, in order to examine the role of the gene in melanoma.

4.5 - Conclusions

In conclusion, the testing of two different transfection methods on melanoma cells showed that Lipofectamine 2000 was overall a preferred method to PCI in SK-Mel28: both the photosensitizer and the 435 nm light treatment used in PCI significantly affected the expression levels of MITF, the master regulator of melanocyte function. Furthermore, we observed a correlation between the levels of MITF in SK-Mel28, and the levels of AP-2a, ERBB3 and AP-2C in these cells. Additionally, MITF knockdown positively affected AKT-signaling. None of the silenced genes affected the levels of ERK or pERK, though that is likely due to the cell line used being BRAF(V600E)-homozygous, which makes the pathway constitutively active and thus largely unaffected by MAPK upstream signaling. It is possible that the effect on AKT signaling is a consequence of the increased level of ERBB3 after MITF knockdown. However, this has to be further investiagted. AP-2a silencing unexpectedly reduced signaling in the PI3K/AKT-pathway. At the moment we have no explanation for this effect, and further studies have to be done to reveal the role of AP-2a upon the PI3K/AKT-pathway.

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Appendix

Appendix A - Buffers and solutions used in the project

Table A1: Complete growth medium supplements

Supplement	**Concentration**	**Per 500 ml RPMI 1640**
Fetal bovine serum	10%	50 ml
glutaMAX	2 mM	5.5 ml of 200 mM stock

Table A2: Contents of prepared protein samples for gel electrophoresis

Compound	**Volume (ul)**
NuPAGE LDS sample buffer (4X)	2,5
NuPAGE Reducing Agent (10x)	1
Protein sample	X
ddH_2O	6,5 - X
Total volume	10

Table A3: Contents of TBS-T 0.1% wash buffer (Tris-buffered saline /w 0.1% Tween 20)

Compound	**Volume (ml)**
5M NaCl	27,4
1M Tris-HCl pH 7,5	20
20% Tween 20	5
ddH_2O	Up to 1000

Table A4: Contents of lysis buffer for protein harvesting

Compound	**Concentration**	**Volume (ml)**
NaCl	150 mM	1,5
Tris pH 7.5	50 mM	5
Nonidet P40	0.10%	0,05 (50 ul)
ddH_2O	------	to 50 ml

Appendix B -Antibody conditions for western blotting

Primary Ab	**Size (kDa)**	**Blocking**	**Primary Ab dilution**	**Secondary Ab + dilution**	**Buffer for Ab dilution**
Histone H3	17	5% dry milk	1:3000	Rabbit, 1:8000	5% dry milk in TBS-T
MITF	50-54	5% BSA	1:2000	Rabbit, 1:5000	5% BSA in TBS-T
AP-2a	48	5% BSA	1:3000	Rabbit, 1:8000	5% BSA in TBS-T
ERBB3	185	5% BSA	1:1000	Rabbit,1:5000	5% BSA in TBS-T
AKT	60	5% BSA	1:1000	Rabbit, 1:5000	5% BSA in TBS-T
pAKT XP	60	5% BSA	1:2000	Rabbit, 1:6000	5% BSA in TBS-T
ERK 1/2	44/42	5% BSA	1:3000	Rabbit, 1:7000	5% BSA in TBS-T
pERK 1/2	44/42	5% BSA	1:2000	Rabbit, 1:7000	5% BSA in TBS-T

