Ghent University
Faculty of Medicine and Health Sciences
Department of Dermatology

Revealing the role of miR-145 as a key player in melanogenesis and melanoma

Peter Dynoodt
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This thesis is submitted as fulfilment of the requirements for the degree of
DOCTOR IN MEDICAL SCIENCES

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Co-promotor: Dr. Mireille Van Gele

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LIST OF ABBREVIATIONS

3'UTR	3'untranslated region
AD	atopic dermatitis
AC	adenylate cyclase
ACTH	adrenocorticotropic
APC	adenomatous polyposis coli
AS	anti-sense
BCC	basal cell carcinoma
bHLH-Lz	basic helix-loop-helix and leucine zipper
cAMP	cyclic adenosine monophosphate
CBP	CREB binding protein
CCND1	cyclin D1
cDNA	copy DNA
CKIα	casein kinase Iα
cKO	conditional knockout
c-Raf	cellular Raf
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
DCT	dopachrome tautomerase
DCR-1	Dicer1
DHI	dihydroxyindole
DHICA	DHI carboxylic acid
DP	dermal papilla
DRU	Dermatology Research Unit
DT	delayed tanning
endo-siRNA	endogenous small-interfering RNA
GDP	guanine diphosphate
GS	Griscelli syndrome
GSK3β	glycogen synthase kinase-3β
GTP	guanine triphosphate
HF	hair follicles
IPD	immediate pigment darkening
IRAK	IL-1R-associated kinase

L-DOPA	3,4-L-dihydroxyphenylalanine
Lef	lymphoid enhancer factor
lncRNAs	long noncoding RNAs
MAP3K	MAP kinase kinase
MAPK	mitogen activated protein kinase
MC1R	melanocortin-1-receptor
MGF	mast cell growth factor
miRNA	microRNA
miRISC	miRNA induced silencing complex
MITF	microphthalmia-associated transcription factor
MLPH	melanophilin
MRE	miRNA recognition element
MYO5A	myosin Va
ncRNAs	non-protein-coding RNAs
nm	nanometer
ORF	open reading frame
PARs	promoter-associated RNAs
piRNAs	PIWI interacting RNAs
PKA	protein kinase A
POMC	pro-opiomelanocortin
pre-miRNA	precursor miRNA
RA	rheumatoid arthritis
RNA	RiboNucleic Acid
RNAi	RNA interference
RT	reverse transcriptase
RTK	receptor tyrosine kinase
RUNX3	runt-related transcription factor 3
SCC	squamous cell carcinoma
SCF	stem cell factor
snoRNAs	small nucleolar RNAs
SOCS-3	suppressor of cytokine signaling-3
SOS	son of sevenless
SOX	SYR-related HMG-box
ssUV	solar simulated UV

STAT3	signal transducer and activator of the transcription 3
Tcf	T-cell factor
TNF-α	tumor necrosis factor-alpha
TRAF6	TNF receptor-associated factor 6
T-UCR	transcribed ultraconserved region
TYR	tyrosinase
TYRP1	tyrosine-related protein 1
TYRP2	tyrosine-related protein 2
UV-A/B/C	ultraviolet-A/B/C
UVR	ultraviolet radiation
qPCR	quantitative PCR
XPO5	exportin-5
α-MSH	alpha-melanocyte stimulating hormone

DANKWOORD

At the end of the road only one person ends up receiving a title. The work behind this was in fact only accomplished with the help of a variety of individuals. Through the confluence of each other's histories and interactions, I feel something very special has happened along this particular path. The way the science was performed and the observations we uncovered on how certain processes work, is so amazing. I feel very fortunate to have played a part during all of this and to have worked with such a fine group of people.

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Thank you for all your support, I shall always wear it.

I dedicate this work to Hendrik De Backer. A dear friend who had to part ways much too early.

CHAPTER 1

INTRODUCTION

1.1 miRNAs

The initial perception, that essential cellular processes are regulated by genetic information, which is translated into functional proteins, was quite straightforward. This conventional view was centered around protein-coding genes, through the common conviction of DNA transcribing into mRNA, conclusively translated into proteins. These points of view had to be adjusted since the discovery of miRNAs, and we have come to realize that this process is a lot more complex than initially perceived. Evidence accumulated from copious amounts of data from high-throughput genomic platforms, led to the conclusion that the complexity of any organism lies mainly in the expansion of the regulatory potential of the non-coding portions of the genome during evolution. These regions were initially perceived as worthless and were therefore termed, junk DNA (Mattick, 2004).

1.1.1 The role of noncoding RNAs

It was generally understood that protein encoding genes encompass the majority of transcribed data required for proper cell function. This was shown to be incorrect, and we have come to realize that the majority of transcribed data are reserved for regulatory non-protein-coding RNAs (ncRNAs). These ncRNAs are classified by size and function, extending from microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), endogenous small-interfering RNAs (endo-siRNAs), PIWI interacting RNAs (piRNAs), promoter-associated RNAs (PARs) and the longer transcribed ultraconserved regions (T-UCRs), and long non-coding RNAs (lncRNAs) (Bartel, 2004; Lagos-Quintana et al., 2001; Taft et al., 2010). See Table 1 for an overview.

Table 1. Overview classes of non-coding RNAs in mammals

NcRNA class	Characteristics
Established ncRNA classes	
Long (regulatory) non-coding RNAs (lncRNAs)	The broadest class, lncRNAs, encompass all non-protein-coding RNA species >~200 nt, including mRNA-like ncRNAs. Their functions include epigenetic regulation, acting as sequence-specific tethers for protein complexes and specifying subcellular compartments or localization
Small interfering RNAs (siRNAs)	Small RNAs ~21–22 nt long, produced by Dicer cleavage of complementary dsRNA duplexes. siRNAs form complexes with Argonaute proteins and are involved in gene regulation, transposon control and viral defence
microRNAs (miRNAs)	Small RNAs ~22 nt long, produced by Dicer cleavage of imperfect RNA hairpins encoded in long primary transcripts or short introns. They associate with Argonaute proteins and are primarily involved in post-transcriptional gene regulation
PIWI-interacting RNAs (piRNAs)	Dicer-independent small RNAs ~26–30 nt long, principally restricted to the germline and somatic cells bordering the germline. They associate with PIWI-clade Argonaute proteins and regulate transposon activity and chromatin state
Promoter-associated RNAs (PARs)	A general term encompassing a suite of long and short RNAs, including promoter-associated RNAs (PASRs) and transcription initiation RNAs (tiRNAs) that overlap promoters and TSSs. These transcripts may regulate gene expression
Small nucleolar RNAs (snoRNAs)	Traditionally viewed as guides of rRNA methylation and pseudouridylation. However, there is emerging evidence that they also have gene-regulatory roles
Other recently described classes	
X-inactivation RNAs (xiRNAs)	Dicer-dependent small RNAs processed from duplexes of two lncRNAs, Xist and Tsix, which are responsible for X-chromosome inactivation in placental mammals
Sno-derived RNAs (sdRNAs)	Small RNAs, some of which are Dicer-dependent, which are processed from small nucleolar RNAs (snoRNAs). Some sdRNAs have been shown to function as miRNA-like regulators of translation
microRNA-offset RNAs (moRNAs)	Small RNAs ~20 nt long, derived from the regions adjacent to pre-miRNAs. Their function is unknown
tRNA-derived RNAs	tRNAs can be processed into small RNA species by a conserved RNase (angiogenin). They are able to induce translational repression
MSY2-associated RNAs (MSY-RNAs)	MSY-RNAs are associated with the germ cell-specific DNA/RNA binding protein MSY2. Like piRNAs, they are largely restricted to the germline and are ~26–30 nt long. Their function is unknown
Telomere small RNAs (tel-sRNAs)	Dicer-independent ~24 nt RNAs principally derived from the G-rich strand of telomeric repeats. May have a role in telomere maintenance
Centrosome-associated RNAs (crasiRNAs)	A class of ~34–42 nt small RNAs, derived from centrosomes that show evidence of guiding local chromatin modifications

The diversity of noncoding RNAs with structural, enzymatic and regulatory functions in cells is extensive. Currently over 1000 human miRNAs have been discovered, making them one of the prominent gene regulators. A repository of miRNAs and miRNA genes from many organisms is available at the miRBase Sequence Database (http://microrna.sanger.ac.uk/sequences), a searchable database of published miRNA sequences and annotations.

MiRNAs are small RNAs, averaging from 20-23 nucleotides in length, performing diverse biological functions, often in a tissue-specific manner. They function by guiding sequence-specific gene silencing at the transcriptional and/or post-transcriptional level (Bartel, 2004). In particular, these natural RNA-silencing processes can be harnessed to induce gene-specific silencing through the supply of non-natural RNA precursors or mimics of natural, small RNA processing intermediates. This approach, known as RNA interference (RNAi), is widely used for the systematic analysis of gene function, and its potential therapeutic applications are currently under intense investigation. However in order to efficiently harness the machinery of RNAi, it is essential to discover how the different types of small RNA molecules are generated.

1.1.2 miRNA Biogenesis

Most miRNAs were found to be transcribed at relatively large distances from coding regions. Therefore, they are likely to be transcribed as independent transcriptional units. On the other hand, some miRNA genes were found to be transcribed within introns of coding regions, and are orientated in the same direction, enabling them to be transcribed simultaneously without the need of a personal promoter. Some miRNAs are located in clusters, and are transcribed as one large multi-cistronic primary transcript, that is further processed into mature miRNAs. The mechanism by which miRNA biogenesis is evolutionarily conserved involves several sequential downprocessing steps performed by endonucleolytic cleavages (Lagos-Quintana et al., 2001; Lau et al., 2001).

MiRNAs are initially transcribed in the nucleus by a RNA polymerase-II or RNA polymerase-III, forming large primary transcripts called pri-miRNAs (500-3000 nucleotides) (Figure 1) (Cai et al., 2004; Lee et al., 2004). They are assembled and processed by different endonucleases. Further processing and maturation of this pri-miR takes place in two sequential steps, which are initially processed in the nucleus, and subsequently exported into the cytoplasm. First, the pri-miR is cleaved by Drosha/DGCR8 into an approximately 60-80 nucleotides long structure, and processed into a smaller precursor miRNA (pre-miRNA), that is typically characterized by a hairpin structure. This hairpin structure is exported out of the nucleus by exportin-5 (XPO5), into the cytoplasm of the cell. The pre-miRNA is then cleaved by Dicer1 (DCR-1) into a small imperfect double stranded miRNA-miRNA* duplex, that

contains both mature miRNA and its complementary strand miRNA*, with an average length of 22 nucleotides. The mature miRNA is incorporated into the miRNA Induced Silencing Complex (miRISC), while the complementary strand is degraded (Bartel, 2004; Lau et al., 2001).

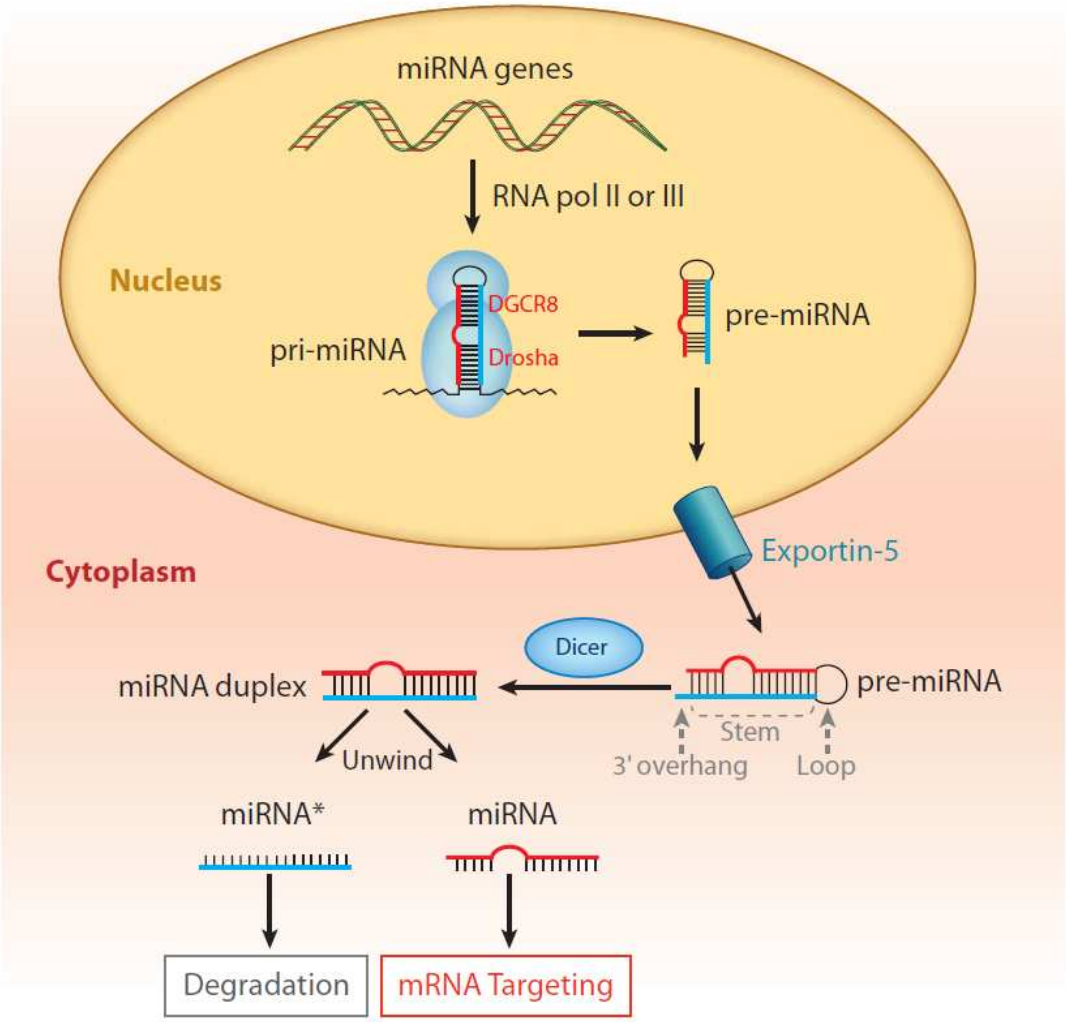

Figure 1. *MiRNA biogenesis.* Pri-miRNA (a 60-80 nucleotide hairpin stem-loop) is transcribed from the miRNA gene by RNA polymerase II or III and then cleaved by Drosha/DGCR8 complex to generate a pre-miRNA (with a 22-bp stem and a 2-nucleotide 3' overhang) in the nucleus. After being exported from the nucleus to the cytoplasm by Exportin-5, pre-miRNA is further cleaved by Dicer to remove the terminal loop. After unwinding, one strand of miRNA acts as the functional guide strand and binds to the target mRNA. The complementary strand (i.e., mRNA*) is rapidly degraded (Sun et al., 2010).

1.1.2.1 Working Mechanism

Once the mature miRNA is loaded into the miRISC, it functions as a guide that directs the miRISC to the complementary sites of the 3'Untranslated Region (3'UTR) transcript sequences, in order to regulate gene expression. The miRISC can regulate target genes in two ways, depending on the degree of complementarity between the miRNA and the 3'UTR of the target mRNA (Figure 2).

In plants these complementarities have to be very specific, meaning that the degree of complementarity has to be exact. This ultimately leads to the degradation of the target sequence. In animals, miRNAs can exercise their mechanism through two different paths depending on the degree of complementarity between miRNA and its target sequence. Translational repression occurs when partial complementarity is established between a miRNA and its 3'UTR of the target sequence. On the other hand, when total complementarity is found, degradation of the mRNA sequence takes place.

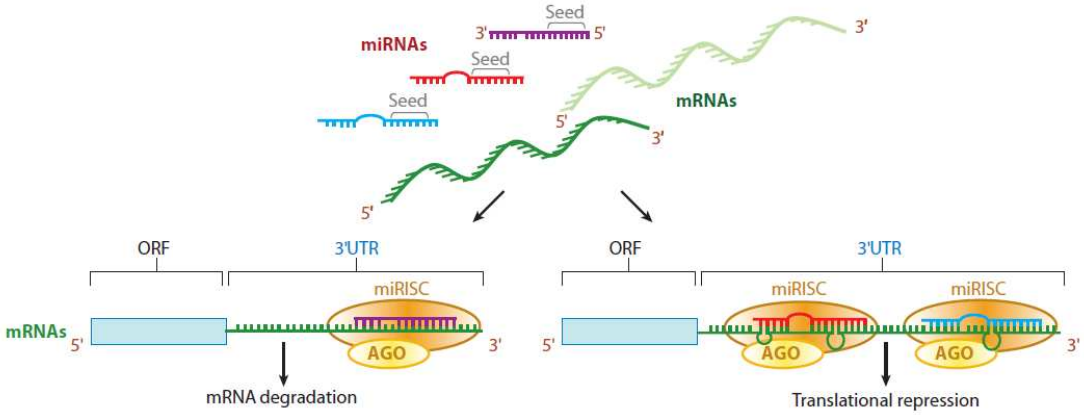

Figure 2. *Mechanisms of functional miRNA targeting.* The 7-nucleotide seed region, which starts at the second nucleotide from the 5' end of the miRNA, is required for miRNA-mRNA interaction. The miRNA-mRNA targeting occurs predominantly at the 3'UTR of the target mRNA, located at the 3' downstream of the ORF. Bulges or mismatches may be present in the middle part of miRNA-mRNA duplex. As shown in the oval on the lower left, perfect base pairing between miRNA and mRNA (mostly plant cells) results in miRISC-mediated endonucleolytic cleavage and hence mRNA degradation. Despite the lack of complete complementarity in animal cells shown in the lower right, base pairing (particularly the 13-16 nucleotides of miRNA) is still important in stabilizing the miRNA-mRNA interaction, which leads to translational repression. Frequently, multiple miRNAs *(red and blue)* can target the same 3'UTR. Conversely, a unique miRNA may target multiple binding sites on the same 3'UTR in animal cells (Sun et al., 2010).

