A study of the protein kinase MK5's effect on melanoma cell proliferation

Master Thesis in Medical Biology

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By

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Abstract

The mitogen-activated protein kinases (MAPKs) play a crucial role in cellular processes such as proliferation, differentiation, apoptosis, metabolism and motility. Both conventional and atypical MAPKs can phosphorylate other proteins referred to as MAPK- activated protein kinases (MAPKAPK) such as MK5 or p38-regulated /activated protein kinase. Mutations in the *MK5* gene have been detected in lung, melanoma and skin cancer tissue. Several studies pointed to MK5 as an important player in mediating senescence in skin and hematopoietic cells and in inhibiting tumor- migration in osteosarcoma U2OS cells. MK5 can also reduce the invasiveness in breast cancer. However, once the tumor is established, MK5 promotes angiogenesis. The latter finding indicates that MK5 may possess oncogenic potentials.

In this study we investigated the possible anti-proliferative role of MK5 in melanoma cells with BRAF (A375 and WM266-4) and NRAS mutation (SK-MEL-2). Melanoma cells that transiently overexpressed wild-type, constitutive active or kinase dead MK5, or cells that were stably transfected with constructs for these MK5 variants were monitored for cell proliferation using MTT assay and BrdU incorporation. The expression of the senescence markers senescence-activated-β-galactosidase, $p21^{Cip1/Waf1}$ and $p16^{INK4A}$ was also tested in these melanoma cell lines.The p21 transcripts were expressed and p21 promoter activity or senescence –activated β-gal activity was elevated in A375 and WM266-4 cells expressing constitutively active MK5, suggesting a possible role for MK5 in mediating senescence in these cells. Transiently activation of MK5 by forskolin treatment of WM266-4 and SK-MEL-2 cells also reduced the proliferation rate compared to non-treated cells. However, others experiments with SK-MEL-2 cells indicated that inactive MK5 reduced proliferation, whereas MK5 wild type overexpression had the opposite effect, suggesting a pro-oncogenic role of MK5 in these cells which depends on the enzymatic activity of the kinase. Further studies are required to elucidate the exact role of MK5 in melanoma. Since skin cancer is one the most aggressive cancers, determining the exact contribution of MK5 in melanoma may be helpful in using MK5 as therapeutic target.

Abbreviations

AKT	Protein kinase B or PKB
Amp	Ampicillin
APAF 1	Apoptotic protease activating factor 1
ARID2 AT	Rich interactive domain containing protein 2
ATM	Ataxia telangiectasia mutated
bp	Base pairs
BrdU	Bromodeoxyuridine
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
CDKN2A	Cyclin- dependent kinase inhibitor 2a or $p16^{INK4}$
CRC	Colo-rectal cancer
DMBA	Dimethylbenzanthracene
EGFP	Enhanced green fluorescent protein
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FSK	Forskolin
GAPs	GTPase - activating proteins
GRIN2A	Glutamate receptor subunit epsilon-1
GEFs	Guanine nucleotide exchange factors
Hrs	Hours
IFN-γ	interferon gamma
JNK	c-Jun N-terminal kinase
Kan	kanamycin
kDa	Kilo Dalton
MAPK	Mitogen-activated protein kinase
MAPKAPK	Mitogen-activated protein kinase (MK)
MAPKK	MAPK kinase (=MAP2K)
MAPKKK	MAPKK kinase (=MAP3K)
MC1R	Melanocortin 1 receptor
MEF	Mouse embryonic fibroblast
MEK	Mitogen –activated protein kinase kinase
MITF	Microphtalmia associated transcription factor
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-dephenyl tetrazolium bromide
NES	Nuclear export signal
NLS	Nuclear localization signal
NH3T3	Mouse embryo fibroblast cells line established at the National Institute of Health
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor
PKA	cAMP-dependent protein kinase or protein kinase A
PRAK	p38-regulated and activated protein kinase
PTEN	Phosphatase and tensin homolog

PPP6C	Serine/threonine phoasphtase6 catalytic subunit
RAC-1	Ras- related C3 botulinum toxin substrate 1
Rb	retinoblastoma
rlu	relative light unit
rpm	Rounds per minute
SCL	Stable cell line
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNAs	small interfering RNAs
SA-β-gal	Senescence –associated β-galactosidase
Ser	Serine
SNX	Sorting nexin protein family
Src	Sarcoma proto oncogene
STK	Serine/threonine kinase
TACC 1	Transforming acidic coiled- coil-containing protein 1
WT	Wild type
YO	Years old

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1. Introduction

1.1 Melanoma biology

Melanoma is the most aggressive skin cancer that originates in melanocytes, specialized pigmented cells, responsible for skin and hair color. Melanocytes arise from the neural crest (pluripotent cells that give rise to neurons, glial cells, adrenal medulla, cardiac cells and craniofacial tissue) during embryonic development, and throughout their maturation migrate widely and proliferate extensively prior to their terminal differentiation and entry into the epidermis and hair follicle. In addition, melanocytes undergo multiple cycles of regeneration, in which melanocyte stem cells give rise to new populations of melanocytes [Marais et al, 2007].

In the skin, melanocytes are located in the basal layer of the epidermis. Melanocytes have a round shape with branch -like extensions named dendrites. Within the melanocytes are unique melanosomes organelles, which produce melanin pigment. The melanosomes are transferred by the dendrites to the keratin layer where they are taken in by receptor- mediated endocytosis and deposited over keratin nucleus to protect the DNA from UV light. Keratinocytes secrete factors that regulate melanocytes survival, differentiation, proliferation and motility, stimulating the melanocytes to produce melanin and resulting in the tanning response [Marais et al., 2005].