Appendix C - Real time PCR data

Table C1: Primer sequences for qPCR

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
TBP	GCC-CGA-AAC-GCC-GAA-TAT	CGT-GGC-TCT-CTT-ATC-CTC-ATG-A
RPLPO	TCG-AAC-ACC-TGC-TGG-ATG-AC	CGC-TGC-TGA-ACA-TGC-TCA-AC
MITF-m	CAT-TGT-TAT-GCT-GGA-AAT-GCT-AGA	GCT-AAA-GTG-GTA-GAA-AGG-TAC-TGC
AP-2a	AGG-GGA-GAT-CGG-TCC-TGA	ACA-TGC-TCC-TGG-CTA-CAA-AAC
ERBB3	CTG-ATC-ACC-GGC-CTC-AAT	GGA-AGA-CAT-TGA-GCT-TCT-CTG-G
AP-2C	GGG-GCT-GTA-GAG-GTG-CTG	CGA-AGA-GGA-CTG-CGA-GGA
ERBB2	GGG-AAA-CCT-GGA-ACT-CAC-CT	CCC-TGC-ACC-TCC-TGG-ATA
MEK1	TTC-TAC-AGC-GAT-GGC-GAG-AT	TCC-AGC-TTT-CTT-CAG-GAC-TTG

Appendix D - MTS data

Figure D1: MTS abs values in absolute values

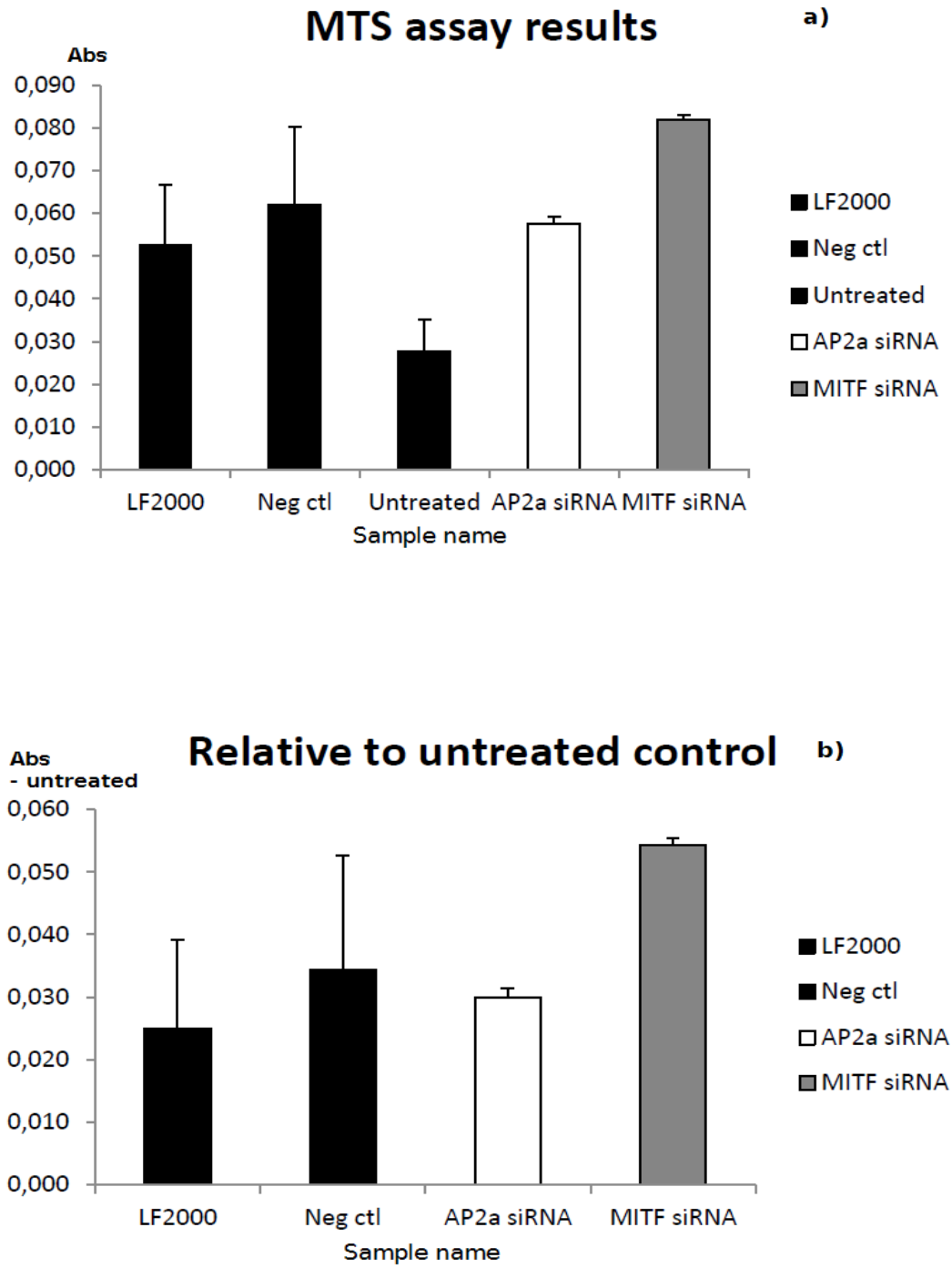