Expert and experimental bioinformaticians have been able to indicate which specific sequences are crucial for the correct target recognition of the miRNA. This region is located at the proximal 5' region of the miRNA, and has been defined as the seed region. This seed sequence is located from the 2nd nucleotide to the 8th nucleotide of the miRNA, and has been shown to be important for biological function and stability. The region located at the 3'UTR of the target mRNA sequence that is complementary to the miRNA seed region, is called the miRNA recognition element (MRE). The MRE can display several degrees of complementarity towards the miRNA seed. When 6 nucleotides on the MRE (hexamer 6m) are complementary towards the seed region of a miRNA, it is considered to generate the least efficient miRNA-target recognition, and thereby least stable binding. Most target-miRNA recognition sites display this complementarity, but have an additional adenosine nucleotide located across from the 3'UTR end of the miRNA (7m-A1), or have an additional nucleotide located at the opposite 5' end (7m-8m). The most efficient miRNA-target interaction was established when both the adenosine at the 3'UTR, as well as the additional nucleotide located at the 5'UTR, are found on the MRE (Bartel, 2009; Sun et al., 2010).

Base pairing between the 3'UTR of the miRNA and the target mRNAs is not always essential for suppressing the target sequence, but if this does occur in this region, it can compensate for weaker seed binding and even increase the suppression. Above all, several MREs can be found on the 3'UTR of target sequences for one specific miRNA, or even several different miRNAs, leading to an intricate and complex manner in which mRNAs can be regulated. This can lead to either synergistic or antagonistic effects during repression of target genes. Finally, sequences surrounding the MRE and their accompanying secondary structures of the 3'UTR, can influence the access of the miRISC to the target region, whereby improper folding of these RNA regions can cause inefficient binding of the miRNA to its target 3'UTR (Liu et al., 2008).

1.1.2.2. Predictions of miRNA target genes

Bioinformatic algorithms have been designed to track down potential targets of miRNAs, and predicted that a single miRNA can influence up to 200 genes (Esquela-Kerscher and Slack, 2006). Other estimations predict that 60% of all protein coding genes are influenced by miRNAs (Friedman et al., 2009). It has been postulated that a large number of genetic pathways are influenced by miRNAs, and that deregulation of miRNA expression could lead to aberrant cell metabolism and disease, including cancer. Till date only a fraction of these predictions have been experimentally confirmed but the exponential rise in miRNA publications might increase confirmations, differentiating the correct predictions from the eratic ones.

The identification of miRNA target genes in plants has proven to be quite straightforward, since these genes need strict complementarity between miRNA and 3'UTR of target genes. This exact complementarity is rarely found in animals, making this approach for finding potential targets almost impossible. The first generation bio-informatic algorithms designed to find potential miRNA target genes, used the complementarity of the seed sequence as the foremost criteria for selecting target genes. This gave rise to a substantial amount of positive hits. Additional methods to enhance the successful prediction analysis were obtained by observing conserved MREs between different species. In this manner, selecting for these conserved sequences between species, an increase of positive hits was obtained (Bartel, 2009).

Even though there has been a progressive increase of experimental validation of miRNA target genes, a majority of miRNA targets still remain unknown. Till date the prediction algorithms remain the sole source for quickly screening a massive amount of genomic data. Such *in silico* analyses have the potential to find *bona fide* miRNA target genes, and have greatly improved the quality of prediction data (Chi et al., 2009; Tan et al., 2009).

1.1.3 Global importance of miRNAs in skin morphogenesis

Skin morphogenesis is the biological process that accounts for the development, cell growth, and cellular differentiation of the largest organ on the human body. According to our genetic "blueprint", this results in establishing the form and structure of a protective barrier that protects the body against environmental hazards, and prevents the loss of essential body fluids.

The initial attempts to determine the significance of miRNA regulation in mammalian skin were aimed at targeting critical components of miRNA biogenesis. This was achieved by ablating a crucial gene that encodes the miRNA-processing enzyme Dicer, thus leading to a global depletion of all mature miRNAs. However, *Dicer* knockout is accompanied by embryonic lethality, and the embryo is rejected around embryonic day 7.5 (E7.5), which is prior to the initiation of skin development. To bypass this problem Cre-Lox recombination technology was employed creating a conditional knockout (cKO) of *Dicer*, which involves splicing out the *Dicer* sequence driven by a *keratin-14* specific promoter (Andl et al., 2006; Yi et al., 2006). Thus, it became possible to remove *Dicer* from embryonic skin stem/progenitor cells starting around E13.5, when the *keratin-14* specific promoter becomes active. Due to the long half life of miRNAs, mature miRNAs are not completely depleted until E17.5, which then enabled scientists to monitor and examine the consequences of complete loss of miRNAs during skin development (Yi and Fuchs, 2009). Additionally, the depletion of miRNAs was only achieved after the formation of the first suprabasal layer in the epidermis between E13 and E15, including the first wave of hair morphogenesis. Mice with the epidermal specific deletion of *Dicer* cKO gave birth to pups indistinguishable from control littermates. However, shortly after birth *Dicer* cKO pups begin to lose weight and neonatally die without developing a hair coat (Andl et al., 2006; Yi et al., 2006). Histological examination of these *Dicer* cKO pups revealed hair follicles (HF) that were underdeveloped, misaligned, and showed increased apoptosis. They also displayed a striking appearance of HFs that evaginate upwards instead of invaginating into the dermis, resulting in the formation of hair cysts. These evaginating HFs attract dermal papilla (DP), which migrate with them, maintaining the physical interaction and communication between HFs and DPs. The inability to maintain normal HFs located at the epithelial side of the epithelial-mesenchymal communication, suggests that these defects originate from deregulation of miRNA gene expression caused by loss of function of *Dicer*. Correspondingly, when examining identical

Dicer cKO in other stratified epithelial tissues such as in the tongue and footpad, similar defects were observed during morphogenesis, suggesting that miRNAs are critical for maintaining the correct morphogenesis of stratified epithelial tissues. In a following study a cKO was achieved through a mutation of another essential cofactor of miRNA processing, *DGCR8*. Identical phenotypes were observed such as those in *Dicer* cKO, including the evaginating HFs, enriched apoptosis in hair bulbs, rough and dehydrated skin and neonatal lethality (Yi et al., 2009). *DGCR8* is known to be essential for the processing of stereotypical miRNAs, whereas *Dicer* was reported to be required for the processing of several classes of small RNAs, including miRNAs and miRNA-derived siRNAs. When analyzing deep sequencing reads of the different classes of small RNAs in *Dicer* cKO and *DGCR8* cKO skins, the production of stereotypical miRNAs was shown to be equivalent in *Dicer* and *DGCR8*. However, the production of a few hairpin miRNAs and miRNA-derived siRNAs is only dependent on *Dicer* and not *DGCR8* processing. This demonstrates more clearly that the acquired *Dicer* cKO phenotypes are indeed the result of loss of function of miRNAs in the skin and that the stereotypical miRNA are the most abundant Dicer products in the skin.

These studies involving *Dicer* and *DGCR8* cKOs in skin development attributed and accentuated the global importance that miRNAs play during skin morphogenesis, maintenance of HFs, epidermal proliferation and apoptosis. Clearly, the next step was to determine which individual miRNA, or which miRNA families, were responsible for these abnormalities during skin morphogenesis.

The skin specific miR-203 is involved in the transition of epidermal cell proliferation from the basal layer into keratinocytes, and the upper stratified layers. During epithelial stratification, which takes place between embryonic days 13.5 to 15.5, miR-203 shows a 25-fold increase in expression in cells laying just above the proliferative basal cell layer. In the basal layer where undifferentiated epidermal stem cells reside, p63 is highly expressed to promote proliferation. Once the epidermal progenitors become suprabasal, miR-203 kicks in to repress the expression of p63. Consequently, these cells are able to exit cell cycle regulation, enabling the terminal differentiation phase (Koster et al., 2004; Yi et al., 2008).

1.1.4 miRNAs in skin diseases

1.1.4.1 miRNA and psoriasis

Psoriasis is the most prevalent human hyperproliferative autoimmune skin disease, characterized by an abnormal accelerated keratinocyte differentiation, reduced apoptosis, as well as pro-inflammatory responses. This is due to the aberrant cross-talk between the immune system and the structural cells of the skin, constituted by the production, infiltration, and inflammation of epidermal lymphocytes, neutrophils and dendritic cells (Lebwohl, 2003; Lowes et al., 2007; Sonkoly et al., 2007). Clinically, this leads to formation of psoriatic lesions that appear as erythematous plaques with silver-white scales. The percentage of the population that is affected by this disease varies from 0.3 to 3% in populations with different ethnic background (Griffiths and Barker, 2007). Psoriasis is a chronic and complex genetic inflammatory skin disease, with lesions displaying dramatically altered transcriptomics, revealing 1300 differentially expressed proteins (Gudjonsson et al., 2010; Zhou et al., 2003). However, little is known about the expression and the role of ncRNAs, such as miRNAs in psoriatic skin.

To date, several miRNA expression patterns have been distinguished in healthy skin and psoriasis. This has indicated a new regulatory mechanism of miRNAs in the pathogenesis of chronic skin inflammation in psoriasis. The keratinocyte expressing miR-203 was the first miRNA to be described as overexpressed in psoriasis. The increased miR-203 coincides with the down-regulation of a predicted target gene known as *suppressor of cytokine signaling-3* (*SOCS-3*). SOCS-3 protein influences the inflammatory responses and keratinocyte proliferation and differentiation, by inhibiting the signal transducer and activator of the transcription 3 (STAT3) pathway. STAT3 is constitutively activated in epidermal keratinocytes of human psoriatic lesions, and is widely expressed by various growth regulating signals and inflammatory cytokines such as interleukin-6, which is present in psoriatic lesions. It is suggested that the SOCS-3 suppression by up-regulation of miR-203, leads to the constant activation of the STAT3 pathway. This results in the subsequent infiltration of immune cells, and development of psoriatic plaques through keratinocyte hyperproliferation (Sonkoly et al., 2007).

In contrast, in mouse skin a hypoproliferative effect is observed when miR-203 is up-regulated. This anti-proliferative effect of miR-203 is puzzling, and leads to questions on target interaction, since miR-203/SOCS-3 target interaction could not be demonstrated (Lena et al., 2008). As stated above, miR-203 is implicated in the induction of cell cycle exit, and in repressing "stemness" in epidermal progenitors by targeting the transcription factor p63. Yet in psoriasis *p63* was not found to be down-regulated at mRNA level. However, when observing protein levels of p63, down-regulation was demonstrated, confirming the likelihood of the miR-203/SOCS-3 interaction. These observations are compelling enough to warrant further investigations into the influence of miR-203 on the pathogenesis of psoriasis. Interestingly, a recent publication reports a novel antisense miRNA derived from the miR-203 locus, which has been designated miR-203-AS (anti-sense) (Joyce et al., 2011). Next generation sequencing techniques were able to detect this new sequence, and even though it is much less abundant than miR-203 in skin, it is the most abundant novel miRNA included in this new data set. These findings suggest a role for miR-203-AS in psoriasis, but do not exclude the possible involvement of miR-203. Further functional characterization of miR-203-AS will help to accommodate any inconsistencies concerning miR-203 and psoriasis.

Two other psoriasis-specific miRNAs, namely miR-146 and miR-125b, show interesting potential for future treatments involving miRNA-based therapies against psoriasis. Both are involved in the tumor necrosis factor-alpha (TNF-α) pathway, with considerable success in contending psoriasis with the use of TNF-α blockers. First, miR-146 was shown to be overexpressed in psoriatic lesions and in patients that have rheumatoid arthritis (Pauley et al., 2008; Sonkoly et al., 2008). Several miR-146a targets have already been identified such as *TNF receptor-associated factor 6 (TRAF6)* and *IL-1R-associated kinase (IRAK)*. These genes are involved in the TNF-α pathway that contributes to psoriatic skin inflammation. Furthermore, it has also been demonstrated that the TNF-α pathway is inhibited by NFkappaB activation, which in turn has been linked to the up-regulation of miR-146a, thus potentially contributing to the disease state (Taganov et al., 2006; Tang et al., 2001). Secondly, miR-125b is down-regulated in psoriasis, and it has been suggested to post- transcriptionally regulate the TNF-α pathway (Tili et al., 2007). Through the loss of function of miR-125b, an increase in TNF-α expression is induced in psoriatic lesions.

For other miRNAs involved in psoriasis we refer to reviews of (Bostjancic and Glavac, 2008; Joyce et al., 2011; Sonkoly et al., 2008) and Table 2.

1.1.4.2 miRNA and atopic dermatitis

Atopic dermatitis is a chronic, relapsing, non-contagious, inflammatory skin disease that is characterized by pruritic (itchy), and eczematoid skin lesions. An initial study, determined to explore the role of miRNAs in this pathogenesis, discovered that in lesional skin of patients with atopic dermatitis, miR-155 is one of the most upregulated miRNAs (Sonkoly et al., 2010). MiR-155 is regulated by T-cell activation signals *in vitro*, and by important trigger factors of atopic dermatitis *in vivo*. Sonkoly *et al*. (2010) were able to show that miR-155 is involved in the regulation of T-cell responses, by suppressing CTLA-4, and by enhancing T-cell proliferation.

In conclusion, these disease-specific miRNAs, together with others that have been identified, may well offer a potential for therapeutic targets in future applications against chronic inflammatory skin diseases.

Table 2. miRNAs in chronic skin disease (**AD:** atopic dermatitis, **RA:** rheumatoid arthritis)

miRNA's	Conditions	Level	Target genes	Function	Cell types
miR-99a	**Psoriasis** (Lerman et al., 2011)	↓	**IGF-1R** (Lerman et al., 2011)	**Elevation of IGF-1R drives tissue towards proliferation**	**KC**
miR-125b	**Psoriasis** (Sonkoly et al., 2007; Xu et al., 2011)	↓	**FGFR2** (Xu et al., 2011) **TNFA** (Tili et al., 2007)	**Potentially reduces keratinocyte proliferation, Innate immune response, TLR signaling**	**Fibroblasts, melanocytes, (KC's)** (Sonkoly et al., 2007)
	AD (Sonkoly et al., 2007)	↓			
miR-146a	**Psoriasis** (Sonkoly et al., 2007)	↑	**TRAF6/IRAK1** (Taganov et al., 2006)	**Regulates TNFA signaling** (Taganov et al., 2006)	**Immune cells (T-Reg, DC's, mast-cells)** (Sonkoly et al., 2007)
	AD (Sonkoly et al., 2007) **RA** (Nakasa et al., 2008)	↑			
miR-155	**AD** (Sonkoly et al., 2010) **RA** (Pauley et al., 2008) **Psoriasis (?)**	↑	**CTLA-4** (Sonkoly et al., 2010) **MMP-3** **Pu.1** (Pauley et al., 2009) **SOCS1** (Lu et al., 2009)	**Innate/adaptive immune responses, germinal center response, IgG Class-Switch** (Pauley et al., 2009)	
miR-203	**Psoriasis**	↑	**SOCS3** (Sonkoly et al., 2007)	**Sustained activation of STAT 3 → inflammation, May increase keratinocyte proliferation**	**KC**
	Normal epithelial cell biology	↓↑	**p63** (Lena et al., 2008)	**Switch between proliferative capacity KCs and commitment to differentiation**	
miR-221/222	**Psoriasis**	↑	**TIMP3** (Zibert et al., 2010)	**Effect on MMP and epidermal proliferation**	
	Tumors (eg. Melanoma) (Felicetti et al., 2008)	↑	**c-kit receptor p27**	**Inducers of proliferation and tumorigenicity** (Mercatelli et al., 2008)	
miR-424	**Psoriasis**	↓	**MEK1, cyclin E3** (Ichihara et al.)	**KC hyperproliferation**	**KC**

1.1.5 miRNAs and UV stress

When cells are exposed to various stresses, multiple mechanisms are induced to cope with such attacks. These include induction of molecular chaperones, rapid clearance of damaged macromolecules, growth arrest, and activation of specific gene expression programs. When cells can no longer deal with the excessive damage that has been induced, apoptotic pathways come into effect to clear out decimated cells. MiRNAs have emerged as key regulators that respond to various stress induced factors, such as ultraviolet radiation (UVR).

Several studies have tried to find the role of miRNAs in responses inflicted by UVR. Studies in plants have established certain miRNAs that respond to such stresses. One such study involving *Arabidopsis Thaliana,* reports that 21 miRNAs genes in 11 miRNA families are differentially up-regulated in response to UVB stress (Zhou et al., 2007). A similar study involving *Populus tremula*, discovered 24 UVB responsive genes, that were classified into 3 separate groups. The first group includes genes that are involved in signal cascades pathways, while the second group was more likely to target transcription factors via prediction databases. The third group of genes was involved in diverse physiological processes (Jia et al., 2009).

Differential miRNA expression profiles have been observed when fibroblast (NIH3T3) received a UVB irradiation, as compared to non-UVB treated cells. A series of different time points associated with different miRNA expression profiles were analyzed (Guo et al., 2009).

All these studies have shown the involvement of miRNAs in UVR. These, and forthcoming studies will enable elucidation of the cellular mechanisms underlying the UVR regulation of gene expressions, mediated by miRNAs.

1.1.6 Delivery of small RNAs into the skin

Successful *in vivo* delivery systems for miRNAs are dependent on their resistance towards the degradation capacities of the skin, specificity to their targeted cells, and high binding affinity to the specific miRNAs in question. To enhance the stability of miRNAs three forms of chemical modifications can be used; 1) 2'-O-methyl group (OMe)-modified oligonucleotides; 2) 2'-O-methoxyethyl-modified oligonucleotides; and 3) locked nucleic acid (LNA). Additionally, several techniques can be applied to aid in the delivery of miRNAs into the skin. Among these penetrating enhancing techniques such as tape-stripping, gene gun, intradermal injection with or without electroporation, and depilatory technology are commonly applied. A direct delivery of these compounds can also be used by intravenous injection or by formulation of RNA oligos together with lipophilic molecules ((Geusens et al., 2011), cf. Addendum 1). A novel, unique and stable formulation was prepared in our lab, which could efficiently deliver siRNAs in cultured primary melanocytes and keratinocytes. These Secosomes were developed for siRNA delivery into the human skin (Geusens et al., 2010), and since siRNA molecules and miRNA molecules differ ever so slightly from each other, experiments are ongoing to assess its application for miRNAs.

1.2 Human skin pigmentation

1.2.1. Ultraviolet Radiation

The terrestrial spectrum of solar UVR is located in the electromagnetic spectrum between ionizing x-rays and non-ionizing visible light, spanning a wavelength region of 100-400 nanometers (nm). UVR is subdivided into three classes: shortwave ultraviolet-C (UVC), mid-wave ultraviolet-B (UVB) and long wave ultraviolet-A (UVA) (Diffey, 2002). The biological effects of each class vary with each accompanying wavelength.