1.2 Types of melanoma

There are two main categories of skin cancer: *non-melanoma skin cancer* (NMSC) which originates in keratinocytes and pluripotent skin cells and *cutaneous melanoma* (CM) which originates from the transformation of melanocytes. Depending on the epidermal layer keratinocytes originate from, NMSC can be subdivided into *basal cell carcinoma* (BCC) and *squamous cell carcinoma* (SCC) [Scherer et al., 2010].

1.3 Risk factors in melanoma

Some factors correlate with increased risk of melanoma development: environmental risk factors such as UV light and increased sun exposure; phenotypic risk factors such as pigmentation (fair skin, blue or green eyes, blonde or red hair, freckles), sun sensitivity, inability

to tan, high number of melanocytic nevi (or) presence of clinically atypical nevi; genetic risk factors such as germline mutations (family history of melanoma) and somatic mutations [Marais et al., 2005].

Melanoma is associated with intermittent high level sun exposure whereas squamous cell carcinoma and basal cell carcinoma are associated with chronic sun exposure [Gilchrest et. al., 1999]. The most common mutations in *B-RAF* and *N-RAS* genes in melanoma do not carry UVB signature mutations (C→T and CC→TT transitions). Only small percentages of melanomas have UVB signature mutation in p53, acquired in time, in advance stage of the cancer. There is evidence that UV can induce immunosuppression and inflammation and furthermore, collaborate with DNA damage, microenvironmental and molecular responses in initiation and/or progression of melanoma [Fisher, 2008]. Zaidi's studies indicate that interferon gamma (IFN-γ) can have, depending of the cellular context, a dual role with anti-tumorigenic or proto-tumorigenic effects. [Zaidi, 2011]. IFN-γ is a macrophage activating factor, associated with survival mechanism and critical for innate and adaptive immunity in tumor control. UV- induced p53 stress response and inflammatory responses prepare keratinocytes for cell cycle arrest and DNA repair, whereas activated melanocytes proliferate and migrate towards the epidermis. It was shown that IFN-γ helps modified melanocytes to survive immune-mediated elimination, as well as melanoma cells within the tumor. Modified melanocytes evolve and accumulate mutations, migrate outside their niche and have aberrant interaction with the inflammatory microenvironment, proliferate and develop in melanoma [Zaidi et al., 2012].

1.4 Progression of melanocytes to melanoma

Melanoma is known to progress in well-defined steps (**Fig. 1.1**):

- benign *common nevus* (mole): disruption of melanocyte regulation by keratinocytes can lead to melanocyte proliferation and spreading and formation of a nevus or common mole. Proliferation can be restricted to epidermis (junctional nevus), dermis (dermal nevus) or can be an overlapping of both components (compound nevus)
- *dysplastic nevus* (atypical mole) with morphologically atypical melanocytes which can progress to
- *radial-growth phase (RGP*) melanoma, an intra-epidermal lesion that can involve also local micro-invasion of the dermis; this stage is considered to be primary malignant stage

- *vertical growth phase* (VGP) primary melanoma associated with nodules or nest of cells invading the dermis and acquisition of metastatic potential, and can lead directly to *metastatic melanoma* - the most dangerous stage, by infiltration and spreading to distal sites in the body or lymph nodes several levels beyond skin area of the primary lesion [Marais et al., 2007].

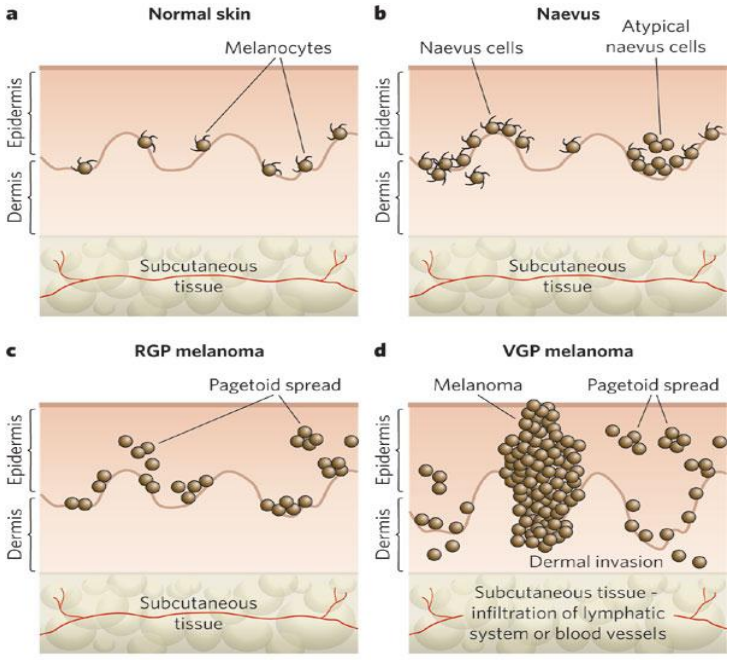


Figure 1.1: Melanocytes progression: a) normal skin with equal distribution of dendritic melanocytes within the basal layer of the epidermis .b) benign nevi with increased number of dendritic melanocytes occur; sometimes some nevi are dysplastic with atypical melanocytes. c) Radial growth –phase (RGP) melanoma considered to be primary malignant stage. d) Vertical –growth- phase (VGP) melanoma with metastatic potential that can lead to metastatic malignant melanoma by spreading to vascular and lymphatic system [Marais et al, Nature 2007].