Figure D1: Absolute values of the 490 nm absorption observed in the MTS assay. D1a) shows raw values, D1b) shows values compared to the untreated sample.

Appendix E - Western blot supplementary data

Table E1: *Standard curve of reference for the calculation of protein concentrations*

Reference protein, µg	**Protein standard solution, µl**	**ddH_2O, µl**
0	0	10
1	0,66	9,33
2	1,32	8,68
3	1,98	8,02
4	2,34	7,66
5	3,00	7,0

The protein standard stock solution used in this project was BioRad Protein Standard, which has a concentration of 1,52 µg/µl.

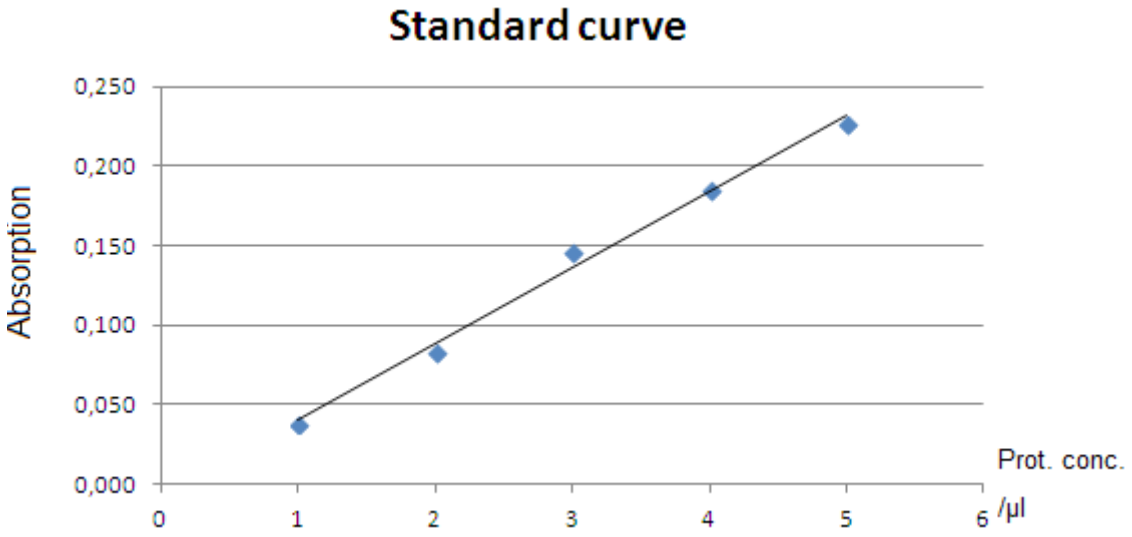

Figure E1: Example of a standard curve used for calculating protein concentration

Appendix F - qPCR-data

Measurements of relative expression in each individual sample, as well as the mean cycle threshold of each gene.