Long-wave UVA radiation ranges from 315-320 nm. UVA is not filtered by the stratospheric ozone layer in the atmosphere, through which approximately 90-99% reaches the earth surface. UVA has a long wavelength and low energy content, enabling it to penetrate deeper through the epidermis. Once considered to be harmless, it is now believed to be harmful if one has undergone excessive and long-term exposure.

Mid-wave UVB spans a wavelength of 280/290-315/320 nm and is filtered by the stratospheric ozone layer in the atmosphere through which only 1-10% reaches the earth's surface. Depletion of the ozone layer, enabling this medium wavelength radiation with its high energy content to reach the earth's surface, this is an increased health concern. UVB penetrates the upper layers of the epidermis, and only 10-20% penetrates the dermis, but it is far more effective in causing sunburns than UVA. This wavelength is also responsible for tanning, wrinkling, photoaging and skin cancer.

Short-wave UVC ranges from 100-200 nm, and is also filtered by the ozone layer in the atmosphere, but does not reach the earth's surface.

UVA and UVB radiation exercise different biological effects on the skin. Due to its high photon energy and shorter wavelength, UVB is more potent and effective in causing biological damage. It is estimated that UVB contributes to up to 80% of the harmful effects of sun exposure, while UVA only takes 20% for its account (Diffey, 1998). As indicated before, the penetration depth of these radiations also account for differences in biological effects.

In order to apply *in vitro* studies with an artificial source, and to measure the effects that occur when human skin cells are exposed to sunlight, it is essential that sources and meters reflect the real world situation. This should provide quality and relevance to any *in vitro* photobiology experiment, and therefore researchers often opt to use a solar simulator as

artificial source of UV. A solar simulator represents the solar spectrum eventually reaching the earth. In particular UVC photons and also some very short UVB photons that never reach the surface of the earth are effectively eliminated by using a UVC blocking filter. Even though solar simulators do not cover the full spectrum, they are more representative than any other UV source (Gasparro and Brown, 2000).

1.2.2 UV Induced Pigmentation

Skin pigmentation in response to UVR has been classified into two time dependent reactions. The first reaction is considered to be the **immediate pigment darkening** (IPD), which is mainly stimulated by UVA radiation. This direct effect occurs within minutes, and persists for several hours, followed by a determined darkening that may last for several days. This tanning response is only transient, and is due to oxidation and polymerization of melanin precursors, including the redistribution of pre-existing melanosomes (Gilchrest et al., 1996; Honigsmann et al., 1986).

The **delayed tanning response** (DT) is considered to be *de novo* synthesis of melanin (melanogenesis) and is mainly stimulated by UVB radiation, and to a lesser extent by UVA exposure (Parrish et al., 1982). This melanogenesis-based tan occurs several days after the initial UV exposure, but is more long-lasting. This tanning response takes more time to develop because of several transcriptional activation processes that are necessary to instigate the downstream melanogenic proteins, which will eventually lead to production of melanin.

1.2.3 Melanogenesis

1.2.3.1 Melanocytes and Melanin

Evolution has entrusted melanocytes with the specific and very important task to protect our surrounding skin cells from the ever abiding presence of sunlight. These specialized cells that originate from neural crest cells, produce a pigmented biopolymer called melanin, which is essentially known as pigment, the primary determinant for our skin, hair, and eye color. These cells are present in the lower layer of the skin epidermis (*stratum basale*), middle layer of the

eye (uvea), inner ear, meninges, bones, and heart. The melanocytes in the skin are dendritic cells that are located at the junction of the dermis and the epidermis. These cells are known for their ability to produce and distribute melanin to the surrounding cells, which then function as a protective barrier against the harmful effects of UV radiation. They function either as a direct shield against UV and visible light radiation, or by absorption of free radicals. The photochemical properties of melanin aid against the stresses induced by UVR gained from sunlight. This photoprotectant absorbs harmful UVR and transforms its energy into heat, thus preventing the formation of free radicals that are accountable for mutagenic effects, responsible for the formation of malignant melanoma and other skin cancers. Inside these melanocytes reside specialized organelles, melanosomes, lysosome-like structures that are in charge of the synthesis of melanin. Melanosomes are translocated from the center of the melanocyte cytoplasm to the tips of its dendrites, where they are then transferred to the surrounding keratinocytes (Van Gele et al., 2009).

Two major types of melanin exist in mammals: **yellow-red pheomelanin** and the **black-brown eumelanin**. These types of melanin differ in size, shape, and packaging of their granules (Slominski et al., 2004). Eumelanosomes are elliptical, while pheomelanin shape is variable with a predominantly rounded contour. Gene mutations that lead to a loss of function are associated with increased pheomelanin production, which leads to lighter skin and hair color. Pheomelanin contains photo-unstable benzothiazines, which are potentially phototoxic, and may contribute to UV-induced skin damage, by generating free hydroxyl radicals and superoxide anions. It is also known to produce histamine, which contributes to erythema and edema. UV exposure reduces the level of glutathione reductase, which is important for pheomelanin synthesis. Eumelanin is considered to be more photoprotective because it has the ability to function as a superoxide dismutase, and is known for its ability to scavenge free radicals, which reduces reactive oxygen to hydrogen peroxide.

Melanin biosynthesis is derived from a common tyrosinase-dependent pathway and is initiated either through hydroxylation of L-phenylalanine to L-tyrosine, or directly from L-tyrosinase. Tyrosinase catalyzes the two rate limiting reactions of melanogenesis by hydroxylation of L-tyrosine, giving the 3,4-L-dihydroxyphenylalanine (L-DOPA), followed by the oxidation of L-DOPA into dopaquinone. From here on the eumelanogenesis and pheomelanogenesis pathways diverge from each other. Eumelanogenesis involves the transformation of dopaquinone into leukodopachrome, and after several subsequent

oxidoreduction reactions produce the intermediates dihydroxyindole (DHI) and DHI carboxylic acid (DHICA), that finally undergo polymerization steps to form eumelanin. Initially, pheomelanogenesis also starts with dopaquinone but is alternatively conjugated with cysteine or glutathione to yield cysteinyldopa and glutathionyldopa, which is further processed and transformed into pheomelanin.

1.2.3.2 Molecules that induce pigmentation

Exposure of skin to UV results in increased synthesis of paracrine factors from keratinocytes and fibroblasts, which can stimulate pigmentation by secreting growth factors that act via receptors located on melanocytes. The activation of the **melanocortin-1-receptor** (**MC1R**) is the start of one of the main signaling pathways, which accompanies UV-induced melanogenesis, and is mediated by an increase in intracellular **cyclic adenosine monophosphate** (**cAMP**) levels.

The **cAMP signaling pathway** is a key physiologic regulator, and has been known to activate protein kinase A and CREB proteins (Im et al., 1998). This eventually upregulates the expression of *MITF*, a key transcriptional regulator that controls the expression of melanogenic enzymes and proteins. The fact that cAMP has been shown to stimulate dendritic formation (Busca et al., 1998), aids in transport of melanosomes into the dendritic tips (Passeron et al., 2004), and increases the gene expression of a known transport protein acting at the peripheral actin filaments, Rab27a , makes it pivotal for UV-induced melanogenesis (Chiaverini et al., 2008).

Certain hormones and chemical agents are commonly used to raise intracellular levels of cAMP. **α-melanocyte stimulating hormone** (**α-MSH**) for example, is a naturally-occurring endogenous peptide hormone, while **forskolin**, is a labdane diterpene that is produced by the tropical perennial plant Indian Coleus (*Coleus forskohlii*). They have the capacity to decrease UV-induced apoptosis of melanocytes, and to increase pH levels (alkalinization) of melanosomes, which aids in the stimulation of enzymatic activity during melanin synthesis (Bohm et al., 2005; Hauser et al., 2006; Kadekaro et al., 2005). Melanogenic stimulators such as forskolin are often used in combination with UV to stimulate melanogenesis (D'Orazio et al., 2006).

One of the important mediators of pigmentation is the large precursor protein **pro-opiomelanocortin** (**POMC**) that is stimulated by p53 and secreted by keratinocytes upon UV irradiation (Cui et al., 2007). POMC produces several derived peptides by proteolytic cleavage, including α-MSH, adrenocorticotropic hormone (ACTH), isobutylmethylxanthine, and dibutyryl cyclic adenosine monophosphate (cAMP) (Chakraborty et al., 1996).

The effects of α-MSH on melanogenesis are regulated via ***tyrosinase*** and ***MC1R***. This receptor gene is one of the most important positive regulators, regulating the intensity as well as the type of melanin produced during melanogenesis. *MC1R* is also a major determining factor in sun sensitivity, and a genetic risk factor for melanoma and non-melanoma skin cancer. Over 30 variant alleles have been identified which correlate with skin and hair color, providing evidence that this gene is an important component in determining normal human pigment variation

MC1R (or ***MSH-R***) is an intronless gene that encodes a receptor protein for α-MSH (see figure 3). The encoded protein contains a seven pass membrane G protein coupled receptor that controls melanogenesis. When binding of MC1R protein on the plasma membrane of melanocytes and α-MSH protein located in extracellular space occurs, the activation of this receptor and stimulation of eumelanin synthesis follows (Goding, 2000; Manne et al., 1995). Inside the cytoplasm, the Gαs protein binds onto the activated MC1R protein (Vongs et al., 2004). Gαs protein increases activation of adenylate cyclase (AC) (Costin and Hearing, 2007). This in turn increases one of the critical factors involved in the signal transduction pathway of melanogenesis, known as cAMP. cAMP is formed by AC through catalyzing the following reaction: ATP -> cAMP (Busca and Ballotti, 2000; Goding, 2000). Due to the increase of intracellular cAMP concentration, activation of protein kinase A (PKA) complexes are induced (Costin and Hearing, 2007). cAMP binds to two sites of the regulatory subunit of PKA, allowing the catalytic subunit to be liberated, and thus activated (Roesler et al., 1998). Activation of PKA allows it to relocate into the nucleus, where it phosphorylates and activates the cAMP responsive element binding proteins (CREB), and CREB binding proteins (CBP) (Costin and Hearing, 2007). CREB proteins activate the expression of certain downstream genes containing consensus CRE (cAMP responsive elements) sequences in their promoter (5'TGACCTCA-3') regions (Karin, 1994). As such, CREB binds to the CRE domain present on the promoter of the important transcription factor *MITF*, which plays a fundamental role in the transcriptional regulation of melanogenesis.

Microphthalmia-associated transcription factor (***MITF)*** is a transcription factor that contains both basic helix-loop-helix and leucine zipper (bHLH-Lz) structural features, which drive important pigmentation genes such as *tyrosinase*. The two major signaling pathways that control *MITF* activity are the MSH/cAMP pathway and Kit signaling pathways (Figure 3).

Another protein that is stimulated by MITF is **tyrosinase-related protein 1** (**TYRP1**), which encodes a melanosomal enzyme that belongs to the tyrosinase family, and plays an important role in the melanin biosynthetic pathway. **Tyrosinase-related protein 2** (**TYRP2**) also known as dopachrome tautomerase (DCT), is a protein that is up-regulated by MITF (Bertolotto et al., 1998).

Genes that regulate the expression of *MITF* include *PAX3* and *SOX10*. *PAX3* is a member of the paired class homeodomain family of transcription factors. *PAX3* both promotes and inhibits melanogenessis through transcriptional regulation of *MITF*, *DCT* and *TYRP1* (Goding, 2000; Vance and Goding, 2004). *SOX10* is a gene that encodes a member of the SOX (SYR-related HMG-box) family of transcription factors involved in the regulation of embryonic development, and in the determination of the cell fate. The encoded protein may act as a transcriptional activator after forming a protein complex with other proteins. In nuclei of melanocytes, SOX10 protein increases expression of MITF protein (Goding, 2000; Vance and Goding, 2004).

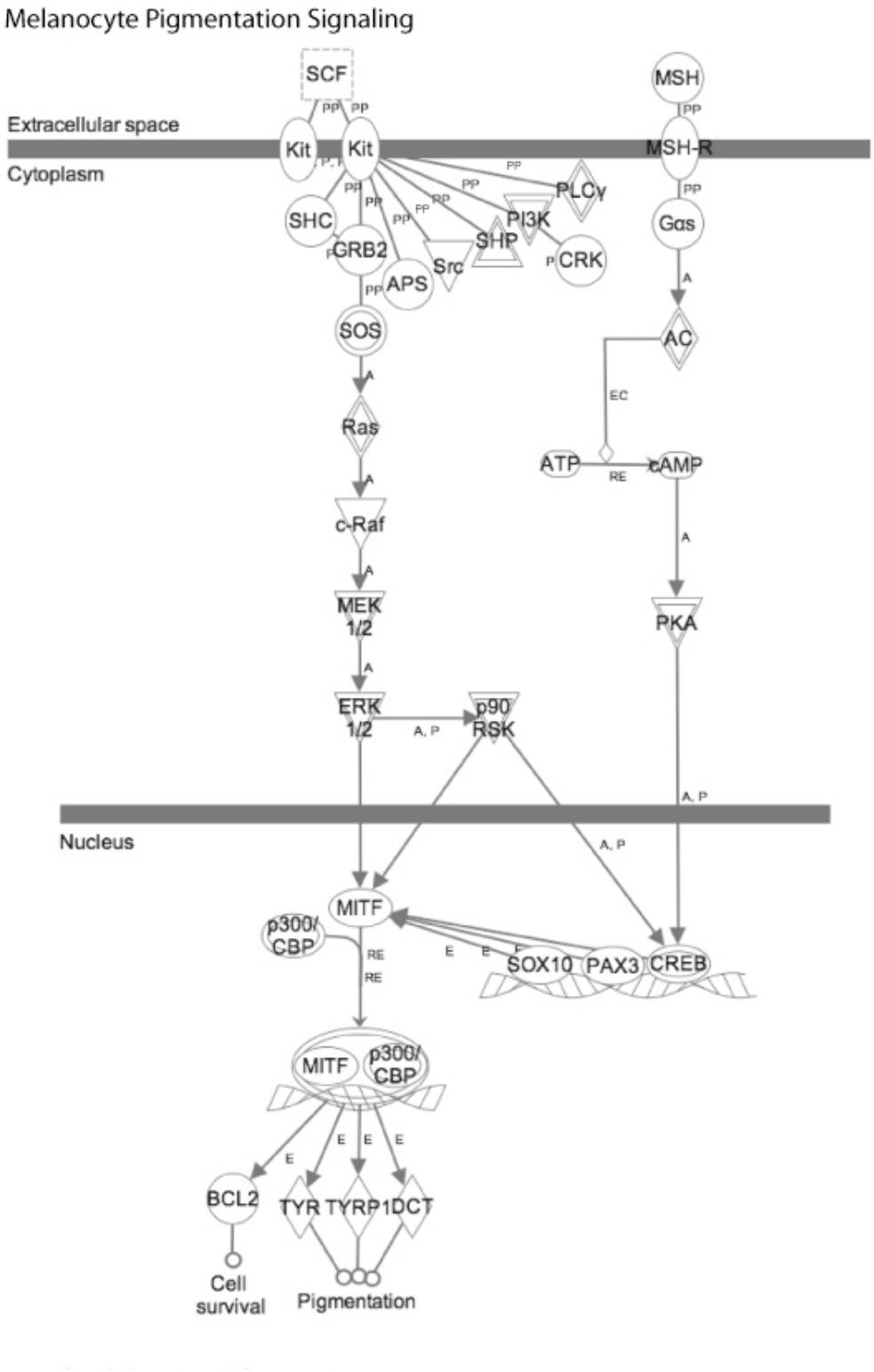

Figure 3. *Melanocyte pigmentation signaling.* Kit signaling pathway and MSH pathway.
(PP) Protein-Protein interactions, A) Activation, (EC) Reaction, (P) Phosphorylation, (RE) Reaction, (E) Expression

Kit signaling up-regulates MITF function through mitogen activated protein kinase (MAPK) phosphorylation. Kit is a membrane bound receptor of the receptor tyrosine kinase (RTK) family, which constitutes a type III transmembrane receptor protein-tyrosine kinase for MGF (mast cell growth factor, also known as stem cell factor (SCF)) (Roskoski, 2005). Kit receptor in plasma membrane from melanocytes is activated by binding to its ligand SCF in extracellular space (Hachiya et al., 2001) (Figure 3). SCF encodes for a ligand of the tyrosine-kinase receptor encoded by the KIT locus. On the cell surface of melanocytes, SCF binds to the extracellular portion of c-KIT receptor, thus mediating dimerization of two subunits located at the inner side, followed by activation of its intrinsic tyrosinase kinase activity, and auto-phosphorylation. The autophosphorylated tyrosine residues serve as docking sites for various signal transduction molecules containing SH2 domains. The activated KIT receptor phosphorylates various substrates and associates with various signaling molecules.

In the cytoplasm of melanocytes, GRB2 binds to the phosphorylated c-KIT present in the plasma membrane of melanocytes. Next, GRB2 binds to the guanine nucleotide exchange factor protein, son of sevenless (SOS), which is then able to bind to the inactive membrane bound protein Ras. Inactive Ras is bound to the nucleotide guanine diphosphate (GDP). In order to activate the Ras protein, SOS catalyses the exchange of the Ras bound guanine GDP against guanine triphosphate (GTP). Due to this exchange to its active GTP bound state, Ras is able to bind to several effector proteins. Hereafter, SOS protein increases the activation of Ras protein (Costin and Hearing, 2007). This Ras protein increases activation of cellular Raf (c-Raf) protein, which is a cellular homolog of viral raf gene, and is one of the important effectors of Ras. c-Raf encoded protein is a MAP kinase kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPase to which it directly binds. Once activated, the c-Raf protein can phosphorylate to activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylate to activate the serine/threonine specific protein kinases, ERK1 and ERK2, which all lead to the activation of the RAS-MAPK pathway (Anjum and Blenis, 2008; Costin and Hearing, 2007). MAPK pathway may directly activate MITF, or it may activate indirectly via p90RSK1 (Goding, 2000; Vance and Goding, 2004). Additionally, p90RSK1 proteins increase phosphorylation and activation of CREB proteins in nuclei from melanocytes.

Activated MITF pairs with the co-activator p300/CPB. Once this complex is formed, several gene expression levels can be regulated. MITF-p300/CBP complex increases expression of anti-apoptotic BCL2 protein, leading to cell survival (McGill et al., 2002). This gene encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death of some

cells. In addition, this MITF-p300/CBP complex increases the expression of tyrosinase (TYR) protein and pigmentation. TYRP1 protein is increased by the same MITF-p300/CBP complex and can subsequently increase pigmentation. Finally, DCT protein is also positively regulated by the MITF-p300/CBP complex, aiding in the increase of pigmentation. MITF phosphorylation also couples transactivation to proteasome mediated degradation. Kit signaling thus triggers short lived MITF activation and net MITF degradation.