If diagnosed early, such RGP, melanoma can be treated by surgical resection, conferring a 90% five years survival rate. Once melanoma cells have gain access to blood and lymphatic system and metastasize, the prognosis is poor (6-9 months median survival rate) due to poor efficiency of current treatments [Marais et al., 2011].

The number of melanoma cases is increasing worldwide, with an estimated doubling incidence every 10-20 years. Melanoma accounts for <5% of skin cancer, but is the most deadly skin cancer, accounting for 80% of skin cancer deaths. It is rare in individuals below age 20 and frequent in young and middle-aged adults [Bloethner et al., 2009]. It is 5th most prevalent cancer and second most common cancer in women under the age of 40 [Zaidi et al., 2012]

1.5 Genes involved in melanoma

Molecular and genome wide analyses of melanoma have identified specific genomic loci involved in the genesis and progression of melanoma. The most frequently mutated genes in melanoma are the *BRAF* and *NRAS* oncogenes. *Cyclin* D_1, $p16^{INK4a}$ and *MITF* genes that function downstream of BRAF have also been identified .Most significant mutations recorded in melanoma are presented in **Table 1.1**[references from Marais et al., 2007; Marais et al., 2005; Barret et al., 2011; Garraway et al., 2012; Franco et al., 2012; Samuels et al., 2011;Flaherty et al., 2012; Krauthammer et al., 2012]. Samuels and colleagues showed that melanoma tumors had alterations in the *GRIN2A* gene encoding members of glutamate signaling pathway, with high prevalence of C→T/G→A transitions. A link between the glutamate pathway and tumorigenesis has been indicated in neural tumors, where excess of glutamate released by glioma cells showed more aggressive growth compared to parental glioma cells [Samuels et al., 2011].

Table 1.1 Mutations recorded in melanoma

Gene type	Gene	Alteration frequency/type(s) in melanoma (%)	Function of the protein /pathway affected
Oncogenes	*BRAF*	*50-70% mutated*	*Protein kinase/MAPK*
	NRAS	*15-30% mutated*	*GTPase/MAPK, PI3K,RALGDS*
	AKT1/AKT2/AKT3	*60-70% overexpressed, <1% point mutation*	*Protein kinase /PI3K*
	CDK4	*5% point mutation or amplification*	*Protein kinase/Cell cycle*
	CCND1	*10% amplified*	*Protein kinase/Cell cycle*
Tumor suppressors	*$CDKN2A^{p16}$*	*30-70% deleted, mutated or silenced*	*Protein kinase/Cell cycle*
	PTEN	*5-20% deleted or mutated*	*Protein tyrosine phosphatase/ PI3K*
	APAF-1	*40% silenced*	*Protease/Apoptosis*
	TP53	*10% lost or mutated*	*Tumor suppressor protein/Cell cycle*
Others	*MITF*	*10-16% amplified*	*Transcription factor /Melanocyte lineage and cell cycle*

	B-catenin	*3% mutation*	*Adherent junction protein/Wnt*
	GRIN2A	*30% mutated*	*Ionotropic glutamate receptors/ Synaptic transmission*
	MYC	*25% amplified or overexpressed*	*Transcription factor /Cell cycle*
Others	*MC1R*	*mutated*	*G-protein coupled receptor/Melanogenesis*
	BAG-3	*mutated*	*Chaperone family/ Autophagy*
	ATM	*mutated*	*Ser/thr protein kinase/ DNA damage checkpoint*
	CASP8	*mutated*	*Caspase/Apoptosis*
	PPP6C	*9% mutated*	*Protein phosphatase/Cell cycle*
	RAC1	*5% mutated, oncogene*	*GTPase/ Cell growth, cytoskeleton reorganization*
	SNX31	*7% mutated*	*Sorting proteins/ Endosomal sorting and signaling*
	TACC1	*7% mutated*	*Unknown*
	STK19	*4% mutated*	*Ser/thr protein kinase/Transcriptional regulation*
	ARID2	*9% LOF mutation*	*Chromatin remodeling protein /Transcriptional activation and repression of several genes*

A recent large scale melanoma exome analysis revealed 6 novel melanoma genes (PPP6C, RAC1, SNX31, TACC1, STK19, and ARID2), three of which-RAC1, STK19, and PPP6C-carring UVB-signature mutations [Hodie et al, 2012].

Because the work presented in this thesis comprises experiments made on melanoma cell lines with *BRAF* and *NRAS* mutations, these genes and their encoding proteins will be presented in more details below.

1.6 RAS genes and their encoding proteins

There are three human *RAS* proto-oncogenes (Harvey (*H)-RAS, Kirsten (K)-RAS* and Neuroblastoma (*N)-RAS*) that reside on chromosomes 11p15, 1p22 and 12p12, respectively and encode four distinct, but highly homologous proteins: HRAS, KRAS4A, KRAS4B and NRAS. The most common mutations in cancer are found in *K-RAS*, followed by *N-RAS*, whereas mutations in *H-RAS* are rare. High frequency of *K-RAS* alterations have been found in pancreatic (90%), colon (50%) and lung cancers (30%), whereas N-RAS mutations are frequent in myeloid leukemia and melanomas (15-30%) [Whitwam et al., 2007]. Moreover, *N-ras* and *H-ras* are dispensable for mouse development, whereas *K-ras* knockout in mice induces embryonic lethality, suggesting that the proteins encoded by these genes have specific functions in different cell types [Whitwam et al., 2007]. Most of the gene alterations are missense mutations in the codons 12, 13 and 61. In melanoma, *N-RAS* mutations concentrate on codon 61 with Q61K (15%) and Q61R (68%) being the most frequent amino acid substitution detected. In contrast, *K-RAS* and *H-RA*S mutations predominantly occur in codons G12 and 13[Bloethner et al., 2009; Medarde et al, 2011].