09.09.2014	Target	Sample	Ctrl	Expression	Mean Cq
PCI	AP2a	1-PS		0,5874	30,58
	AP2a	2-NC		0,50702	30,68
	AP2a	3-Light	*	1	30,04
	AP2a	4-AP2a		0,05284	33,86
	AP2a	5-MITFm		0,30531	31,39
	AP2a	Untreated		1,36754	29,44
	MITF-m	1-PS		0,2826	22,63
	MITF-m	2-NC		0,1813	23,16
	MITF-m	3-Light	*	1	21,03
	MITF-m	4-AP2a		0,15421	23,31
	MITF-m	5-MITFm		0,04852	25,04
	MITF-m	Untreated		3,09493	19,25
	RPLPO	1-PS		N/A	17,31
	RPLPO	2-NC		N/A	17,19
	RPLPO	3-Light	*	N/A	17,4
	RPLPO	4-AP2a		N/A	17,06
	RPLPO	5-MITFm		N/A	17,07
	RPLPO	Untreated		N/A	17,04
	TBP	1-PS		N/A	24,22
	TBP	2-NC		N/A	24,12
	TBP	3-Light	*	N/A	24,58
	TBP	4-AP2a		N/A	24,08
	TBP	5-MITFm		N/A	24,2
	TBP	Untreated		N/A	24,64

16.09.2014	Target	Sample	Ctrl	Expression	Mean Cq
PCI	AP2a	Untreated		3,9793	28,6
	AP2a	1-PS		0,64851	30,25
	AP2a	2-NC		0,43692	30,51
	AP2a	3-Light	*	1	29,45
	AP2a	4-AP2a		0,04624	33,89
	AP2a	5-MITFm		0,33958	31,13
	MITFm	Untreated		6,97348	19,19
	MITFm	1-PS		0,31865	22,68
	MITFm	2-NC		0,18467	23,15
	MITFm	3-Light	*	1	20,86
	MITFm	4-AP2a		0,24435	22,89
	MITFm	5-MITFm		0,05076	25,27
	RPLPO	Untreated		N/A	17,12
	RPLPO	1-PS		N/A	17,49
	RPLPO	2-NC		N/A	17,18
	RPLPO	3-Light	*	N/A	17,18
	RPLPO	4-AP2a		N/A	17,11
	RPLPO	5-MITFm		N/A	17,23
	TBP	Untreated		N/A	26,55
	TBP	1-PS		N/A	24,25
	TBP	2-NC		N/A	23,95
	TBP	3-Light	*	N/A	24,22
	TBP	4-AP2a		N/A	24,29
	TBP	5-MITFm		N/A	24,4

01.09.2014	Target	Sample	Ctrl	Expression	Mean Cq
PCI	AP2a	4-AP2a		0,06824	34,68
	AP2a	3-Light	*	1	31,2
	AP2a	5-MITFm		0,50416	31,72
	AP2a	2-NC		0,76784	31,16
	AP2a	Untreated		1,41728	30,27
	MITF-m	AP2		0,44192	23,02
	MITF-m	K-minus	*	1	22,24
	MITF-m	MITF-m		0,15337	24,48
	MITF-m	NegControl		0,44066	23
	MITF-m	UtenLys		1,22669	21,52
	RPLPO	AP2		N/A	17,27
	RPLPO	K-minus	*	N/A	17,38
	RPLPO	MITF-m		N/A	17,21
	RPLPO	NegControl		N/A	17,32
	RPLPO	UtenLys		N/A	17,23
	TBP	AP2		N/A	25,18
	TBP	K-minus	*	N/A	25,87
	TBP	MITF-m		N/A	25,1
	TBP	NegControl		N/A	25,08
	TBP	UtenLys		N/A	25,16