The **Wnt signaling** pathway has also been associated to melanogenesis, and initially works through binding of Wnt proteins to the cell surface receptor of the Frizzled family. This causes the receptors to activate Dishevelled family protein, which is a key component of the membrane-associated Wnt receptor complex. This activation subsequently inhibits a multiprotein complex containing axin, adenomatous polyposis coli (APC), casein kinase Iα (CKIα) and glycogen synthase kinase-3β (GSK3β). This protein complex promotes the continuous ubiquitin mediated degradation of an intracellular signaling molecule, β-catenin, leading to a low abundance of this molecule in the cytoplasma (Rubinfeld et al., 1996). Due to the deactivation of the complex containing GSK3β, the accumulation of β-catenin is permitted. One of the key factors of β-catenin is its stability. If stable enough it is translocated into the nucleus and binds to transcription factors of the T-cell factor Tcf/lymphoid enhancer factor (Lef) family, which then directly leads to the positive transcriptional regulation of MITF (Behrens et al., 1996; Peifer and Polakis, 2000).

It has been demonstrated that the intracellular accumulation of cAMP levels resulting in activation of PKA can rapidly phosphorylate GSK3β, leading to its deactivation. Additionally, the direct phosphorylation of β-catenin by this cAMP/PKA signaling, and thus the inhibition of β-catenin degradation, increases Tcf-dependent transcriptional activity, followed by increase in *MITF* transcription (Hino et al., 2005; Suzuki et al., 2008). This shows how cross talk between different pathways during melanogenesis is ever present.

1.2.4 Intracellular melanosome transport

Introduction

Melanosome transport in melanocytes is considered to be a model system for the study of cytoskeletal regulation of intracellular transport and has contributed to a better understanding of the molecular mechanisms involved in vesicle transport and membrane trafficking processes. Melanosomes are transported from the nucleus to the cell periphery of the melanocytes by coordination between bidirectional microtubule-dependent movements and the unidirectional actin-dependent movement.

Here we provide an in depth overview of all the essential players that help regulate this type of transport to date. We discuss how the gathered knowledge about the RAB27A-MLPH-MYO5A tripartite complex can be translated into a possible therapeutic application to reduce (hyper)pigmentation of the skin. Mutations within any of the *RAB27A-MLPH-MYO5A* tripartite complex cause one of the three different subtypes of a rare autosomal recessive hereditary disease, known as Griscelli syndrome (GS), which develops early in life. A common characteristic found in these patients is hypopigmentation of skin and hair (Rees, 2003).

Review

M. Van Gele, ***P. Dynoodt*** *and J. Lambert.*

Griscelli syndrome: a model system to study vesicular trafficking.

Pigment Cell Melanoma Res 2009; 22: 268-282.

1.2.5 Melanosome transfer

To date, several hypotheses on how melanocytes transfer melanosomes into keratinocytes have been developed. Unfortunately, none of these described modes of transfer can be considered as conclusive. In the following we shall briefly describe these four modes of action (Figure 4) (Van Den Bossche et al., 2006).

A. Cytophagocytosis is defined as the cellular engulfment of particles that have been pinched off from the dendritic tips of melanocytes. The dendritic tips containing mature melanosomes form protrusions into the keratinocytes' cell membrane. These protrusions are engulfed by the closure of the keratinocytes membrane leading to the dispersion of melanin granules throughout its cytoplasm.

B. Exocytosis is based on the cellular secretion of melanin out of the melanocytes followed by fusion of the melanosomal membrane with the plasma membrane of the keratinocyte, either through endocytosis or phagocytosis.

C. The fusion method of the plasma membranes of both melanocytes and keratinocytes suggests the formation of pores or channels between both cell types. Melanosomes are transported through this connection.

D. Membrane vesicles containing melanosomes are shed by the melanocytes followed by either fusion with the membrane of keratinocytes or ingestion through phagocytosis.

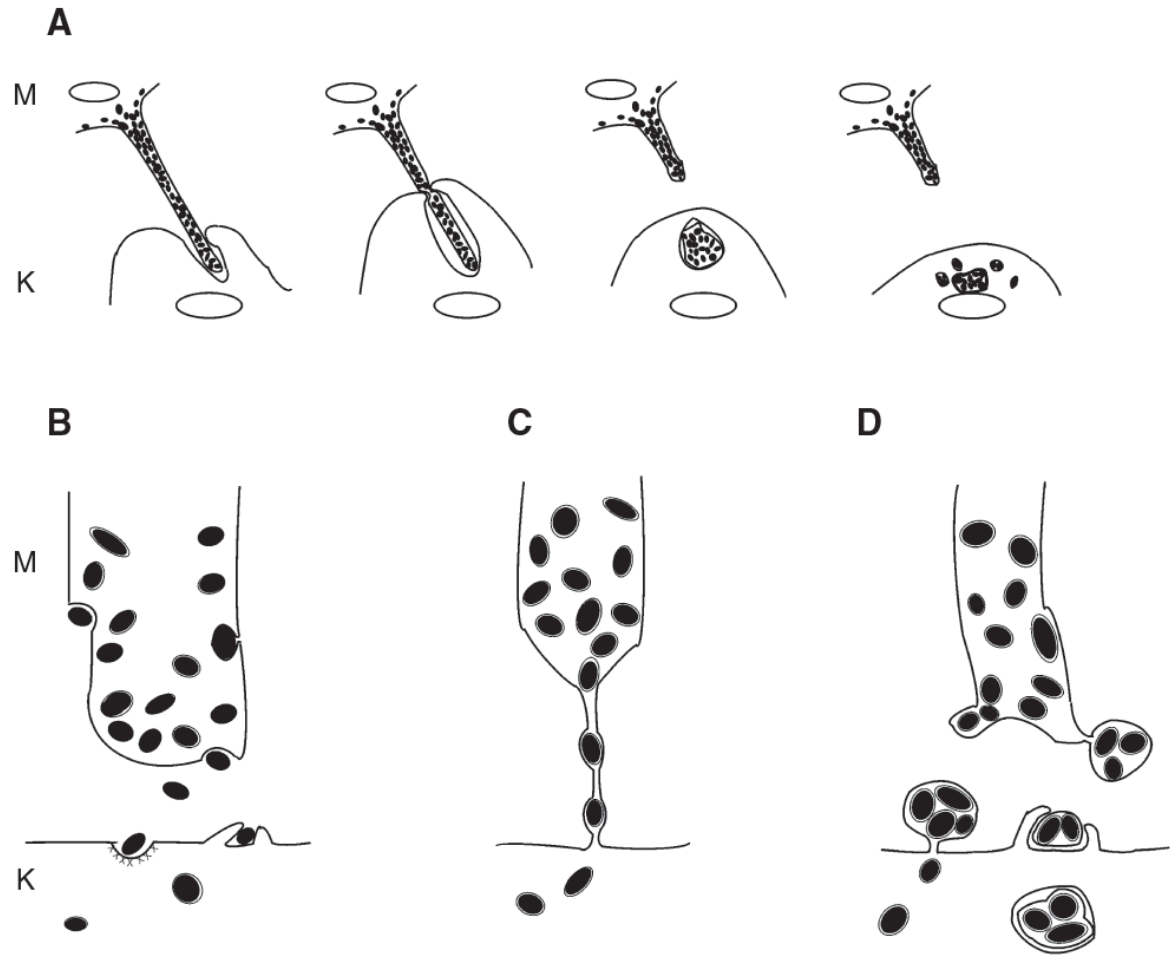

Figure 4. Different modes of melanin transfer
A) Cytophagocytosis. B) Exocytosis. C) Fusion. D) Membrane vesicles.
M: melanocyte, K: keratinocyte

1.3 Skin cancer

Cancer is a disease characterized by malfunctioning of growth, differentiation and loss of sustainable tissue integrity. Cancer cells proliferate in an uncontrollable manner, and thus begin to invade into surrounding tissues, becoming metastatic. During metastases the cancer cell is able to loosen itself from the tumor, thus migrating freely through the blood stream and lymph nodes towards other locations of the body (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011).

Skin cancers are a collection of cancers that originate from epithelial cells of the skin (basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)), or from melanocytes / pigment

cells of the skin (melanoma). Melanoma is the most aggressive type of skin cancer. It is a malignant injury that originates from melanocytes that are found at the basal layer of the epidermis. The melanin produced by melanocytes functions as a physiological barrier against UVR. In this manner melanocytes deposit the pigment into the surrounding keratinocytes, which then protect the DNA that is situated in the nucleus. This results in the protection of the keratinocytes from the influence of UVR.

The incidence of all types of skin cancers in Western Europe has increased considerably during the past half century and it is expect that this incidence will only increase with the continuing aging of the population (Erb et al., 2008; Samarasinghe and Madan, 2012).

BCC represents 70% of all skin cancers. Although the occurrence of this form of skin cancer is most prevalent, it is not registered in the cancer registration system because of its low mortality rate. BCC is characterized by an extensive local growth, which can damage the skin tissue. Surgery is the most efficient treatment, although there are alternative treatments for certain subtypes of BCC. SCC represents 20% of all skin cancers (Samarasinghe and Madan, 2012). This skin cancer is caused by UVR, or in the case of immune suppression, can be found in transplant patients. This form of skin cancer has a metastatic potential, usually by first migrating to the lymph glands, and then further towards the internal organs (Samarasinghe and Madan, 2012).

1.3.1 Melanoma

Melanoma is the most aggressive skin cancer. Melanoma represents only 10% of all skin cancers and belongs to the five most frequent cancer type found in Belgian women. The incidence of melanoma has increased considerably in the second half of the 20th century. Although the incidence increases with age, it still affects a relatively young population compared to other cancer types. The prognosis of melanoma significantly correlates with the tumor thickness, therefore an early detection is crucial (Samarasinghe and Madan, 2012). Since observation of the skin by clinical surveillance with additional inspection by use of a dermatoscope, is relatively easy, and a noninvasive technique, it subjects melanoma to the early detection by screening preferably towards people at risk. With men melanoma is mainly found in the region of the torso, while with women the legs are the more prone sites.

Different forms of melanoma are known. The superficial spreading melanoma and the nodular melanoma are the most frequent types. Lentigo maligna melanoma and acral lentiginous melanoma are less frequent subtypes. Superficial spreading melanoma initially shows a horizontal growth phase with the possibility to grow inwards. Nodular melanoma is at a very early stage already growing vertically. Both subtypes are mainly caused by intermittent sun exposure (high UV exposures on a non regular basis). Lentigo maligna melanoma is a slow growing melanoma located on the surface of the skin, and is mostly associated with elderly who have been through chronic exposure to sun light, for example to the face. Finally, the acral lentiginous melanoma is mostly found at the exterior regions of the body for example the hand palms, foot soles, and nail beds.

1.3.2 Role of miRNAs in melanoma

Cancer is a complex genetic disease with structural chromosomal deviations and disturbed expression of coding genes as well as non-coding genes. Hanahan and Weinberg postulate that healthy cells that develop into cancer cells have to attain essential capacities to enable them to develop into a cancer cell; for example subsistence in growth signals, loss of growth inhibition substances, resistance to apoptosis, increase in angiogenesis, unlimited division capacity and metastasis (Hanahan and Weinberg, 2000). At the basis of these new properties lie the genetic deviations that are present through genetic hereditary or can be additionally induced by exogenous factors that are either chemical, physical or of an infectious nature (Weinberg, 2007). For example smoking of tobacco products can cause lung cancer, the exposure to UVR can contribute to the development of skin cancer, and cervix cancer arises after the infection of the human papilloma virus.

The function of genes that affect cancer can be divided into two categories: the oncogenes, which play an active role during the progression of cancer by accelerating the growth controlling signaling pathways, and the tumor suppressors, which play a role during cell growth and attend to the DNA integrity and reparation of DNA damage. Gain-of-function by point mutations, amplification of chromosomal rearrangements, and the loss-of-function by point mutations or deletions of the respective oncogenes and tumor suppressors, insure that a cell may attain the aforementioned capacities and enable it to transform into a cancer cell (Calin et al., 2004).

MiRNA expression profiling studies have shown that normal tissues have distinguished miRNA expression signatures compared to their different tumor counterparts, including the different stadia that tumors go through. At this moment different diagnostic signatures have been made for lung tumors, breast cancer, glioblastoma, thyroid cancer, hepatocellular carcinoma, pancreatic cancer, colorectal cancer, and chronic lymphocytic leukemia (Calin and Croce, 2006). Expression of one or several miRNAs has been shown to correlate with general survival rates, post operative survival, and response to treatment and risks associated to metastasis (Calin and Croce, 2006). The involvement of miRNAs in tumor formation and the way they act as potential oncogenes or tumor suppressors has aroused great interest in the use of these molecules as potential therapeutic targets for cancer therapies. The general therapeutic strategies utilizing antisense mediated inhibition of oncomiRs and the substitution of microRNA mimics through viral vector coding miRNAs are interesting strategies that are actually being studied (Trang et al., 2008).

In 2008, the first link between the deregulated expression of a specific miRNA and its function during oncogenesis of melanoma was established (Bemis et al., 2008). MiR-137 is located in the chromosomal region 1p22 and is known to carry alleles susceptible to melanoma. Increased expression of miR-137 represses the expression of *MITF*, which was also significantly decreased in uveal melanoma cells, with an additional decrease in cell growth through inducing G1 cell cycle arrest (Chen et al., 2011). MITF is an important regulator in development, maturation, apoptosis and pigmentation in melanocytes. Induction of MITF, for example when exposed to UV, protects the skin against DNA damage (Cui et al., 2007). MiR-182 also works as a negative regulator of MITF and FOXO3 expression. Increased expression of miR-182 has been associated with a rise in invasiveness of melanoma, increasing its metastatic potential (Segura et al., 2009). MiR-340 is also capable of binding to the 3'UTR of *MITF* causing mRNA degradation (Goswami et al., 2010).

MiR-532-5p has been shown to be an oncomiR by directly targeting *runt-related transcription factor 3* (*RUNX3*), which is a known tumor suppressor during the transition of a melanocyte into a metastatic melanoma (Kitago et al., 2009). It has also been shown that miR-210 is upregulated in melanoma and breast cancer metastases that suffer from hypoxia (Zhang et al., 2009). MiR-210 targets *MNT*, which is a gene known as one of the *Myc* antagonists. The miR-200 family is increased in melanoma cell lines compared to normal melanocytes. The difference between each member of the miRNA family is the mode of invasion that each

miRNA instigates. MiR-200a for example, results in a protrusion-associated elongated mode of invasion by reduced actomyosin contractility, while miR-200c adopts a more rounded, amoeboid-like mode of invasion by reduced expression of myristoylated alanine-rich protein kinase C substrate (Elson-Schwab et al., 2010).

MiR-34b, miR-34c, and miR-199a* have been identified as tumor suppressors in melanoma cells because they target an oncogene called *MET*, which encodes for a tyrosine kinase receptor for hepatocyte growth factor (Migliore et al., 2008). It has also been reported that miR-34 expression is aberrantly expressed due to CpG methylation of its promoter region (Lodygin et al., 2008). MiR-196a is significantly repressed in malignant melanoma cell lines and tissue samples, and when re-expressed *in vitro*, it is able to significantly reduce the invasive behavior of melanoma cell lines (Braig et al., 2010; Mueller and Bosserhoff, 2010). It has also been reported that the overexpressing miR-193b in melanoma cell lines repressed proliferation by down-regulating cyclin D1 (CCND1) (Chen et al., 2010).

Recent developments that have given vast amounts of data are the miRNA expression profile studies that have been performed in melanoma. Through these studies miRNA signatures in melanoma have been defined. These data have clinical relevance as potential biomarkers as well as prognostic value into determining at what stage the melanoma has progressed (Segura et al., 2010).

These miRNA signatures can not only be found in cancer tissues but also in blood. The possibility to detect which miRNAs are found in the patient's blood offers perspectives for determining the present stage of the melanoma in a non-invasive manner (Leidinger et al., 2010).

It is beyond the scope of this PhD thesis to review all miRNAs that have been reported in melanoma. We have summarized most of the described miRNAs in Table 3 (Li and Xi, 2011).

Table 3. MicroRNAs in melanoma progression

Progression		miRNA	Target(s)	Regulatory Factor	Associations
		let-7a	ITGB3		↓ Migration, invasion
		let-7b	CCND1		↓ Proliferation, differentiation
		miR-137	MITF		↓ Cell migration, invasion and survival
	↓	miR-155			↑ Proliferation
Melanocyte		miR-324-5p			
		miR-34a	MET	Promoter methylation	↓Proliferation
↓		miR-106a			
		miR-126			
Primary Melanoma		miR-133a			
		miR-141			
	↑	miR-145			
		miR-15b			↑ Proliferation, survival
		miR-200c			Migration style transition
		miR-27b			
		miR-210	MNT		↑ Proliferation
		miR-126			
		miR-200c			
		miR-141			
	↓	miR-133a			
Primary Melanoma		miR-34a			
		miR-199a*	c-Met		↓ Cell migration, invasion and survival
↓		miR-34b/c	c-Met		↓ Cell migration, invasion and survival
Metastasis		miR-106a			
		miR-133a			
		miR-199a*			
	↑	miR-182	MITF, FOXO3		↑ Migration, invasion and survival
		let-7b			
		miR-133a			
		miR-155			↓ Proliferation, survival
	↓	miR-193b			
Melanocyte		miR-196a	HOX-C8 HOX-B7		↓Invasion
		miR-133a			
↓		miR-17-5p			
		miR-18a			
Metastasis		miR-19a/b			
	↑	miR-221/222	c-Kit, p27	PLZF	↑ Proliferation, invasion; ↓ differentiation
		miR-532-5p	RUNX3		↑Invasion
		miR-20a			
		miR-92a			

1.4 References

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CHAPTER 2

AIMS AND OUTLINES

Aims

Previous studies of the Dermatology Research Unit (DRU), at the Ghent University Hospital, focused on the role of motor proteins, such as Myosin VA (MYO5A), in primary human melanocytes and pigmentation. The DRU previously developed a method based on RNAi to reduce the expression of specific isoforms of the motor protein MYO5A, known to be involved in intracellular melanosome transport. The DRU succeeded in blocking melanosome transport in human melanocytes cultured *in vitro*, and thus reducing pigmentation by means of an *exon F MYO5A* siRNA formulation (Van Gele et al., 2008). In line with these studies a new emphasis was placed in this thesis on the discovery of new molecules, which could be manipulated in order to promote or reduce pigmentation. This was carried out by miRNA expression profiling of UV-irradiated melanocytes.