RAS proteins serve as transducers that couple cell surface receptors to effectors of intracellular pathways. The proteins alternate between "on" and "off" conformation by binding to GTP or GDP, respectively. Extracellular signals are received by membrane and tyrosine kinase receptors that activate guanine nucleotide exchange factors (GEFs), which promote activation of RAS by binding to GTP. RAS signaling is terminated by GTPase- activating proteins (GAPs) that mediate RAS-GTP-hydrolysis. The outcome of oncogenic mutations in *RAS* is the consistent RAS-GTP-bound state that continuously activates downstream effector pathways [Gupta et. al., 2011]. It was suggested that NRAS and BRAF activation occur at early stage during melanoma development since mutations were present in nevi, radial growth phase and persist through the metastatic spread. *N-RAS* mutations are found in 10% of common acquired nevi and 28-56% congenital nevi [Bloethner et al., 2009]. Rare cases of melanoma with *N-RAS* mutations were detected in skin unexposed to sun, indicating that UV –radiation may play a role in genesis of *N-RAS* in melanoma [Jiveskog et al., 1998]. *N-RAS* mutations in melanoma activate CRAF instead of BRAF in the RAF-MEK-ERK MAPK pathway [Dumaz et al., 2006]. *B-RAF* and *N-RAS* mutations are mutually exclusive and have different pathological and clinical behavior. Tumors

with *NRAS* mutations are more likely to be thicker, more frequently located in the extremities and have a higher mitotic rate, whereas *BRAF* mutations have higher rate of ulceration [Devitt et al., 2011; Ellerhorst et al., 2011].

1.7 RAF genes and their encoding proteins

The ARAF, *BRAF* and *CRAF* genes reside on chromosomes Xp11, 7q34 and 3p25, respectively and encode for 3 structurally related RAF proteins in humans: ARAF, BRAF and CRAF (also called RAF-1). *ARAF* and *CRAF* appear in single spliced variant, whereas *B-RAF* undergoes alternative splicing, with more than 10 isoforms of the protein [Heath et al., 2011]. Studies with *raf* genes knockout mice revealed that neither *b-raf* nor *c-raf* deficient embryos are viable. This is due to neuronal or vascular defects in the placenta in mice lacking *b-raf* gene [Marais et al., 1997] or fetal liver apoptosis in mice lacking *c-raf* gene [Wan et al., 2004], whereas *a-raf* deficient embryos die soon after birth of neurological and intestinal defects [Weber et al., 2001]. These studies indicate that not only RAS, but also RAF proteins have distinct function in embryonic development [Galabova-Kovacs et al., 2006].

RAS recruits inactive cytosolic RAF to the plasma membrane where it is activated through a number of phosphorylation events. BRAF is activated by RAS alone, whereas A-RAF and C-RAF require both RAS and the tyrosine kinase Src for full activation [Sahai et al., 2001; Garraway et al., 2005]. This may explain why B-RAF has elevated basal kinase activity compared to the other two RAF proteins [Sahai et al., 2001]. Importantly, these differences make B-RAF more susceptible for mutations and may explain why B-RAF is the most frequently mutated RAF isoform in cancer.

BRAF plays a crucial role in development and maintenance of melanoma phenotype. About 60% of cutaneous melanomas have *BRAF* mutations, 20% have *RAS* mutations and initiating oncogenic event in the remaining 10-20% of melanomas is unknown. BRAF mutations are also found in papillary thyroid (40% to 70%) colorectal (18%) and ovarian (14%) cancers [Flaterhy et al., 2010; Davies et al., 2002]. Until now, more than 75 different mutations have been described in the *B-RAF* gene with valine to glutamic acid substitution (V600E) the most common one in melanoma, accounting for 90% of the BRAF mutations.

The $BRAF^{V600E}$ mutation is present in 6 to 8% of human cancers and interestingly, 5% of human lung cancers carry BRAF mutations, but not V600E [Dankort et al., 2007]. It was

suggested that the V600E mutation in melanoma is an indirect consequence of UV exposure, since T>A transversion is distinct from C>T or CC>>TT pyrimidine mutations common in UV-induced DNA damage and furthermore, other forms of skin cancer such as basal or squamous cell carcinoma do not contain B-RAF mutations. Other mutations such as V600K and V600D /V600R account for 16% and 3% respectively of all BRAF mutations identified in melanoma [Long et. al., 2011]. *BRAF* is mutated in up to 80% of benign nevi, but rarely progress to melanoma, indicating that BRAF alone cannot induce oncogenic properties of Ras, therefore additional loss of important cellular check points are needed [Pollock et al., 2003].

1.8 The oncogenic RAS/RAF effect on signaling pathways

The mitogen activated protein kinase (MAPK) RAS-RAF-MEK-ERK pathway is associated with cell proliferation, but also regulates differentiation, senescence and apoptosis/cell survival. This pathway is hyper-activated in up to 90% in human melanomas [Tsao et al., 2004].

RAS activates the three closely related RAF proteins (A-, B- and C-RAF).While RAF acts mainly through MAPK signaling pathway, RAS acts through several additional effector pathways (**Figure 1.2**). RAS can also interact with phosphoinositide-3 kinases (PI3Ks) in processes involved in cell survival, and with RAC and RHO protein family implicated in cytoskeleton signaling. Moreover, RAS interacts with RAS-related proteins (RAL) via RAL guanine nucleotide dissociation stimulators (RALGDS) which affects vesicle trafficking. Finally, RAs activates Protein Kinase C (PKC) and calcium mobilization via phospholipase C [Platz et al., 2008].