PS-only control lacking

05.09.2014	Target	Sample	Ctrl	Expression	Mean Cq
PCI	AP2a	Untreated		1,35309	29,94
	AP2a	1-PS		0,46192	31,4
	AP2a	2-NC		0,51602	31,12
	AP2a	3-Light	*	1	30,46
	AP2a	4-AP2a		0,04408	34,55
	AP2a	5-MITFm		0,39531	31,57
	MITF-m	Untreated		3,12294	19,32
	MITF-m	1-PS		0,27867	22,72
	MITF-m	2-NC		0,18119	23,22
	MITF-m	3-Light	*	1	21,05
	MITF-m	4-AP2a		0,16092	23,27
	RPLPO	Untreated		N/A	16,96
	RPLPO	1-PS		N/A	17,25
	RPLPO	2-NC		N/A	17,08
	RPLPO	3-Light	*	N/A	17,31
	RPLPO	4-AP2a		N/A	16,94
	RPLPO	5-MITFm		N/A	17,07
	TBP	Untreated		N/A	24,56
	TBP	1-PS		N/A	24,11
	TBP	2-NC		N/A	24,03
	TBP	3-Light	*	N/A	24,39
	TBP	4-AP2a		N/A	23,92
	TBP	5-MITFm		N/A	24,17

MITF-levels of MITF-transfected sample lacking

16.09.2014	Target	Sample	Ctrl	Expression	Mean Cq
LF2000	AP2a	4-AP2a		0,11495	30,98
24t	AP2a	5-MITFm		0,17166	30,43
	AP2a	1-LF2000		0,60153	28,49
	AP2a	2-NC		0,69665	28,06
	AP2a	3-Untreated	*	1	27,59
	MITFm	10-AP2a		0,6451	19,4
	MITFm	11-MITFm		0,15224	21,51
	MITFm	7-k+		0,68863	19,2
	MITFm	8-NC		0,68951	18,98
	MITFm	9-k-	*	1	18,49
	RPLPO	10-AP2a		N/A	16,96
	RPLPO	11-MITFm		N/A	16,98
	RPLPO	7-k+		N/A	17,15
	RPLPO	8-NC		N/A	16,81
	RPLPO	9-k-	*	N/A	16,82
	TBP	10-AP2a		N/A	24,8
	TBP	11-MITFm		N/A	24,85
	TBP	7-k+		N/A	24,4
	TBP	8-NC		N/A	24,3
	TBP	9-k-	*	N/A	24,4

14.01.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP2a	4-AP2a		0,49066	22,53
24t	AP2a	3-Untreated	*	1	21,42
	AP2a	1-LF2000		0,69004	22,15
	AP2a	5-MITFm		0,62721	22,11
	AP2a	2-NC		0,66236	22,18
	MITF	4-AP2a		0,87093	20,96
	MITF	3-Untreated	*	1	20,67
	MITF	1-LF2000		0,69097	21,4
	MITF	5-MITFm		0,40747	21,98
	MITF	2-NC		0,57744	21,63
	RPLPO	4-AP2a		N/A	17,73
	RPLPO	3-Untreated	*	N/A	17,68
	RPLPO	1-LF2000		N/A	17,67
	RPLPO	5-MITFm		N/A	17,39
	RPLPO	2-NC		N/A	17,7
	TBP	4-AP2a		N/A	24,95
	TBP	3-Untreated	*	N/A	24,83
	TBP	1-LF2000		N/A	25,21
	TBP	5-MITFm		N/A	25,15
	TBP	2-NC		N/A	25,13

21.05.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF2000	AP2a	1-LF2000		1,01214	21,59
24t	AP2a	2-NC		1,09518	21,45
	AP2a	3-Untreated	*	1	21,59
	AP2a	4-AP2a		0,2069	24,04
	AP2a	5-MITFm		0,5167	23,04
	MITFm	1-LF2000		0,9532	20,97
	MITFm	2-NC		0,86723	21,07
	MITFm	3-Untreated	*	1	20,88
	MITFm	4-AP2a		1,49321	20,48
	MITFm	5-MITFm		0,13245	24,29
	RPLPO	1-LF2000		N/A	17,61
	RPLPO	2-NC		N/A	17,48
	RPLPO	3-Untreated	*	N/A	17,53
	RPLPO	4-AP2a		N/A	17,65
	RPLPO	5-MITFm		N/A	17,76
	TBP	1-LF2000		N/A	24,52
	TBP	2-NC		N/A	24,59
	TBP	3-Untreated	*	N/A	24,56
	TBP	4-AP2a		N/A	24,8
	TBP	5-MITFm		N/A	25,32