Manipulation of miRNA expression or function, either by mimicking or inhibiting them, is emerging as an innovative, and highly promising therapeutic strategy. Moreover, intrinsic drawbacks with siRNA-induced gene silencing (including off-target effects, elicitation of interferon response, and interference with endogenous miRNA biogenesis) are eliminated when using miRNAs, due to their specific biogenesis and mechanism of action.

The first objective of this thesis is **to identify miRNAs involved in the pigmentary process in melanocytes**, either by modulating the expression of miRNAs, decreasing pigmentation and thus potentially forming a basis for treatment against hyperpigmentation and melasma, or increasing pigmentation, and thus offering a better protection against the harmful effects of UV and melanoma development. This might lead to new insights into miRNA-based treatments, offering an attractive alternative by specifically targeting key genes in melanogenesis.

In order to determine, which biological effects miRNAs have on the pigmentary process, and as a second objective, **functional studies** were performed with the **most prominently influenced candidate miRNA** from a miRNA profiling study, following an experimental model system (ssUV/forskolin). MiR-145 came out as a key player during melanogenesis, but has also frequently been reported to be down-regulated in various cancer types including prostate cancer (Fuse et al., 2011), bladder cancer (Chiyomaru et al., 2010), colon cancer (Akao et al., 2007b), and B-cell malignancies (Akao et al., 2007a). Accordingly, various miRNA profiling studies performed on malignant melanoma tissues have suggested a tumor suppressor role of miR-145 in melanoma (Segura et al., 2010). This miRNA is located on

chromosome 5q, which is a common deletion region found in melanoma (Lin et al., 2008). Therefore, as a third objective a possible **key role of miR-145 in melanoma and its tumor suppression response** on cell growth, invasion and migratory activity, was considered and explored.

Outlines

The specific role of miRNAs in pigmentation is not yet defined. In order to study the pigmentary process, a robust *in vitro* model system that monitors this process is required. An experimental model system that studies the effect of ssUV and forskolin was developed, based on the model system using a mouse cell line of normal immortal melanocytes, melan-a (Lei et al., 2002). This system was slightly modified in order to fit the desired responses. This model was used, in order to obtain a more complete understanding of this complex pigmentation process. After confirmation of several important upregulated pigmentation genes, we defined an experimental model system, ultimately capturing and registering differentially expressed miRNAs involved in pigmentation.

By comparing miRNA profiles of treated melan-a cells (ssUV and forskolin), *versus* control melan-a cells, miRNA profiling was obtained through a qPCR-based method, using stem-loop primers. To obtain cDNA from all miRNAs present, a megaplex reverse-transcriptase primer pool of 10 ng of total RNA was used. The expression of 540 miRNAs, was then quantified through real-time qPCR, containing a Taqman probe library. After screening for miRNAs that affect the cellular pigmentary process in our model system, 16 differentially expressing miRNAs were retained. After consulting target prediction databases, one specific miRNA caught our attention, presenting not only several potential target binding sites to a gene involved in melanogenesis, but additionally showing a 15-fold knockdown compared to its control counterparts. Gain- and loss-of-function experiments involving miRNA precursors and miRNA inhibitors were used to mimic or inhibit this specific miRNA in an attempt to uncover biological effects that are influenced by miR-145. Western blotting was used to investigate the impact of this miRNA on protein expression, and real-time qPCR was used to quantify the gene expression levels of important genes during the pigmentation process. Luciferase target assays were designed and used to determine which direct targets are influenced by this miRNA. The results enabled us to confirm that this one specific miRNA plays a key role during the pigmentation process.

After establishing the role of miR-145 in melanocytes, and confirming its key role during the pigmentary process, we decided to investigate the potential tumor suppressor role of this miRNA in melanoma. MiR-145 has already been studied in several cancer types, and has been described as having a tumor suppressor role. Three cell lines were chosen with a different metastatic potential, and the expression level of miR-145 was quantified. Two metastatic cell lines, BLM and FM3P, demonstrated an extremely low expression level of miR-145, while a third and last primary melanoma cell line showed a slightly increased expression of this miRNA, as compared to normal human primary melanocytes. After overexpressing the miRNA in these melanoma cell lines, several functional studies were performed in order to investigate the tumor suppressor role of miR-145 in melanoma. Wound healing assays were performed in order to study the effect of the miR-145 on cell migration and cell interactions, while the Trypan Blue Exclusion Test of Cell Viability was used to determine if this miRNA influences cell proliferation. A collagen invasion assay was performed in order to determine the invasiveness of the melanoma cell lines into a collagen type-1 matrix. To uncover the mechanism of miR-145 as a tumor suppressor, knockdown studies on a direct target, *FSCN1*, of this miRNA were performed.

In conclusion, the outlines of this thesis describe a useful and comprehensive strategy on how to uncover the involvement of miRNAs during melanogenesis and melanomagenesis. Moreover, we have established a strong platform to guide future research and promote further in depth studies.

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CHAPTER 3

RESULTS

3.1 Screening for potential miRNAs involved in pigmentation

Introduction

Production of melanin is controlled by multiple players whereby more than 150 genes have been identified. Knowledge of which specific role miRNAs execute during the regulation of the pigmentary process is very limited, till date. The following study is the first to perform an in depth miRNA expression profiling analysis in an attempt to unravel the role that miRNAs play during melanogenesis. The obtained results confirm a new pivotal layer of regulation, wherein a single miRNA is able to modulate both mRNA and protein levels of several important pigmentation genes. The compelling evidence that has been gathered indicate a key role in melanocyte pigmentation for miR-145, and thus in the regulation of melanogenesis. Interestingly, melanocytes overexpressing miR-145 display a perinuclear accumulation/retention of melanosomes followed by a hypopigmentary phenotype. Discovery of this important pigmentation factor may expand the therapeutic potential of miRNAs in pigmentary disorders.

Paper:

Peter Dynoodt, Pieter Mestdagh, Gert Van Peer, Jo Vandesompele, Karen Goossens, Luc Peelman, Barbara Geusens, Reinhart Speeckaert, Jo Lambert and Mireille Van Gele.
Identification of miR-145 as a key regulator of the pigmentary process.
JID 2013: 133,* 201-209. *doi: 10.1038/jid.2012.266.

Supplementary Material

Supplementary Material and Methods

Luciferase reporter assay for human myosin VA

Taking into account that the 3' UTR of human *myosin VA* (NM_000259) contains three putative miRNA binding sites (position 129-136; seed 1, position 731-737; seed 2, position 781-787; seed 3) for hsa-miR-145 we decided to investigate if an interaction occurs between hsa-miR-145 and *myosin VA* (*MYO5A*) by utilizing an *in vitro* luciferase assay (Lal *et al.*, 2009; Mestdagh *et al.*, 2010). The 3'UTR, containing the three miRNA binding sites of *MYO5A*, was PCR amplified using cDNA derived from primary human melanocytes and the primers listed in Table S3. The primers are flanked by either an XhoI or a NotI restriction site located at their 5' end to allow ligation into the multiple cloning region of the psiCHECK™-2 Vector (Promega Benelux B.V., Leiden, The Netherlands) containing a downstream stop codon of a SV40 promoter-driven *Renilla* luciferase gene. This resulted in a wild-type (WT) 3'UTR *MYO5A* luciferase reporter construct. In order to determine which binding site (or a combination) is responsible for the miR-145_MYO5A interaction, single mutants (Mut) were created by introducing four point mutations in the seed region of each of the three hsa-miR-145 binding sites of *MYO5A*. More specific, each miRNA binding site of *MYO5A* was first mutated using the Quikchange II XL Site-directed mutagenesis kit (Agilent, Diegem, Belgium) according to the manufacturer's instructions. Primers for site-directed mutagenesis (Table S4) were designed as described by Mavrakis *et al*. (2011) using the Quikchange Primer Design Program available on the Agilent website (www.agilent.com/genomics/qcpd) and taking into account the company's recommendations. Four point mutations were introduced into the seed region of each putative miR-145 binding site namely at positions 1, 3, 4 and 5 as shown in Figure S2. Presence of the mutated nucleotides was confirmed by sequencing and alignment against the sequence of the WT construct and the reference sequence NM_000259.

Next, HEK293T cells were seeded in RPMI medium supplemented with 10% FCS (Life Technologies Europe B.V., Ghent, Belgium) at a density of 10 000 cells/well in an opaque 96-well plate (Thermo Fisher Scientific, Erembodegem, Belgium). Twenty-four hours after seeding, cells were co-transfected with either the WT or single mutant (Mut1, Mut2 or Mut3) 3'UTR psiCHECK-2 vector construct (100 ng) (Promega Benelux B.V., Leiden, The Netherlands) and 50 nM of the miR-145 mimic (Life Technologies Europe B.V., Ghent, Belgium) or an equal amount of the Pre-miR negative control 2 (NC) (AM17111, Life Technologies Europe B.V., Ghent, Belgium) using 0.4 µl DharmaFECT Duo (Thermo Fisher Scientific, Erembodegem, Belgium). All transfections were conducted three times in triplicate. Cells were lysed and luminescence was quantified 48 hours after transfection using the Dual-Glo Luciferase assay system (Promega Benelux B.V., Leiden, The Netherlands) on a FLUOstar OPTIMA microplate reader (BMG LABTECH, Isogen Lifescience, Sint-Pietersleeuw, Belgium). Data were normalized against the activity of the *Firefly* luciferase gene. The results were reported as an average luciferase activity +/- SEM and statistically evaluated using an unpaired t-test.

Pre-miR / anti-miR transfections and miRNA quantification

To achieve upregulation of miR-145, chemically modified, ds nucleic acids are transfected with minimal cellular stress to mimic endogenous mature miRNAs (pre-miR-145, PM11480, Life Technologies Europe B.V., Ghent, Belgium). This enables detailed study of miRNA biological effects via gain-of-function experiments. Pre-miR negative control 2 (AM17111, Life Technologies Europe B.V., Ghent, Belgium) are random sequence pre-miR molecules that have been extensively tested in cell lines and tissues and validated not to produce identifiable effects on known miRNA function. To induce downregulation of miR-145, small, chemically modified ssRNA molecules, designed to specifically bind to and inhibit

endogenous miRNA molecules, were transfected into the cells (anti-miR inhibitor anti-145, AM111480, Life Technologies Europe B.V., Ghent, Belgium). This enables detailed study of miRNA biological effects via loss-of-function experiments. An anti-miR inhibitor negative control #1 (AM17010, Life Technologies Europe B.V., Ghent, Belgium) was used as a negative control.

For qPCR and western blot analysis, melan-a cells were plated into P60 dishes at a density of 500 000 per dish in RPMI medium without TPA. Twenty-four hours later the medium was replaced with 2.4 ml fresh medium. The final pre-miR-145 or anti-miR-145 concentration was 50 nM after adding 18 µl HiPerFect Transfection Reagent (Qiagen, Hilden, Germany). This solution was mixed by vortexing and incubating for 10 minutes at room temperature to allow formation of transfection complexes. These complexes were added in a drop-wise manner onto the cells. The medium was replaced 24 hours later and gene silencing was monitored at the desired time points by harvesting the cells for either RNA isolation, or used to prepare whole-cell lysates (see below). All transfections were performed in triplicate.

Quantification of miR-145 by TaqMan Real-Time PCR was carried out as described by the manufacturer (Life Technologies Europe B.V., Ghent, Belgium). Briefly, 800 ng of template RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit and the multiplex RT primer pools containing miRNA-specific stem-loop primers. A five times diluted (5x) RT-product was introduced into a 5 µl PCR reaction. Reactions were run in 384-well plates on a 7900HT RT-qPCR system (Applied Biosystems Europe, Halle, Belgium) at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. MiR-145 expression was normalized between different samples based on the values of U6 small nucleolar RNA expression.

Western blotting

Lysates of melan-a cells were made and immunoblotting was performed as previously described by Van Gele *et al*. (2008). Primary antibodies included the purified rabbit anti-Myosin Va exon F (1/5000)) (Van Gele *et al.*, 2008), a rabbit polyclonal antibody against Rab27a (H-60) (1/200, sc-22756, Santa Cruz, Heidelberg, Germany), a polyclonal antibody against Fascin1 (S-18) (1/200, Santa Cruz: sc-16578), a polyclonal antibody PEP7 for the detection of Tyrosinase, provided by Vincent Hearing (Jimenez *et al.*, 1991), and a mouse monoclonal antibody GAPDH clone B-5-1-2 (1/10000, Sigma-Aldrich, Bornem, Belgium). The latter was used to control loading and to perform normalisation. The blots were incubated with the appropriate HRP-conjugated secondary antibody (1/3000, Amersham Biosciences Ltd, Buckinghamshire, UK). Detection was performed with an ECL detection system kit (Amersham Biosciences Ltd, Buckinghamshire, UK). Quantification software (TotalLab, Life Science analysis essentials, TotalLab™ TL100, version 2006c nonlinear dynamics) has been used to quantify the loading of the different proteins after detection with an Ultima 16si (Isogen Life Science, Temse, Belgium).

Immunohistochemistry

To co-immunostain myosin Va and melanosomes, fixed melan-a cells were incubated overnight at 4°C with a polyclonal myosin Va exon F antibody (1/500) (Van Gele *et al.*, 2008) and the monoclonal mouse anti-human melanosome marker HMB45 (1/40, Dako, Heverlee, Belgium). Human primary melanocytes were incubated for 2 hours at RT with a mouse monoclonal NKI-beteb antibody directed against the (pre) melanosomal *silver* protein (1/40, Melanoma-associated antigen, 100kD, (Monosan, Uden, The Netherlands)), together with the myosin Va exon F antibody. The appropriate secondary antibodies were used: an Alexa 594-conjugated mouse anti-rabbit (1/1000) or an Alexa 488-conjugated rabbit anti-

mouse (1/1000) antibody (Life Technologies Europe B.V., Ghent,, Belgium). 4',6-diamidino-2-phenylindole (DAPI) was added during the washing steps to detect cell nuclei. Cells were mounted on glass microscope slides with Glycergel (DakoCytomation, Carpinteria, CA, USA) and confocal images were captured with a Leica Sp5 AOBS confocal microscope (Leica, Mannheim, Germany). Images were taken by using a 63× HCX PL Apo 1.4 oil objective. DAPI was excited with a UV diode laser at 405 nm, Alexa 488 by using the 488 line of a Multi Argon laser and TRITC (or Alexa 594) by a HeNe laser at 543 nm. For actin labelling, cells were incubated with the fluorescent F-actin probe TRITC phalloidin (Sigma-Aldrich, Bornem, Belgium).

Quantification of hypopigmented pellets of human primary melanocytes

In order to follow up on any hypopigmentation effect of miR-145 on human primary melanocytes, harvested cell pellets were closely observed and photographed. This experiment was performed on four individual human primary melanocyte donors. These cells were either transfected with negative (pre-miR) controls, pre-miR-145 or a siRNA aimed against tyrosinase and were harvested 6 days post transfection. Equivalent amounts of cells were spun down in separate wells for each condition and quantified. Quantification was performed by measurement of mean intensities of various regions of interest with the use of Fiji software. The mean values of the two controls were rescaled to one and compared to the treated conditions, which finally resulted in quantification of reduced pigmentation.

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Supplementary Tables

Table S1. List of 16 differentially expressed miRNAs after ssUV and forskolin treatment of melan-a cells. Average down- or upregulation represents the mean (+/-SEM) of three independent treated melan-a cells *versus* non-treated cells (*P*-values were determined by unpaired t-test).

	Average downregulation	+/- SEM	*P*-value
miR-125b	1.7297	0.0564	0.001046
miR-139-5p	1.9895	0.0424	0.000081
miR-145	15.7547	0.8445	0.000013
miR-155	2.5090	0.2547	0.013583
miR-193*	6.0061	1.3398	0.017236
miR-206	3.3674	0.1817	0.000435
miR-218	2.7893	0.1655	0.000983
miR-221	2.7950	0.0381	0.000003
miR-222	3.2741	0.0876	0.000026
miR-28	2.3109	0.1330	0.001939
miR-335*	2.7718	0.0536	0.000012
miR-365	2.4574	0.1444	0.001745
miR-455	3.4144	0.5351	0.020630
	Average upregulation	+/- SEM	*P*-value
miR-130b	2.1078	0.1145	0.0027
miR-182	3.0727	0.1256	0.0002
miR-9	2.7486	0.2019	0.0028

Table S2. Sequences (forward (F) and reverse (R)) of 3'UTR luciferase reporter constructs for mouse *Myo5a*.

Position 123-130 of *Myo5a* 3' UTR (NM_00259) mmu-miR-145 F (site 1) TCGAGAAGTACTGATTATCTCTAAAGGAAGATGTT**AACTGGAA**ATCCACCCCTCCCCCCAGAATC CTTTCGATGC miRNA binding site with 8mer seed (black) mmu-miR-145 R (site 1) GGCCGCATCGAAAGGATTCTGGGGGGAGGGGTGGAT**TTCCAGTT**AACATCTTCCTTTAGAGATA ATCAGTACTTC
Scrambled (Scr) sequence mmu-miR-145_Scr. F (site 1) TCGAGAAGTACTGATTATCTGATTATGCAATAAAA**GATGCGAG**ATCCACCCCTCCCCCCAGAATC CTTTCGATGC scrambled miRNA binding site with 8mer seed (black) mmu-miR-145_Scr. R (site 1) GGCCGCATCGAAAGGATTCTGGGGGGAGGGGTGGAT**CTCGCATC**TTTTATTGCATAATCAGATA ATCAGTACTTC

Table S3. Overview of the primers used to make the 3'UTR construct for human *Myosin VA* for the luciferase reporter assays. The primers amplify a 1.273 kb part of the 3'UTR region of *MYO5A*, containing the three miRNA binding sites. The forward primer (F) is at the 5' end linked to the XhoI restriction site and the reverse primer (R) is at the 5' end linked to the NotI restriction site (highlighted in bold) for ligation into the multicloning site of the psiCHECK™-2 Vector.

Gene	Accession N°	Primer 5'→3'	Annealing Temp	Product size
MYO5A	NM_000259	F: **CTCGAG**TCTTGCCCGAAATAAGAACCCA R: **GCGGCCGC**TCTCTCTCTGTCACCCAGGC	57 °C	1273 bp

Table S4. Primers used for site-directed mutagenesis to create mutant reporter constructs used in the luciferase reporter assays for human *myosin VA*. The miRNA binding seed is underlined and the mutated nucleotides are highlighted in bold.