The major downstream substrates of RAS/RAF are **m**itogen –activated protein kinase or **e**xtracellular signal- regulated **k**inase kinase 1 and 2 (MEK1/2). RAF phosphorylates and activates MEK1/2, which in turn bind, phosphorylate and activate the **e**xtracellular signal-regulated **k**inases 1 and 2 (ERK1/2) (**Figure1.3**). When activated, ERK 1/2 phosphorylate numerous cytosolic and membrane –localized cytoskeletal proteins, thereby regulating cell shape and migration [Arkenau et al., 2011]. ERK1/2 can also translocate to the cell nucleus and activate gene expression by interaction with various transcription factors that are required for the cell cycle entry into G_1 –phase. In turn, the transcription factors stimulate the expression of cyclin D_1 and suppression of cyclin –dependent kinase inhibitors, such as p27 and p21 [Gupta et

al., 2011]. The level of ERK1/2 signaling must be carefully modulated to stimulate proliferation. A low signaling is not enough to induce proliferation, whereas too much signaling can induce other responses such as differentiation or senescence [Mikula et al., 2001].

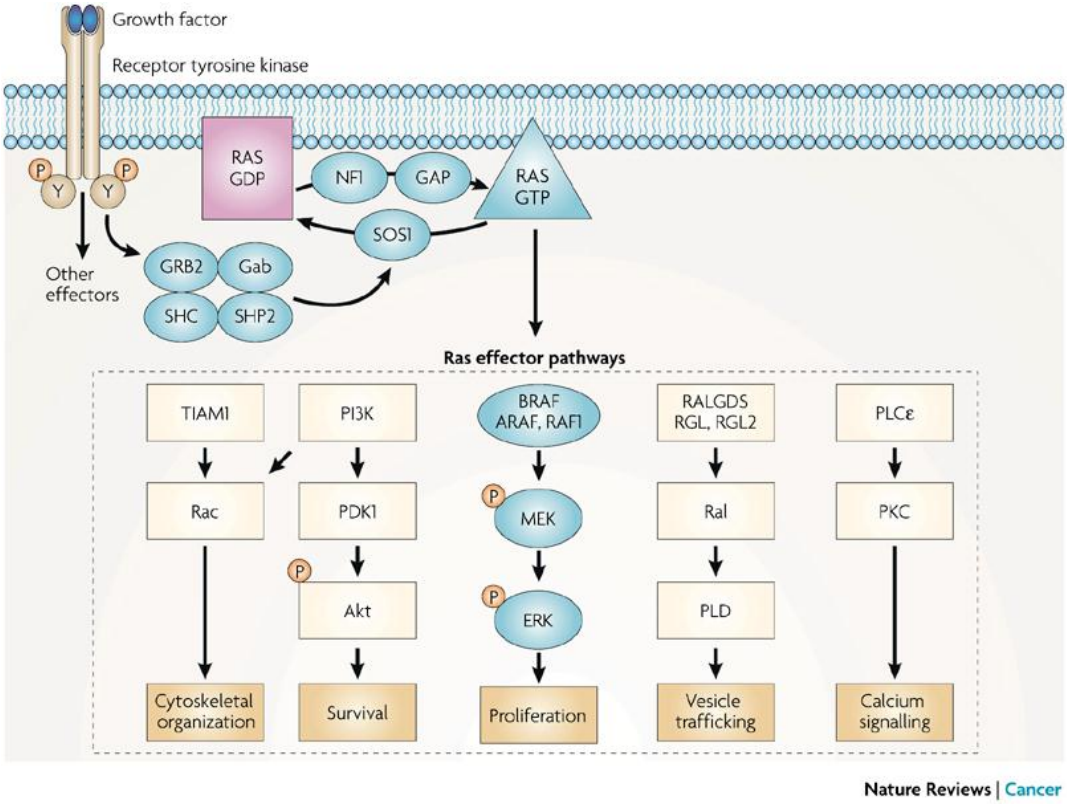


Figure 1.2: RAS signaling pathway. Growth factors activate cell-surface receptor complexes, which contain adaptors such as SHC (SH2-containing protein), GRB2 (growth- factor- receptor bound protein 2) and Gab (GRB2-associated binding) proteins. These proteins recruit SHP2 and SOS1, the latter mediating activation of RAS-GDP bound to RAS-GTP bound. The GTP- activating protein (GAP) and neurofibromin(NF1) accelerates activation of RAS which in turn activates several pathways: BRAF-MEK-ERK pathway involved in proliferation, PI3K-AKT pathway involved in cell survival. RAS also activates exchange factors of RAL and the downstream target phospholipase D (PLD), an enzyme that regulates vesicle trafficking, whereas activation of RAC regulates actin dynamics and cytoskeleton organization. RAS binds and activates the enzyme phospholipase Cε (PLCε) which regulates the PKC family and calcium signaling [Schubbert et. al., Nature Reviews/Cancer 2007]

In human primary melanocytes oncogenic BRAF induces expression of $p16^{INK4a}$ and senescence, whereas mouse melanocytes with inactive $p16^{INK4a}$ can be transformed by oncogenic RAS. These data suggest that inactivation of $p16^{INK4a}$ pathway is required for RAS/RAF-induced melanomagenesis [Marais et al., 2007]. Ha and colleagues underlined that, at least in melanocytes, senescence is regulated by the tumor suppressor Arf , which is transcribed from an alternative reading frame of the INK4a locus, and less by p53.They showed that activated NRAS and deficient Arf can lead to melanoma in a p53- independent manner [Ha et al., 2008].