09.05.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2C	1-K+		1,12707	24,33
24t	AP-2C	2-NC		0,93805	24,98
Sekundærgener	AP-2C	3-K-	*	1	24,23
	AP-2C	4-AP2a		1,26427	24,12
	AP-2C	MITF-m		1,57032	23,35
	ERBB2	1-K+		1,28583	26,16
	ERBB2	2-NC		0,98617	26,93
	ERBB2	3-K-	*	1	26,25
	ERBB2	4-AP2a		1,44649	25,94
	ERBB2	MITF-m		1,00218	26,01
	ERBB3	1-K+		1,31467	23,35
	ERBB3	2-NC		1,12771	23,96
	ERBB3	3-K-	*	1	23,47
	ERBB3	4-AP2a		1,3244	23,29
	ERBB3	MITF-m		2,1261	22,15
	RPLPO	1-K+		N/A	18,82
	RPLPO	2-NC		N/A	19,03
	RPLPO	3-K-	*	N/A	18,58
	RPLPO	4-AP2a		N/A	18,69
	RPLPO	MITF-m		N/A	18,1
	TBP	1-K+		N/A	25,93
	TBP	2-NC		N/A	26,49
	TBP	3-K-	*	N/A	25,63
	TBP	4-AP2a		N/A	25,97
	TBP	MITF-m		N/A	25,65

28.04.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2C	1-K+		1,29496	24,21
24t	AP-2C	2-NC		1,02767	24,84
Sek	AP-2C	3-K-	*	1	24,2
	AP-2C	4-AP2a		1,37444	24
	AP-2C	5-MITF-m		1,8135	23,15
	ERBB2	1-K+		1,37975	26,15
	ERBB2	2-NC		0,98228	26,94
	ERBB2	3-K-	*	1	26,22
	ERBB2	4-AP2a		1,50129	25,9
	ERBB2	5-MITF-m		1,21667	25,75
	ERBB3	1-K+		1,35197	23,34
	ERBB3	2-NC		1,14029	23,88
	ERBB3	3-K-	*	1	23,38
	ERBB3	4-AP2a		1,27132	23,3
	ERBB3	5-MITF-m		2,02063	22,18
	RPLPO	1-K+		N/A	18,72
	RPLPO	2-NC		N/A	18,97
	RPLPO	3-K-	*	N/A	18,42
	RPLPO	4-AP2a		N/A	18,71
	RPLPO	5-MITF-m		N/A	18,03
	TBP	1-K+		N/A	26,01
	TBP	2-NC		N/A	26,35
	TBP	3-K-	*	N/A	25,53
	TBP	4-AP2a		N/A	25,77
	TBP	5-MITF-m		N/A	25,54

24.04.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2C	1-K+		0,99191	22,96
24t	AP-2C	2-NC		0,8759	23,51
Sek	AP-2C	3-K-	*	1	23,22
	AP-2C	4-AP2a		0,78801	23,12
	AP-2C	5-MITF-m		1,10911	22,48
	ERBB2	1-K+		0,97636	25,12
	ERBB2	2-NC		0,87612	25,65
	ERBB2	3-K-	*	1	25,36
	ERBB2	4-AP2a		0,71501	25,4
	ERBB2	5-MITF-m		0,8089	25,07
	ERBB3	1-K+		1,08741	22,93
	ERBB3	2-NC		1,03617	23,37
	ERBB3	3-K-	*	1	23,33
	ERBB3	4-AP2a		1,14971	22,68
	ERBB3	5-MITF-m		2,47237	21,43
	RPLPO	1-K+		N/A	17,76
	RPLPO	2-NC		N/A	18,15
	RPLPO	3-K-	*	N/A	18,06
	RPLPO	4-AP2a		N/A	17,45
	RPLPO	5-MITF-m		N/A	17,41
	TBP	1-K+		N/A	24,88
	TBP	2-NC		N/A	25,23
	TBP	3-K-	*	N/A	25,13
	TBP	4-AP2a		N/A	24,84
	TBP	5-MITF-m		N/A	24,59