Gene *MYO5A*	Primer 5'→3'
Site 1	F:GTACTGATTATCTCCCAAATGAGAAGTCATT**C**A**AGT**GAAATCTCCCTAGAATACTTTCATCACTTTG R:CAAAGTGATGAAAGTATTCTAGGGAGAT<u>TTC**ACT**T**G**</u>AATGACTTCTCATTTGGGAGATAATCAGTAC
Site 2	F:TTCTGTTTACAATTCAATCAGATCACAGTTTT<u>**C**A**AGT**GA</u>TTATATGCAAATACCTACAGATTCACCTGC R:GCAGGTGAATCTGTAGGTATTTGCATATAA<u>TC**ACT**T**G**</u>AAAACTGTGATCTGATTGAATTGTAAACAGAA
Site 3	F:TACAGATTCACCTGCACAAGTAGCAGAC<u>**C**C**GTT**AA</u>AGTCATGTAGTAATATGACAAAATGC R:GCATTTTGTCATATTACTACATGACT<u>TT**AAC**G**G**</u>GTCTGCTACTTGTGCAGGTGAATCTGTA

Supplementary Figures

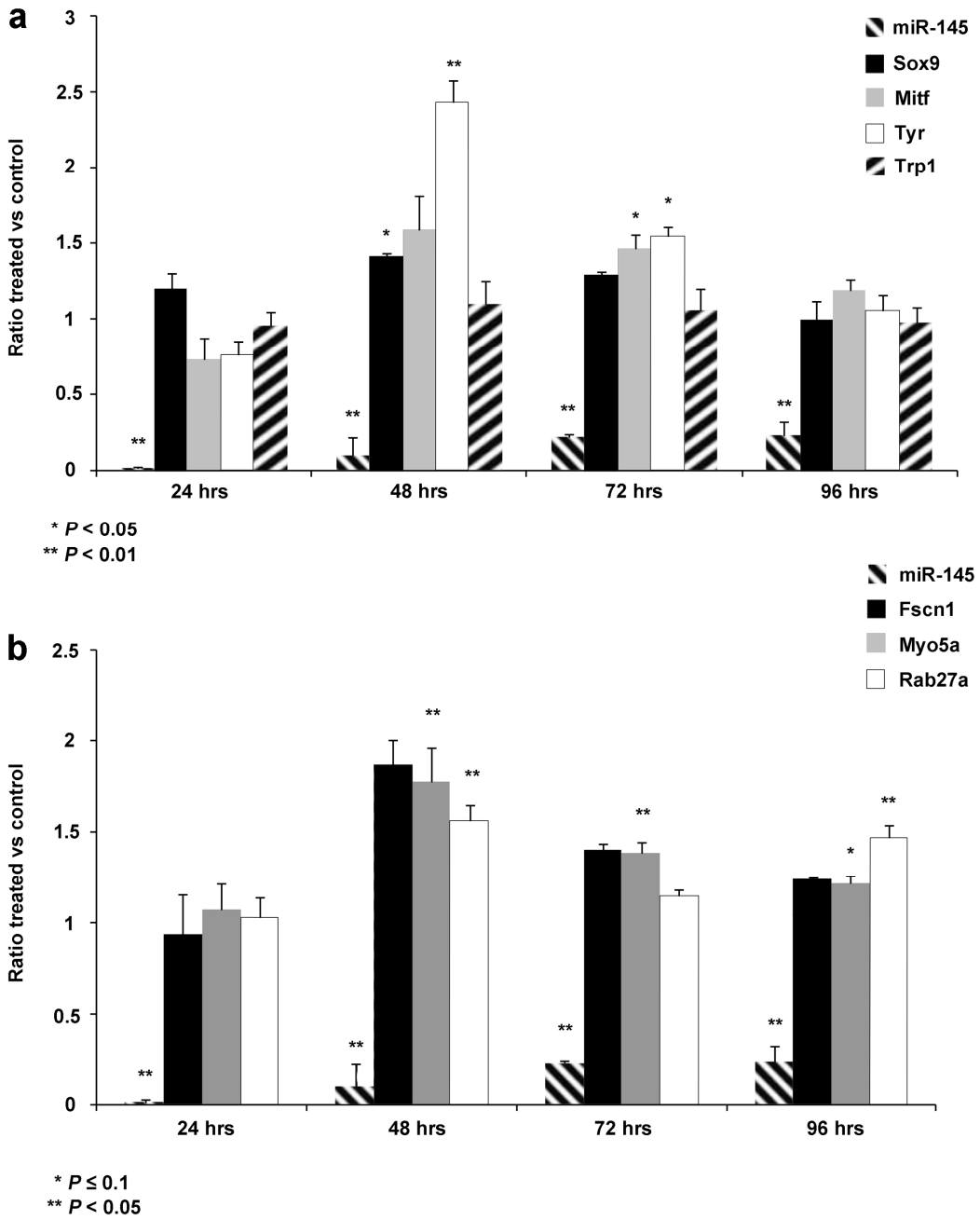

Figure S1. Downregulation of miR-145 induces an upregulation at the mRNA expression level of genes involved in the pigmentary process.
Melan-a cells were transfected with 50 nM anti-miR-145 or with an equal amount of anti-miR negative control #1. Cells were harvested for RNA isolation at 24, 48, 72 and 96 hours post transfection (PT). The relative expression levels of miR-145 and several pigmentation genes were determined by RT-qPCR. The mean ratio of the treated samples *versus* the mean ratio of negative controls was plotted for miR-145 and each analyzed gene at the different time-points. MiR-145 expression was downregulated at each time-point. A maximum increase in gene expression level (ratio >1) was observed at 48 hours PT for genes involved at the onset of pigmentation, except for *Trp1* (**a**) and for genes involved in the transport and transfer of melanosomes to the cell periphery (*Fscn1*, *Myo5a* and *Rab27a*) (**b**). The ratio (treated *versus* control) is shown as the mean (+/-SEM) of three independent experiments. *P*-values were determined with an unpaired t-test.

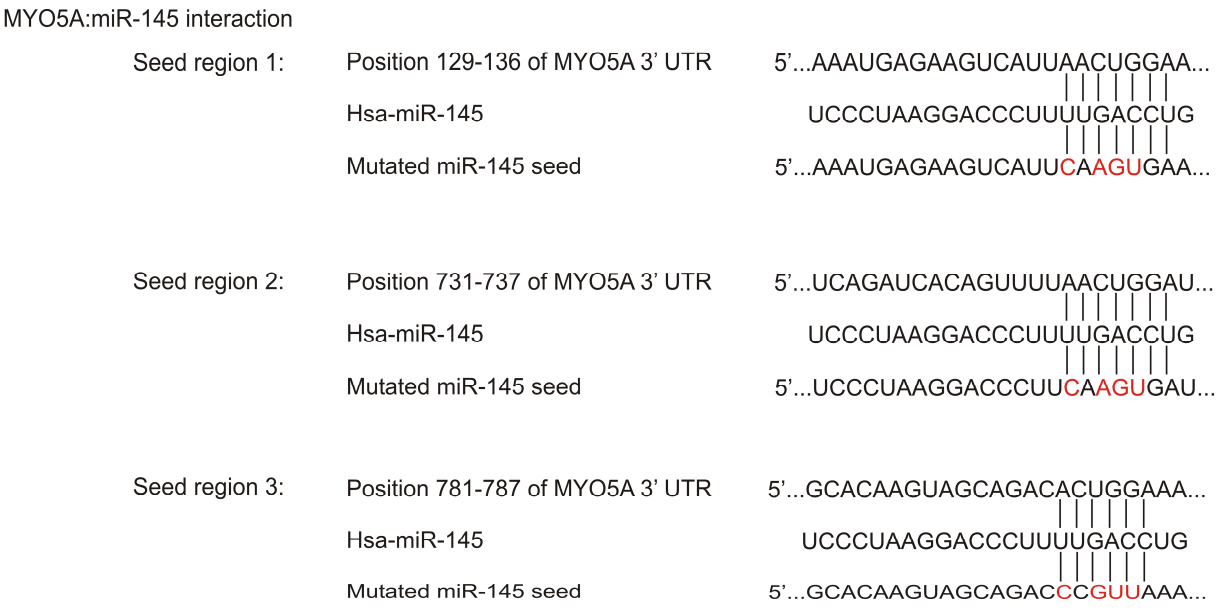

Figure S2. Overview of the three putative miR-145 binding sites in the 3'UTR of human *Myosin VA*. The position of the seed regions is based on the NCBI Reference Sequence NM_000259. Predicted consequential pairing of target region and hsa-miR-145 is indicated by vertical lines. The nucleotides indicated in red were mutated in the mutant 3'UTR constructs.

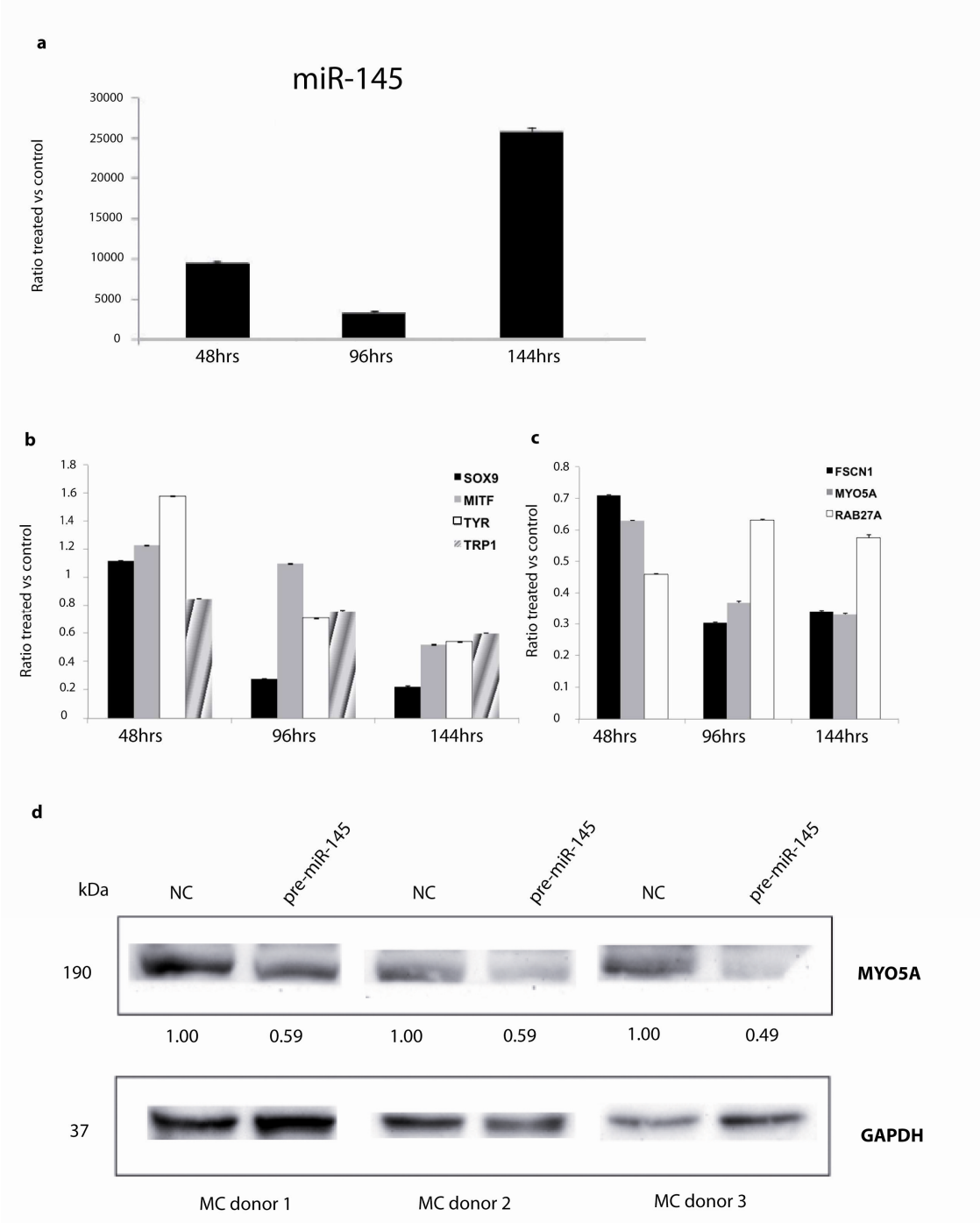

Figure S3. Overexpression of miR-145 in human primary melanocytes results in downregulation of *MYO5A* and other pigmentation genes. Human primary melanocytes (MCs) were transfected with 50 nM pre-miR-145 or with pre-miR negative control. Cells were harvested for RNA isolation at 48, 96 and 144 hours PT. The relative expression levels of miR-145 and a set of pigmentation genes was determined by RT-qPCR. The mean ratio of the treated samples *versus* the mean ratio of negative controls was plotted for miR-145 and each analyzed gene at the different time-points. A clear overexpression of miR-145 was observed (**a**). A time-dependent decrease in gene expression level (ratio <1) was observed for genes involved at the onset of pigmentation (**b**) and for genes involved in the transport and transfer of melanosomes to the cell periphery (**c**). Ratios (treated *versus* control) are the mean (+/-SEM) of qPCR duplicates of human MCs derived from MC donor 1. Data are representative for three independent experiments (i.e. analysis performed on MC derived from other donors). (**d**) Western blots and densiometry values showing downregulation of MYO5A protein in pre-miR-145 transfected MCs *versus* control samples (NC) in three different MC donors.

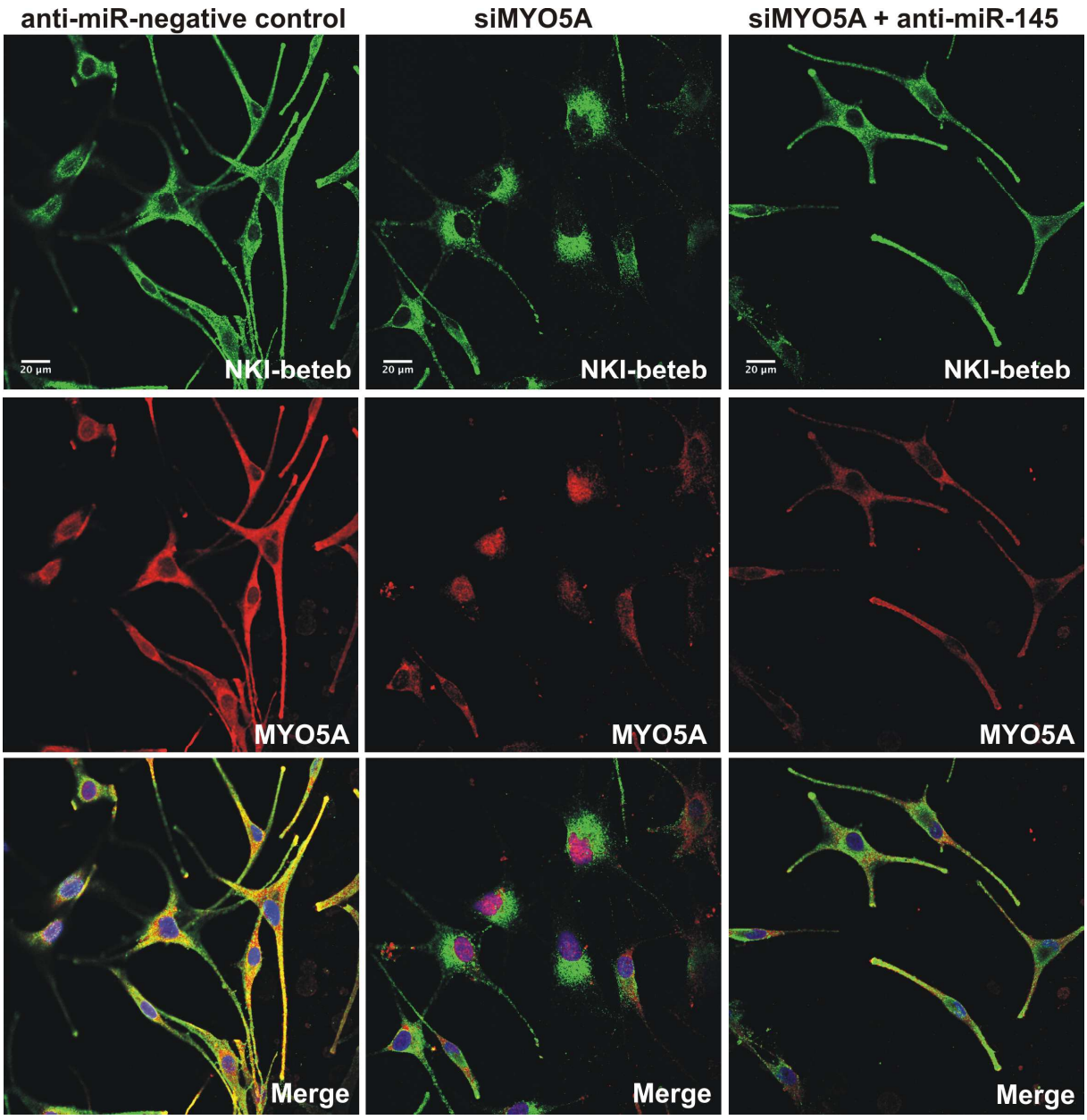

Figure S4. Expression of anti-miR-145 restores the perinuclear accumulation of melanosomes in primary human melanocytes. Human melanocytes were transfected with anti-miR negative control (left), siMYO5A exon F (50 nM) (middle) or co-transfected with 50 nM siMYO5A exon F and 50 nM anti-miR-145 (right). Cells were fixed 6 days after transfection and co-immunostained with the melanosomal marker NKI-beteb (green) and a rabbit polyclonal anti-Myosin Va (MYO5A) exon F antibody (red). Expression of MYO5A is strongly reduced in siMYO5A transfected melanocytes and melanosomes (NKI-beteb) accumulate around the nucleus whereas melanosomes show a peripheral distribution in control-transfected cells. Co-transfection of siMYO5A and anti-miR-145 into human melanocytes results in co-localization of NKI-beteb and MYO5A and a redistribution of the melanosomes to the cell periphery and dendritic tips due to elevated expression of (endogenous) MYO5A. Bars = 20µm.