Another important pathway in melanoma is PI3K pathway (**Figure 1.3)** with role in survival, proliferation, growth and motility. Phosphoinositides are membrane lipids that are converted to second messengers through hyperphosphorylation of PI3Ks. PI3K stimulates activation of the protein kinase B (PKB or Akt) pathway and this is negatively regulated by PTEN (phosphate and tensin homologue). NRAS can activate both ERK1/2 and PI3K signaling pathways, whereas loss of PTEN can induce phosphorylation of MEK and ERK1/2 via feedback loops [Flaherty et al., 2010].

The differential tumorigenic potential of RAS isoforms was studied on INK4a/Arf deficient melanocytes. Although both KRAS and NRAS can activate the MAPK pathway in melanocytes and cooperate with the transcription factor c-MYC, only NRAS can effectively activate PI3K/AKT/MYC pathway and inhibiting MYC -mediated glycogen synthase kinase-3 (GSK3) phosphorylation and degradation [Whitwam et al., 2007].

The tumor suppressor PTEN is inactivated in 10-30% of BRAF mutant melanoma cell lines and 10% of human tumors [Madhunapantula et al., 2009]. Transgenic mice studies have shown that the BRAF (V600E) mutation in collaboration with PTEN loss induce metastatic melanoma [Dankort et al, 2009].

The $p38^{MAPK}$ pathway is also involved in inhibition of RAS- proliferative signaling. Studies in NIH3T3 cells revealed that RAS activity stimulates the p38 pathway and p38, through its downstream targets MK2 (MAPK-activated protein kinase 2) and PRAK (p38-related/activated kinase), provides a negative feedback to the biological effects of RAS signaling by blocking JNK (also called stress activated protein kinase SAPK) activation. Both MK2 and PRAK inhibit RAS-induced JNK activation by 85% [Chen et al., 2000].Therefore inhibition of RAS- proliferative signaling by the MKK6/p38/PRAK pathway may be used as strategy against RAS-related cancer.

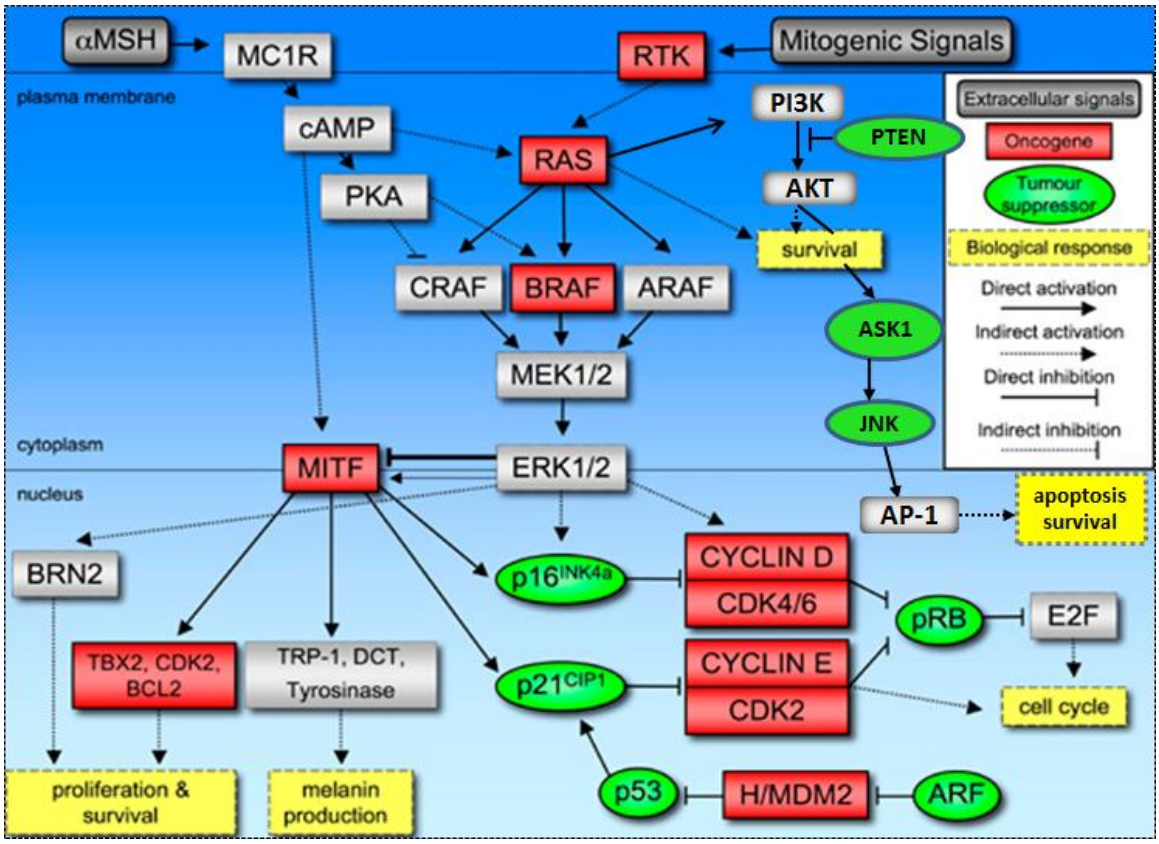


Figure1.3: Oncogenic BRAF-signaling pathway involved in tumorigenesis. Shematic representation of RAS/RAF/MEK/ERK signaling pathway with various effector processes, including those governing cell proliferation and survival. Oncogenic RAS interacts with PI3K-AKT-JNK pathway, mediating survival of the cells, whereas downstream BRAF is the p16 ^{INK4a}/CDK4/pRb/E2F cell cycle pathway which in melanocytes is downstream of microphthalmia- associated transcription factor (MITF). The CDK inhibitor p21^{CIP} acts as a nodal point connecting the pRb pathway to the p53 tumor suppressor and MITF. In addition, in melanocytes the MITF is under (both positive and negative) control of BRAF-(and cAMP-) signals to regulate melanin production in response to α-MSH. Proteins are color-coded as explained in the insert [Michalaglou et al., Oncogene 2008]