27.12.2014	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP2a	4-AP2a		0,40504	31,71
72t	AP2a	3-Untreated	*	1	30,37
Primær	AP2a	1-LF2000		1,09019	30,91
	AP2a	5-MITFm		0,82	30,88
	AP2a	2-NC		0,86389	30,61
	MITFm	4-AP2a		1,16794	20,74
	MITFm	3-Untreated	*	1	20,94
	MITFm	1-LF2000		0,96454	21,65
	MITFm	5-MITFm		0,54328	22,04
	MITFm	2-NC		1,0049	20,95
	RPLPO	4-AP2a		N/A	17,21
	RPLPO	3-Untreated	*	N/A	17,09
	RPLPO	1-LF2000		N/A	17,87
	RPLPO	5-MITFm		N/A	17,2
	RPLPO	2-NC		N/A	17,16
	TBP	4-AP2a		N/A	24,46
	TBP	3-Untreated	*	N/A	24,51
	TBP	1-LF2000		N/A	25,05
	TBP	5-MITFm		N/A	24,84
	TBP	2-NC		N/A	24,49

14.06.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP2a	1-K+		1,22025	21,85
72t	AP2a	2-NC		1,09561	21,9
Primær	AP2a	3-K-	*	1	22,14
	AP2a	4-AP2a		0,81675	22,46
	AP2a	5-MITF		0,99666	22,06
	MITF	1-K+		1,2547	20,7
	MITF	2-NC		1,08918	20,8
	MITF	3-K-	*	1	21,03
	MITF	4-AP2a		1,43351	20,55
	MITF	5-MITF		0,6296	21,61
	RPLPO	1-K+		N/A	17,5
	RPLPO	2-NC		N/A	17,27
	RPLPO	3-K-	*	N/A	17,26
	RPLPO	4-AP2a		N/A	17,4
	RPLPO	5-MITF		N/A	17,3
	TBP	1-K+		N/A	25,13
	TBP	2-NC		N/A	25,16
	TBP	3-K-	*	N/A	25,38
	TBP	4-AP2a		N/A	25,3
	TBP	5-MITF		N/A	25,16

28.07.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP2A	4-AP2a		0,39776	22,55
72t	AP2A	5-MITFm		1,14874	21,26
Prim+sek	AP2A	1-LF2000		0,82864	21,24
	AP2A	2-NC		0,85423	21,73
	AP2A	3-Untreated	*	1	22,15
	AP2C	4-AP2a		0,62269	23,51
	AP2C	5-MITFm		1,23197	22,77
	AP2C	1-LF2000		0,78101	22,94
	AP2C	2-NC		0,86361	23,33
	AP2C	3-Untreated	*	1	23,76
	ERBB3	4-AP2a		0,49789	23,78
	ERBB3	5-MITFm		2,09182	21,95
	ERBB3	1-LF2000		0,69803	23,05
	ERBB3	2-NC		0,99413	23,07
	ERBB3	3-Untreated	*	1	23,71
	mitf	4-AP2a		0,77991	19,56
	mitf	5-MITFm		0,52585	20,37
	mitf	1-LF2000		0,86734	19,16
	mitf	2-NC		0,77857	19,85
	mitf	3-Untreated	*	1	20,13
	RPLPO	4-AP2a		N/A	16,21
	RPLPO	5-MITFm		N/A	16,5
	RPLPO	1-LF2000		N/A	16,05
	RPLPO	2-NC		N/A	16,55
	RPLPO	3-Untreated	*	N/A	17,18
	TBP	4-AP2a		N/A	23,86
	TBP	5-MITFm		N/A	24,05
	TBP	1-LF2000		N/A	23,52
	TBP	2-NC		N/A	24,1
	TBP	3-Untreated	*	N/A	24,75