3.2 Studying the role of miR-145 in melanoma cell lines

Introduction

MiRNAs are known to influence genes in cancer that mediate processes during tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and invasion. The observations made in the previous study strongly suggest that miR-145 is a major, if not the only miRNA regulator that can shut down the entire pigmentation process causing hypopigmentary effects in pigment forming cells (melanocytes). Transformation of melanocytes leads to uncontrolled growth and eventually gives rise to malignant melanoma. Previous studies in other cancer types demonstrated a tumor suppressor function for miR-145. Therefore, an investigation to uncover a potential link, which involves miR-145 in both melanogenesis and melanomagenesis was relevant. The functional analysis involving our melanoma cell lines has provided additional evidence that miR-145 indeed has an invasion suppressor role in malignant melanoma.

Paper:

Peter Dynoodt, Reinhart Speeckaert, Olivier De Wever, Inès Chevolet, Lieve Brochez, Jo Lambert and Mireille Van Gele.
MiR-145 overexpression counteracts migration and invasion of metastatic melanoma cells. Int J of Onc., 7 dec 2012, doi: 10.3892/ijo.2013.1823. (Early online publication)

CHAPTER 4

DISCUSSION AND FUTURE PERSPECTIVES

4.1 Identification of miRNAs involved in pigmentation

miR-145; a novel target to influence pigmentation

The mechanism of formation of skin pigmentation, which is the production of melanin, is notably complex, and involves a multitude of consecutive steps. The pigmentation of human skin results from the synthesis of melanin by dendritic cells located at the basal layer of the skin, called melanocytes. These cells contain organelles, called melanosomes, which are miniature factories that synthesize melanin. Melanosomes are transported to the exterior regions of the cytoplasm, and migrate along the dendrites to be transferred from the melanocytes into keratinocytes, which are the predominant cell type in the outermost layer of the skin. The keratinocytes are transported to the surface of the skin during the epidermal differentiation process (Gilchrest et al., 1996; Hearing and Tsukamoto, 1991).

Since hyperpigmentation disorders result from an overproduction of melanin, which is related to sun exposure, hormonal changes, prescription drugs or acne scarring, it is important to develop novel therapeutic approaches, the rationale of which is based on the inhibition of certain key genes involved during pigmentation. It therefore appears promising to develop novel inhibiting compounds, that are both highly effective, and exhibit good tolerance. Of late, the use of miRNA therapeutics enabled specific applications, not only in the cosmetic field, such as skin or hair care, but also in dermatological, and pharmaceutical fields.

Most synthetic skin-lightening compounds are cytotoxic with respect to melanocytes, potentially causing permanent depigmentation of the skin. These agents are commercially available, and are administered to patients, often with disappointing results. Even the use of hydroquinone, the gold standard product for treatment of hyperpigmentation, is still subject to controversy, due to a bad safety profile (Draelos, 2007; Toombs, 2007). Therefore, there is a high need for novel therapies with a better efficiency and less side effects.

MiRNAs are tiny non-coding, regulatory RNAs, involved in several fundamental cellular processes such as differentiation, proliferation, and apoptosis (Ambros, 2004). They exert their function by binding to a subset of target mRNAs, resulting in translational repression and/or increased degradation of mRNAs. The use of miRNAs makes it possible to obtain a specific inhibition of the synthesis of a target protein by degradation of the mRNA encoding

the protein. The degradation of the target mRNA is obtained through activation of the RISC complex (RNA Induced Silencing Complex), which acts through the binding of the anti-sense strand of the dsRNA to the mRNA (Sun et al., 2010).

Manipulation of miRNA functions, either by mimicking or inhibiting them, is emerging as a highly promising therapeutic strategy. Such developments can contribute to our understanding of melanogenesis and melanoma progression and reveal new targets for selective therapy. Compared to the single-target knock down achieved by siRNA, the miRNA solution simply utilizes naturally occurring components that are inherent to the melanocyte machinery. Depending on the sequence complementary to their targeted gene transcripts, miRNAs can induce either mRNA degradation or suppression of protein synthesis, or both, while siRNAs primarily trigger mRNA degradation. As a result, the effect of miRNAs is generally broader, moderate, specific, and less adverse than siRNAs. Moreover, intrinsic drawbacks of siRNA-induced gene silencing (including off-target effects, elicitation of the interferon response, and interference with the endogenous miRNA biosynthesis) are eliminated when using miRNAs, due to their specific biogenesis and mechanism of action (Judge et al., 2005; Sledz et al., 2003).

The specific objective of this work was to investigate and identify miRNAs involved in the tanning process. Several reports revealed that the expression levels of miRNAs may change in response to different stress factors such as hypoxia and UV treatment (Kulshreshtha et al., 2007; Pothof et al., 2009). In order to investigate this *in vitro*, an experimental pigmentation model was developed, in which we utilized a UV radiation source and addition of a melanogenic stimulator, forskolin, to monitor melanogenesis. Treated mouse melanocytes (melan-a) were irradiated with a solar simulator (UVB+UVA), while untreated cells were used as controls. Using a RT-qPCR based miRNA expression profiling technology, we identified a miRNA signature containing 16 miRNAs, separating differentially expressed miRNAs in treated melan-a cells *versus* untreated cells. In total, 13 miRNAs were found to be downregulated, while only 3 were upregulated. This suggests that the role of miRNAs in melanogenesis is associated with a widespread miRNA repression, rather than an activation. Closer inspection of the 16 differentially expressed miRNAs showed a remarkable expression level of miR-145, which was 15 times downregulated in treated mouse melanocyte cells (melan-a), compared to non-treated controls. Subsequently, functional studies involving the overexpression of this specific miRNA show that a negative moderation of key genes taking

part in melanogenesis, is feasible. This was also confirmed at the protein level. Overexpressing miR-145 in melan-a cells additionally causes the redistribution of melanosomes from the peripheral to the perinuclear region. When transfections of miR-145 mimics are performed in human primary melanocytes, a clear accumulation/retention of melanosomes around the nucleus is observed. A hypopigmentary effect of harvested cell pellets distinguishes miR-145 transfected melanocytes from non-transfected counterparts.

We presume miRNA-145 attacks the melanogenesis pathway along two fronts (Figure 1). One is located at the forefront of melanogenesis, attacking the *SOX9* gene, partially responsible for the initiation of melanogenesis. The other front is situated towards the end of the melanogenesis pathway, including the correct transfer of the melanosomes from the melanocytic dendrites into the keratinocytes. These genes located at the periphery of the melanocytes, are *MYO5A*, *RAB27A*, and *FSCN1*. We propose a working model for miR-145, in which these two fronts cooperate, pulling all the remaining melanogenesis genes (*MITF, TYR and TRP1*,) downwards, leading to a hypopigmentary phenotype.

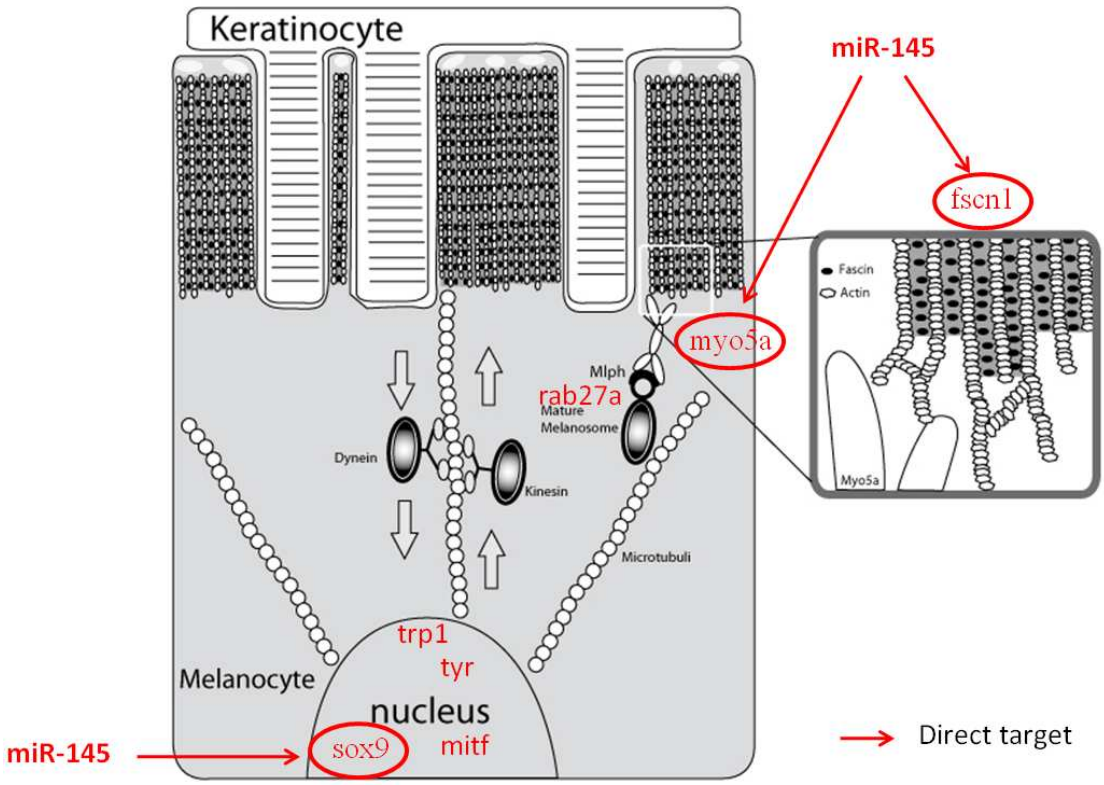

Figure 1: miR-145 mechanism of action

With regard to the experiments using antagomiRs, an increase of the same genes influenced by the miRNA "mimics" was observed. This was only temporally, and gene expression levels returned to basal levels 72 hours after transfections. Knockdown of miR-145 is insufficient to switch on the melanogenesis process *in vitro*. In order to switch "ON" these important processes, subsequent key regulators probably also need to be switched on to further proceed along the melanogenesis pathway. We hypothesize that it is easier to shut down certain processes, than to enable a complex pathway, which involves a multitude of consecutive steps to run smoothly.

Parallel with this miRNA signature, it would be interesting to integrate mRNA signatures which could broaden the overall understanding of how these players interact. We were able to determine that miR-145 can downregulate *Sox9, Mitf, Tyr, Trp1, Myo5a, Rab27a,* and *Fscn1*. As such, we only determined a subset of genes that are directly or indirectly regulated by this specific miRNA. To obtain a comprehensive understanding of the genes that are influenced by miR-145, a genome wide expression analysis to dissect and analyze the genes that are involved in, or controlled by, the transcriptional regulatory function of this specific miRNA is essential. This could also be a way of establishing what kind of side effects to expect when utilizing miR-145 for potential further treatments.

With regard to the miRNA signature containing 16 differentially expressed miRNAs, we opted for the study of a single miRNA most likely to have the largest biological effect. The choice for miR-145 was obvious. The role that other miRNAs play during the pigmentation process still needs to be clarified. Synergistic or antagonistic effects must be taken into consideration when multiple miRNAs cooperate to repress a gene or pathway. Overexpressing one of these miRNAs might not produce any measurable effect, leading to the false conclusion that this miRNA is not involved in the pigmentary process.

Topical application of miRNAs into the skin is one of the last and major hurdles that need to be taken in order to accomplish a miRNA-based treatment against skin disorders. The barrier of the *stratum corneum* needs to be breached in order to enable nucleic acids to reach the melanocytes located at the basal layer of the skin. A non-viral lipid-based carrier for cutaneous gene therapy has been optimized and characterized in our lab. This liposomal formulation, termed SECosomes is highly efficient, not only in cultured skin cells, but also in excised human skin explants (Geusens et al., 2009). An *ex vivo* 3D pigmented human

reconstructed skin model, suitable for functional testing of RNAi-induced depigmentation is available, and investigations into translating a miRNA-based depigmentation agent into a proof-of-principle are warranted (Van Gele et al., 2011). One previous study reported the development and use of a specific miRNA (miR-434-5p) product, able to reduce pigmentation successfully *in vitro* as well as *in vivo,* without any detectable cytotoxic effects (Wu et al., 2008). This illustrates the feasibility of the use of miRNA-mediated skin lightening agents. Additionally, skin pigmentation models are regarded as excellent tools to study the efficiency/potential of newly developed "small RNA" delivery vehicles, and can be combined with any other easy to follow miRNA expression. Eventually, these delivery vehicles can then be combined with other miRNA mimics/antagomirs, leading to novel miRNA-mediated topical therapies to treat skin disorders such as psoriasis, atopic dermatitis and melanoma. Applied research introducing miRNAs to the clinical realm is not only a big challenge for therapeutic companies, but also for academic researchers and dermatologists (Baker, 2010).

It is recommended to view the potential ethical implications that are associated with developing pigment-altering therapeutics and the bioethical debates that have sparked up through these endeavors. Certainly in the context of recent discoveries relating to genes associated with the pigmentation process, which could suggest opportunities into creating effective skin-lightening products. Various societies consider the urge to use skin whitening products, roots from a racism perspective, while other societies regard this as reaching out to a definition of beauty or attractiveness. Considering another undertone where the degree of some ones skin tone can be associated to unequal distributions of income, job opportunities, and education, consequently leading to prejudiced social, cultural, and economic positions in society, seems highly unfair (Dadzie and Petit, 2009). In line with this it has even been confirmed that skin tone can directly affect health outcomes through differences in the quality of health care services provided to some racial minorities and indirectly through the aforementioned inequalities (Daniels, 2009; Hersch, 2008).

Isn't it is the obligation of society to provide treatments and enhancements for the purpose of meeting general needs and removing barriers that restrict equal opportunities, thus promoting the equality of opportunity? Well, by developing an appearance-altering skin-lightening therapeutic, one could eventually have a positive impact on the health of its recipients seen in the light of race and health issues. On the other hand, skin-lightening technology is controversial and could further undermine racial differences and public support for cooperative healthcare systems due to racial and financial reasons. Justice systems should

overlook the values that affect opportunity as well as prioritizing what the healthcare needs. Justice may even be required to restrict access to race-related enhancements if it creates even more differences by unfairly distribution. These restrictions should be coordinated at an international level, which encompasses all countries once certain products have been considered dangerous for health. This should be done to avoid over-the-counter sales of skin-lightening products in certain countries while they are banned in others, due to health concerns (e.g.; hydroquinone). It is also important to fore come potential health risks by educating the public that it is important to be safe than sorry when using these products. In line with these statements it seems highly relevant that the right centers are set up with the right specialists who can inform the public what the right choices are after the appropriate research has been performed. These bioethical challenges are by no means restricted to skin-lightening technologies but can also be applied to a broad scope of enhancement technologies that are currently growing.

4.2 Unraveling the role of miR-145 in melanoma

miR-145 overexpression counteracts migration and invasion of metastatic melanoma cells

Several factors such as heritable predisposition and genetic mutations increase the risk to melanoma. Ultraviolet radiation such as that emitted by the sun is directly absorbed by DNA, which causes genetic mutation, and is a major risk factor for all types of skin cancers including malignant melanoma. Genetic mutations in melanocytes are associated with histological changes along a continuum that may accommodate an invasive malignancy. MiRNAs are known to be aberrantly expressed in many cancer types, and can either exert tumor suppressive or oncogenic functions. Loss-of-function or gain-of-function of tumor suppressor miRs or oncomiRs, respectively could influence the progression of melanoma. Manipulation of these molecules as potential targets for cancer therapies has generated great interest. They can also serve as diagnostic markers to predict patient prognosis and outcome. General therapeutic strategies in relation to antisense-mediated inhibition of oncogenic miRNAs, and miRNA replacements with miRNA mimics or viral vector-encoded miRNAs, are currently under investigation (Trang et al., 2008).

By overexpressing miR-145 in melanoma cell lines with different metastatic potential, and performing several functional studies we aimed to investigate the tumor suppressor role of

miR-145. Three melanoma cell lines were examined in gain-of-function experiments involving miR-145. The results from these experiments show that by overexpressing miR-145 the metastatic potential and progression of malignant melanoma cells can be decreased thereby demonstrating an invasion suppressor function of this miRNA. Combined with an appropriate (topical) delivery system this miRNA is potentially a useful target in the therapy of melanoma. To uncover the mechanism on how miR-145 accomplishes its invasion suppressor role we examined the expression levels of target genes *FSCN1*, *MYO5A*, *SOX9* and of one indirect target (*RAB27A*) after overexpression of miR-145. Neither *SOX9*, *MYO5A* nor *RAB27A* were involved in the biological effects caused by miR-145 mimics.

In several previous cancer studies knockdown of *FSCN1* by miR-145 has demonstrated its contribution to oncogenesis and progression of these cancer types (Chiyomaru et al., 2010; Fuse et al., 2011). We therefore focused on the role of *FSCN1* and its interaction with miR-145 in melanoma. In contrast to these previous studies, where the knockdown of *FSCN1* leads to a reduced cell migration, we observed an increase in cell invasiveness, and thus an increase in metastatic potential in two of our cell lines, namely, BLM and WM793. We hereby hypothesize that *FSCN1* exerts a different role in melanoma compared to other tumor types and that other target genes of miR-145 that do not take part in the FSCN1 pathway such as *MUC1*, *MMP-11*, *FLI1* and *JAM-A* could be involved in cell migration and invasion (Gotte et al., 2010; Sachdeva and Mo, 2010; Zhang et al., 2010). In breast cancer cell lines it has been suggested that miR-145 leads to suppression of invasion, and metastasis by directly targeting *MUC1*, which in turn causes the downregulation of *β-catenin*, *cyclin D1*, and *cadherin 11* (Sachdeva and Mo, 2010). While in another study the inhibition of breast cancer cell invasiveness is attributed to the direct targeting of miR-145 towards JAM-A, a membrane protein involved in cell-cell adhesion and cell motility regulation, and to the knockdown of the actin-bundling protein fascin (Gotte et al., 2010). Further in depth studies, by using microarray analysis for example between miR-145 transfected and control melanoma cells which could eventually lead to identification of invasion targets of miR-145, will be necessary to uncover the precise mechanism on how miR-145 performs its invasion suppressor role in melanoma.

The transition of a normal melanocyte to metastatic melanoma may involve several histological intermediates including an atypical/dysplastic nevus of varying stages of severity, or melanoma *in situ* and invasive melanoma. Indeed, melanoma diagnosed and treated during

the radial growth phase have been shown to have an excellent prognosis. Currently, there is no cure for late stage melanoma. As a result, early detection plays a critical role in reducing melanoma morbidity and mortality. MiR-145 may be a potential prognostic marker to predict if melanoma may become metastatic. New prognostic tools to complement the visual examination of lesions, especially early-stage melanoma and borderline lesions, and those that lack the classic appearance of melanoma could significantly enhance the detection process, thereby increasing survival rates of patients.