1.9 Mitogen activated protein kinase-activate protein kinase-5(MK5)

The mitogen-activated protein kinases(MAPKs) are part of signaling pathways involved in cell proliferation, differentiation, gene expression, apoptosis, cell survival, metabolism and motility [Kostenko et al., 2011a]. The MAPK pathways can be grouped in conventional and atypical pathways (**Figure 1.4**). The first group consists of three consecutive phosphorylation events exerted by MAPK kinase kinases (MAPK3), MAPK kinase (MAPK2), and MAPK [Kostenko et al., 2011a].The conventional MAP kinase pathways are represented by the MEK1/2-ERK1/2, JNK, p38^{MAPK}, and MEK5/ERK5 pathways, whereas atypical MAPK pathways are not organized in three partite modules and include the ERK3/4, ERK7/8, and nemo-like kinase

(NLK) pathways. Both conventional and atypical MAPK pathways can phosphorylate non-protein kinase substrates, or yet other protein kinases referred to as MAPK- activated protein kinases (MAPKAPK). Such a MAPKAPK is MK5 or p38-regulated /activated protein kinase (PRAK - human homolog).

MK5 is highly conserved and ubiquitously expressed in vertebrates and it seems to be most abundant in brain, heart and platelets [Gaestel M, 2006].The human *mapkapk5* gene encodes for two differently spliced transcript variants, resulting in a 471 and 473 amino acid PRAK variant, respectively [Kostenko et al., 2011a]. It is not known if these two isoforms have different functions.

MK5 shows amino acid sequence and structural similarities with MK2 and MK3 (approximately 33% homology), but there are several unique properties that distinguish MK5 from MK2/MK3 [Kostenko et al., 2012]. In contrast to MK2 and MK3, MK5 belongs to atypical MAPK pathways and contains a unique C-terminal sequence that is lacking in MK2 and MK3 [Cargnello et al., 2011].

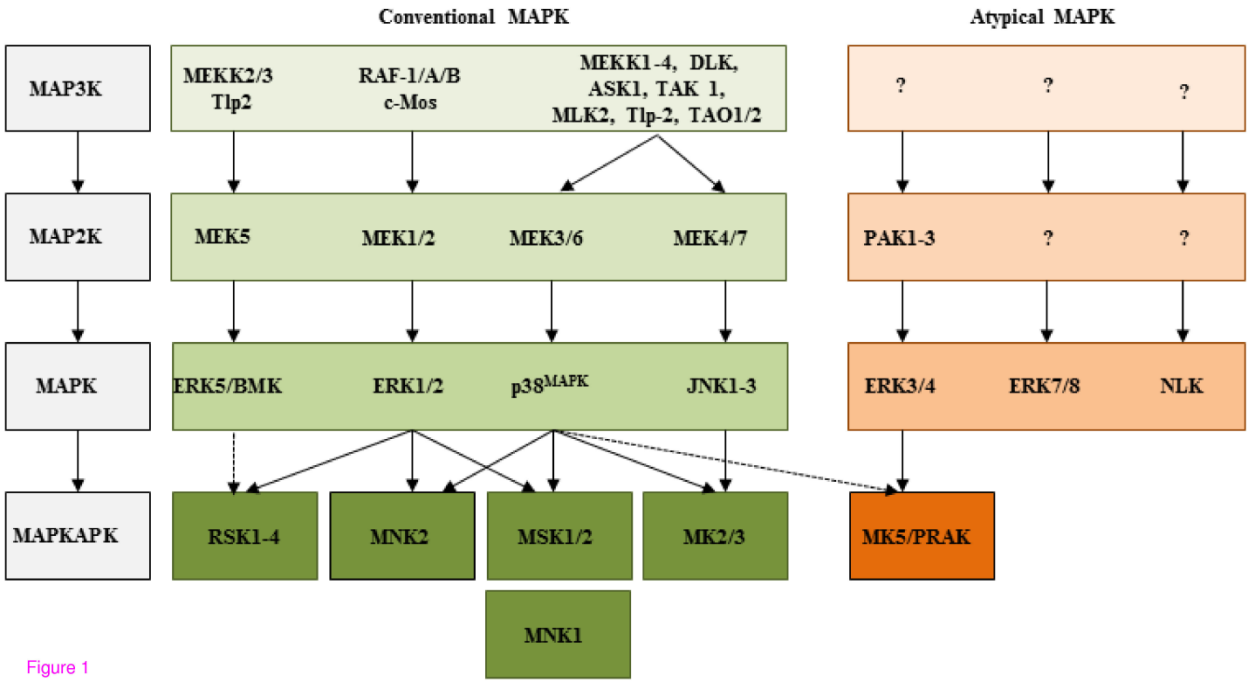


Figure 1.4: The conventional and atypical mammalian MAP kinase pathways.
The conventional MAP kinase pathways are represented by the MEK1/2-ERK1/2, JNK, p38MAPK, and MEK5/ERK5. The MAP3Ks phosphorylate MAP2K, which in turn phosphorylates MAPK. Downstream of MAPKs are substrates including other protein kinases referred to as MAPK-activated protein kinases (MAPKAPK). The

atypical pathways include ERK3, ERK4, ERK7, ERK8, and NLK. MAPKs can converge to different MAPKAPK as shown in this figure [Kostenko et al. J Mol Signaling Rev 2012].