13.08.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2C	1-K+		0,84077	25,41
72t	AP-2C	2-NC		1,2059	24,38
sek. gener	AP-2C	3-K-	*	1	24,65
	AP-2C	4-AP2a		1,13757	24,32
	AP-2C	5-MITF		1,52623	24,01
	ERBB3	1-K+		0,77397	24,64
	ERBB3	2-NC		1,06739	23,66
	ERBB3	3-K-	*	1	23,76
	ERBB3	4-AP2a		0,94721	23,69
	ERBB3	5-MITF		2,36922	22,49
	RPLPO	1-K+		N/A	18,44
	RPLPO	2-NC		N/A	18,28
	RPLPO	3-K-	*	N/A	18,12
	RPLPO	4-AP2a		N/A	18,14
	RPLPO	5-MITF		N/A	18,24
	TBP	1-K+		N/A	26,21
	TBP	2-NC		N/A	25,33
	TBP	3-K-	*	N/A	25,5
	TBP	4-AP2a		N/A	25,19
	TBP	5-MITF		N/A	25,32

09.08.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2c	1-K+		1,07371	24,54
72t	AP-2c	2-NC		1,23468	24,36
sek gener	AP-2c	3-K-	*	1	24,49
	AP-2c	4-AP-2a		1,22803	24,07
	AP-2c	5-MITF		1,5073	23,79
	ERBB3	1-K+		0,65836	24,17
	ERBB3	2-NC		1,10326	23,45
	ERBB3	3-K-	*	1	23,42
	ERBB3	4-AP-2a		0,96503	23,34
	ERBB3	5-MITF		1,86046	22,42
	RPLPO	1-K+		N/A	18,22
	RPLPO	2-NC		N/A	18,32
	RPLPO	3-K-	*	N/A	18,11
	RPLPO	4-AP-2a		N/A	18,14
	RPLPO	5-MITF		N/A	18
	TBP	1-K+		N/A	25,42
	TBP	2-NC		N/A	25,38
	TBP	3-K-	*	N/A	25,24
	TBP	4-AP-2a		N/A	24,96
	TBP	5-MITF		N/A	25,13

11.05.2015	Target	Sample	Ctrl	Expression	Mean Cq
LF	AP-2C	1-K+		0,7559	23,16
24t	AP-2C	2-NC		0,66796	23,82
Sek	AP-2C	3-K-	*	1	23,26
	AP-2C	4-AP2a		0,70673	23,26
	AP-2C	MITF-m		1,05339	22,63
	ERBB2	1-K+		0,79992	25,3
	ERBB2	2-NC		0,78482	25,81
	ERBB2	3-K-	*	1	25,48
	ERBB2	4-AP2a		0,80118	25,3
	ERBB2	MITF-m		0,81683	25,21
	ERBB3	1-K+		0,87233	22,94
	ERBB3	2-NC		0,93159	23,32
	ERBB3	3-K-	*	1	23,24
	ERBB3	4-AP2a		1,00154	22,74
	ERBB3	MITF-m		2,26531	21,5
	RPLPO	1-K+		N/A	17,73
	RPLPO	2-NC		N/A	18,16
	RPLPO	3-K-	*	N/A	18,33
	RPLPO	4-AP2a		N/A	17,6
	RPLPO	MITF-m		N/A	17,58
	TBP	1-K+		N/A	24,81
	TBP	2-NC		N/A	25,34
	TBP	3-K-	*	N/A	25,21
	TBP	4-AP2a		N/A	24,93
	TBP	MITF-m		N/A	24,83