General Conclusion

We were able to identify miR-145 as a key player during melanogenesis. Attention should be paid to potential side effects, and more research is needed on the possible invasion suppressor role that miR-145 might possess in melanoma.

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CHAPTER 5

SUMMARY-SAMENVATTING-RÉSUMÉ

5.1 Summary

Melanogenesis is a complex process that takes place when melanocytes are activated by UVR emitted by the sun, followed by the generation of melanin. In a response, melanocytes protect the skin against the harmful effects of UVR by producing melanin-containing melanosomes, and by transferring them to the neighbouring keratinocytes where the melanin pigment serves as a shield against UV damage. Like most of these processes, distinct signaling pathways, which result in the activation of specific transcription factors, and a whole range of subsequent reprogramming of gene expression, will help the melanocyte to overcome the harmful UV effects. How these processes are delegated and controlled has not yet been completely elucidated. MiRNAs have recently been shown to be regulators of gene expression, and are known to be involved during stress induced responses; but until to date, no miRNAs have been associated with UV stress resulting in the augmentation of ultraviolet-induced pigmentation. It would therefore be interesting to find a link between melanogenesis and miRNA gene regulation, adding a new layer of control during pigmentation. Additionally, this can open new paths towards the discovery and development of innovative therapies for the treatment of patients with skin pigmentation disorders.

The primary aim of this thesis is to determine which miRNAs are involved during the pigmentation process, and to analyze their biological function in melanocytes. We investigated the effects of solar simulated UV (ssUV), and forskolin, on pigmentation in mouse melanocytes (melan-a), following an experimental model, involving several consecutive treatments. These treatments stimulate the processing and transport of melanosomes, which play an essential role during pigmentation. Melanocytes that did not receive these treatments were used as controls. Next, total RNA, containing all miRNAs, was isolated from these melanocytes. To determine the expression levels of these miRNAs, a high-throughput and sensitive miRNA expression profiling platform was subsequently applied to measure the miRNA expression levels of melanocytes treated with ssUV and forskolin *versus* untreated melanocytes. By comparing the miRNA profiles of treated melanocytes *versus* control melanocytes, we were able to profile 540 miRNAs. Based on these data, a miRNA signature (consisting of 16 miRNAs) associated with ssUV and forskolin treatments was established for the first time. Among the miRNAs that were differentially regulated one specific miRNA showed a marked knockdown in the treated melanocytes: miR-145 displayed a 15-fold downregulation. Functional studies were performed wherein we assessed whether the absence or overexpression of this miR-145 altered cell function (phenotypical effect).

Overexpression studies (gain-of-function) of miR-145, in mouse and human melanocytes, showed that several important pigmentation genes were influenced by this miRNA, both at the gene and protein expression level. One of them was the motor protein myosin VA (MYO5A) involved in intracellular melanosome transport in melanocytes through the formation of a RAB27A-MLPH-MYO5A tripartite complex. By use of a luciferase reporter assay we were able to demonstrate direct targeting of *MYO5A* by miR-145 in mouse and human melanocytes. In addition, gain-of-function studies clearly demonstrated a biological response in primary human melanocytes cultured *in vitro*, whereby the melanocytes obtained a hypopigmentary effect in a time dependent manner, as a result of perinuclear accumulation/retention of melanosomes. In conclusion, our results reveal that miR-145 is a key regulator of the pigmentary process. Consequently, miR-145 could possibly become a potential target in the treatment of skin pigmentation disorders.

In the second part of the thesis we studied the role of miR-145 in melanoma cell lines. Previous studies have already mentioned miR-145 as a tumor suppressor in other tumor types. Due to the fact that melanoma is generated from malignant melanocytes, and that miR-145 plays such a prominent role during the pigmentation process, we decided to investigate the effects of miR-145 in several melanoma cell lines, and to examine if the same tumor suppressor functions could be observed in melanoma. Several functional studies involving wound healing assays, proliferation assays, and matrix cell invasion assays, were performed after overexpressing miR-145 in the melanoma cell lines. These gain-of-function experiments in invasive melanoma cell lines resulted in a suppression of cell migration and invasive potential, while an anti-proliferative effect was observed in a primary, non-invasive melanoma cell line. In previous studies, involving other tumor types, it was shown that miR-145 exerts its tumor suppressor role via *FSCN1*, but surprisingly when knocking down *FSCN1* in our melanoma cell lines, this did not inhibit cell proliferation and migration. On the contrary, *FSCN1* knockdown increased cell invasion leading us to suggest that miR-145 does not necessarily act via targeting *FSCN1*. Further studies will be needed to clarify the mechanism of action that miR-145 has as a tumor suppressor. The overall results from these experiments show that by overexpressing miR-145, a decrease in cell migration, and invasive potential of malignant melanoma is observed, showing a reduced invasion suppressor function of this miRNA.

5.2 Samenvatting

Melanogenese is een complex proces dat plaatsvindt wanneer melanocyten worden geactiveerd door UV radiatie (UVR) van de zon, met als gevolg de productie van melanine. Als reactie op UVR, beschermen melanocyten de huid tegen de schadelijke effecten van UVR door het produceren van melanine bevattende melanosomen, en door hen over te brengen naar aangrenzende keratocyten, waar het melanine pigment dient als schild tegen UV schade. Zoals bij de meeste van deze processen worden de melanocyten bij het overwinnen van de schadelijke UV effecten geholpen door verschillende signaleringsroutes die de activatie van specifieke transcriptiefactoren tot gevolg hebben, en door een hele reeks van daaropvolgende herprogrammaties van genexpressie, Hoe deze processen worden gedelegeerd en gecontroleerd is nog niet volledig duidelijk. Recent werd aangetoond dat miRNAs regulatoren zijn van genexpressie, en wij weten dat zij betrokken zijn bij stress geïnduceerde respons, maar tot op heden werden geen miRNAs geassocieerd met UV stress, of met een toename van ultraviolet-geïnduceerde pigmentatie. Het zou daarom interessant zijn een verband te vinden tussen melanogenese en miRNA genregulatie, om aldus een nieuw controleniveau aan het pigmentatieproces toe te voegen. Dit kan tevens perspectieven bieden voor de ontdekking en ontwikkeling van innovatieve therapieën voor de behandeling van patiënten met huidpigmentatie aandoeningen.

Het hoofddoel van deze thesis was het bepalen van welke miRNAs betrokken zijn bij het pigmentatieproces, en het analyseren van hun biologische functie bij melanocyten. Wij onderzochten de effecten van gesimuleerde zon UV (ssUV), en forskoline, op pigmentatie in muis melanocyten (melan-a), volgens een experimenteel model met verschillende opeenvolgende behandelingen. De behandelingen stimuleren de productie en het transport van melanosomen, die een essentiële rol spelen tijdens pigmentatie. Melanocyten die geen behandeling hadden ondergaan werden gebruikt als controle. Vervolgens werd totaal RNA, dat alle miRNAs omvat, geïsoleerd uit deze melanocyten. Om het expressieniveau van deze miRNAs te bepalen, werd een hoge doorvoer en een gevoelige miRNA expressie profilering platform toegepast, ten einde de miRNA expressieniveaus van met ssUV en forskoline behandelde, *versus* niet behandelde melanocyten te meten. Door het vergelijken van miRNA profielen van behandelde *versus* controle melanocyten, konden 540 miRNAs worden geprofileerd. Steunend op deze gegevens werd voor de eerste keer een miRNA signatuur

(bestaande uit 16 miRNAs) geassocieerd met ssUV en forskoline behandelingen opgesteld. Eén van de onder de in behandelde melanocyten differentieel gereguleerde miRNAs, vertoonde een uitgesproken knockdown effect: miRNA-145 was 15-voudig neergereguleerd. Functionele studies werden uitgevoerd waarbij we inschatten of de afwezigheid of overexpressie van dit miR-145 de celfunctie wijzigt (fenotypisch effect). Overexpressiestudies (gain-of-function) van miR-145, in melanocyten afkomstig van zowel muis als mens, toonden aan dat verschillende belangrijke pigmentatiegenen werden beïnvloed door dit miRNA, zowel op gen- als proteïne expressieniveau. Eén van deze was het motorproteïne myosine VA (MYO5A), betrokken bij intracellulair melanosoomtransport, via de vorming van een RAB27A-MLPH-MYO5A driedelig complex. Door middel van een luciferase reporter test konden we directe targeting van MYO5A door miR-145, in melanocyten afkomstig van zowel muis als mens, aantonen. Tevens demonstreerden gain-of-function studies een duidelijke biologische respons *in vitro*, in primaire humane melanocyten, waarbij de melanocyten een tijdsafhankelijk hypopigmentair effect verwierven, ten gevolge van accumulatie/retentie van melanosomen. Uiteindelijk tonen onze resultaten aan dat miR-145 een centrale regulator is van het pigmentatieproces. Bijgevolg kan miR-145 mogelijks een beloftevol doelwit worden in de behandeling van huidpigmentatie aandoeningen.

In het tweede deel van deze thesis bestudeerden we de rol van miR-145 in melanoom cellijnen. Vroegere studies vermeldden reeds miR-145 als een tumor suppressor in andere tumortypes. Doordat melanoom ontstaat vanuit kwaadaardige melanocyten, en door het feit dat miR-145 een dermate belangrijke rol speelt tijdens het pigmentatieproces, werd besloten de effecten van miR-145 op verschillende melanoom cellijnen te onderzoeken, ten einde vast te stellen of dezelfde tumor suppressor functies konden worden vastgesteld in melanoom. Verschillende functionele studies, waaronder wondheling- en proliferatietesten, en matrix cel invasietesten, werden uitgevoerd na overexpressie van miR-145 in de melanoom cellijnen. Deze gain-of-function experimenten in invasieve melanoom cellijnen resulteerden in suppressie van celmigratie en invasief potentieel, terwijl een anti-proliferatief effect werd waargenomen in een primaire, non-invasieve melanoom cellijn. In vroegere studies met andere tumortypes, werd aangetoond dat miR-145 zijn tumor suppressor rol vervult via *FSCN1*, maar bij een knockdown van *FSCN1* in melanoom cellijnen, konden wij nochtans geen inhibitie van celproliferatie en migratie vaststellen. Daarentegen verhoogde *FSCN1* knockdown celinvasie, hetgeen er ons toe aanzet te suggereren dat miR-145 niet noodzakelijk

via *FSCN1* targeting werkt. Verdere studies zullen nodig zijn om het mechanisme van miR-145 als tumor suppressor te ontrafelen. In het algemeen tonen deze resultaten aan dat door overexpressie van miR-145, een afname in celmigratie, en invasief potentieel van kwaadaardig melanoom kan worden vastgesteld, duidend op een verminderde invasie suppressor functie van dit miRNA.

5.3 Résumé

La mélanogenèse est un mécanisme complex qui se déroule quand les mélanocytes sont activées part le UVR émis par le soleil, suivi par la génération de mélanine. Dans une réaction, les mélanocytes protègent la peau contre l'impact nuisible des UVR, en produisant des mélanosomes contenants de la mélanine, et en les transférants aux kératocytes envoisinantes, où le pigment mélanine sert de bouclier contre les dégâts causés par l'UV. Comme la plupart de ces mécanismes, des sentiers de transmission particuliers, qui résultent dans l'activation de facteurs de transcription et tout une gamme de reprogrammation d'expression de gènes ultérieures, aideront le mélanocyte à vaincre les effets nuisibles de l'UV. La manière dont ces mécanismes sont délégués et contrôlés, n'a pas encore été entièrement élucidé. Les MiRNA ont récemment été reconnus comme des régulateurs d'expression de gènes, et sont connus comme impliqués dans des processus provoqués par le stress, mais jusqu' aujourd'hui, aucun miRNA n'a été associé avec le stress causé par l'UV, résultant dans une augmentation de pigmentation provoquée par l'ultra-violet. Ainsi il serait intéressant de trouver un lien entre la mélanogenèse et la régulation des gènes par le miRNA, ajoutant une nouvelle couche de contrôle pendant la pigmentation. En plus, ceci pourrait ouvrir de nouveaux sentiers dans la découverte et le développement de thérapies innovatives pour le traitement de personnes affectées par des troubles de pigmentation de la peau.

Le but prinipal de cette thèse est de déterminer quels miRNA sont impliqués dans le processus de pigmentation, et d'analyser leur fonction biologique dans les mélanocytes. Nous avons étudiés les effets d'UV solaire simulé (ssUV), et de forskoline sur la pigmentation dans des mélanocytes de souris (melan-a), suivant un modèle expérimental, impliquant différents traitements consécutives. Ces traitements stimulent le développement et le transport des mélanosomes qui jouent un rôle essentiel pendant la pigmentation. Des mélanocytes sans traitement étaient utilisés comme contrôle. Ensuite, la totalité d' RNA, contenant tous les miRNA, était isolée de ces mélanocytes. Pour déterminer les niveaux d'expression de ces miRNA, un plate-forme profilé à haut débit et sensifif à l'expression du miRNA était appliqué pour mesurer les niveaux d'expression du miRNA des mélanocytes traités avec ssUV et forskoline, versus des mélanocytes non traités. En comparant les profiles des mélanocyrtes traités versus les mélanocytes de controle, nous étions capables de profiler environ 540 miRNA. Basé sur ces données, une signature miRNA (comportant 16 miRNA) associée avec

les traitements ssUV et forskoline, était établie pour la première fois. Parmis les miRNA régulés différentiellement, un miRNA spécifique montrait un knock-down prononcé dans les mélanocytes traités: miR-145 étalait une régulation de 15 fois à la baisse. Des études fonctionnelles étaient effectuées pour évaluer si l'absence ou la surexpression de ce miR-145 changait la fonction cellulaire (effet phénotypique). Des études de surexpression (gain d'expression) du miR-145, dans des mélanocytes de souris et humains, démontraient que plusieurs gènes de pigmentation importantes étaient influenencées par cet miRNA, autant sur le niveau d'expression génétique que sur le niveau des protéines. Parmis ces derniers se trouvait la protéine moteur myosine VA (MYO5A), impliquée dans le transport intracellulaire des mélanosomes par la formation d'un complexe tripartite RAB27A-MLPH-MYO5A. En utilisant un essai reporter luciferase, nous étions capables de montrer un ciblage direct du MYO5A par le miR-145 dans des mélanocytes de souris et humains. En plus, des études de gain de fonction démontraient clairement une réponse biologique dans des mélanocytes primaires humains, cultivés in vitro, par lequel les mélanocytes obtenaient un effet hypopigmentaire en fonction du temps, à la suite d'accumulation/rétention périnucléaire des mélanosomes. En conclusion, nos résultats démontrent que le miR-145 est un régulateur clé du processus de pigmentation. Par conséquent, le miR-145 pourrait se montrer un cible potentiel dans le traitement de troubles de pigmentation de la peau.

Nous avons étudié le rôle de miR-145 dans des lignées cellulaires de mélanomes dans la deuxième partie de la thèse. Des études antérieures ont déjà mentionné miR-145 comme un suppresseur de tumeurs dans d'autres types de tumeurs. Par le fait que le mélanome est généré à partir de mélanocytes malignes, et que miR-145 joue un rôle tellement important dans le processus de pigmentation, nous avons choisi d'examiner les effets de miR-145 dans plusieurs lignées cellulaires de mélanomes, et d'examiner si les mêmes fonctions de suppression de tumeurs puissent être observés dans des mélanomes. Plusiers études fonctionnelles impliquant des essais de cicatrisation de plaies, des essais de prolifération, et des essais d'invasion de cellules matrices, étaient effectuées après avoir surexprimé miR-145 dans les lignées cellulaires de mélanomes. Ces essais de gain de fonction dans des lignées cellulaire de mélanomes envahissantes, résultaient dans une suppression de migration cellulaire et de potentiel envahissant, tandis qu'un effet anti-proliférative était observé dans une lignée cellulaire primaire, non-envahissante de mélanomes. Dans des études antérieures, impliquant d'autres types de tumeurs, il était établie que miR-145 déploie son rôle de suppresseur de

tumeurs par FSCN1, mais surprenant, en knocking down FSCN1 dans les lignées cellulaires de mélanomes, ceci n'inhibait pas la prolifération cellulaire et la migration. Au contraire, *FSCN1* knockdown augmentait l'invasion cellulaire, nous menant à suggérer que miR-145 n'agit pas nécessairement par le ciblage du *FSCN1*. Des études ultérieures seront nécessaires pour clarifier le mécanisme d'action du miR-145 comme suppresseur de tumeurs. Le résultat global de ces essais montre qu'en surexprimant miR-145, une réduction de migration cellulaire et du potentiel envahissant de mélanome maligne étaient observé, démontrant une fonction de suppression envahissante réduite du miRNA.

ADDENDUMS

CURRICULUM VITAE

Curriculum Vitae

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Education

1996-1998: High School – KTA Bruges - Sport-Sciences

1998-2004: Hogeschool Ghent - **Industrial Engineer in Agriculture and Biotechnology** -
Major: Applications in cell and gene techniques
Minor: Horticulture

Dissertation: In vitro regeneration of shoot meristems with the aid of new benzyladenine-derivatives
Promotor: Prof. Dr. ir. S. Werbrouck

2004-2005: Ghent University, Faculty of Science - **Master of Molecular Plant Biotechnology**

Dissertation: Molecular and morphological characterization of the nodule senescence process in the model legume *Medicago truncatula*
Promotor: Prof. Dr. M. Holsters

Publications

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Title: miR-145 for use to modulate skin pigmentation
Inventors: Jo Lambert, Mireille Van Gele, Peter Dynoodt and Pieter Mestdagh
Provisional Patent Application for the United States:
United States Patent and Trademark Office (USPTO) confirm priority date 03/08/2012 and attributed submission number (US 61/679,126).

Conferences

November 3rd-5th, 2008 – RNAi – The RIGHT Track to Therapy – Brussels, Belgium

September 21st-24th, 2011 – 21st International Pigment Cell Conference (IPCC) – Bordeaux, France

"Skin and Other Pigment Cells: Bridging Clinical Medicine and Science"

Courses

November 27th-28th, 2008 – Biogazelle accelerating your analysis – Ghent, Belgium

"Course on qPCR experiment design and data-analysis"

May 9th-13th, 2011 – VIB – VUB workshop on Entrepreneurship in Life Sciences – Brussels, Belgium

Abstracts/Posters

September 4th-7th, 2010 – 16th Meeting of the European Society for Pigment Cell Research - Hinxton, United Kingdom

"Development of a 3D pigmented skin model to evaluate RNAi-induced depigmentation."

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