The main upstream activators of MK5 are ERK3, ERK4 and $p38^{MAPK}$ (isoform β) that can induce phosphorylation of MK5 at Thr-182 [Kostenko et al., 2012].Another activator of MK5 is cAMP-dependent protein kinase (PKA) which phosphorylates MK5 at Ser-115[Kostenko et al.,2011c]. In resting cells, MK5 is found predominantly in the nucleus, but it can shuttle between the nucleus and the cytoplasm due to a functional nuclear localization signal (NLS) and a nuclear export signal (NES). The latter partially overlaps with the $p38^{MAPK}$ docking site [Kostenko et al., 2011a].

The atypical MAP kinase ERK3/4-MK5 pathway is involved in both oncogenic and anti-oncogenic processes, depending on the cell context (**Figure 1.5**). Mutations in *MK5* gene have been detected in lung, melanoma and skin tissue, but it remains to be determined whether they are driver or passenger mutations. The implications of these mutations on MK5's functions have not been examined [Kan et al., 2010; Bell et al., 2011; Berger et al., 2012]. As several studies have indicated a tumor-suppressive role for MK5, this thesis evaluates MK5's possible role in modulating cell proliferation of melanoma cell.

1.10 Association of MK5/PRAK with cancer

Premature senescence can be induced by oncogenes such as *ras*, and serves as defensive mechanism against tumor development [Serrano et al, 1997]. Mice with deficient MK5are more susceptible to skin carcinogenesis induced by dimethylbenzanthracene (DMBA) mutagen, thus suggesting that MK5 is a tumor suppressor [Sun et al., 2007]. In primary murine and human fibroblasts a cascade of events is triggered in the RAS-RAF-MEK-ERK-MAPK pathway, in which PRAK mediates senescence upon activation of p38 by oncogenic *ras*. Furthermore, PRAK phosphorylates p53 at Ser-37and stimulates the transcriptional activity of p53 which in turn increases $p21^{WAF1}$ expression and induces cell cycle arrest [**Figure.1.4, (8)**]. Full activation of p53 during premature senescence may require phosphorylation of multiple sites (Ser15, Ser33, Ser37, Ser46) [Sun et al., 2007]. It was previously shown by Li and colleagues that overexpression of PRAK suppresses NH3T3 cell proliferation and PRAK nuclear localization is essential to its inhibitory effect [Li et al, 2008]. PRAK also inhibits oncogenic H-RAS induced proliferation of NIH3T3 cells [Chen et al, 2000].

MK5 functions as tumor suppressor not only in skin, but also in hematopoietic cells. Studies of Yoshizuka and his colleagues on $MK5^{-/-}$ mice expressing activated *Eμ- N-Ras*^{G12D} oncogene in hematopoietic cells revealed that MK5 deletion accelerated the development of hematopoietic tumors, both T-lymphoid and myeloid origin through hyper-activation of JNK pathway. Moreover, when present in the cells, MK5 suppresses JNK activity in both normal and cancer hematopoietic cells [Yoshizuka et al., 2012b] (**Figure 1.5 (10)**). It was previously reported that activated *ras* induce senescence in primary splenocytes as a suppression mechanism to lymphoma development [Braig et al., 2005]. In Yoshizuka study, splenocytes with activated ras failed to induce proliferative arrest. Splenocytes with oncogenic *ras* and wild-type MK5 induce the expression of senescence markers such as, DcR_2, $p16^{INK4a}$ and $p19^{ARF}$. The exact mechanism by which MK5 suppresses the JNK pathway remains to be established but the expression of the leukocyte specific adaptor protein Grap2 was reduced in $MK5^{-/-}$ hematopoietic cells. Grap2 enhances the activity of hematopoietic progenitor kinase 1 (HPK1), which in turn activates JNK. Hence knockout of MK5 may reduce JNK activation by negatively interfering with Grap2/HPK1 [Yoshizuka et al., 2012b].

In cells with depleted MK5 the senescence markers expression was either abolished or reduced. The senescence –associated β-galactosidase (SA-β-gal) was poorly expressed in cells with wild-type and depleted MK5, indicating that MK5 could be partly involved in senescence mechanism, at least in hematopoietic cells [Yoshizuka et al., 2012b].

Another study elegantly highlighted the tumor -suppressor role of MK5 [Kress et al., 2011]. The authors used a siRNA screen of human kinome in osteosarcoma cells to identify MK5/PRAK as negative regulator of c-Myc expression at translational level [**Figure 1.4, (7)**].

The mechanism might be used in DNA damage control. Aberrant levels of c-Myc were observed during carcinogenesis, therefore downregulation of Myc upon DNA damage is important for cell cycle arrest and DNA repairing. *Myc* mRNAwas previously shown to be target of the micro RNA miR34b/c. Kress and colleagues showed that MK5 controls the levels of miR34b/c via FoxO3a transcription factor [Kress et al., 2011]. MK5 phosphorylates and activates FoxO3a which binds to the pre-miRNA promoter and increases the expression of miR34b/c and reduces Myc expression. Interestingly, MK5 stimulates expression of miR34b/c in a p53-independent manner, although MK5/PRAK can phosphorylate and activate p53 [Sun et al., 2007].

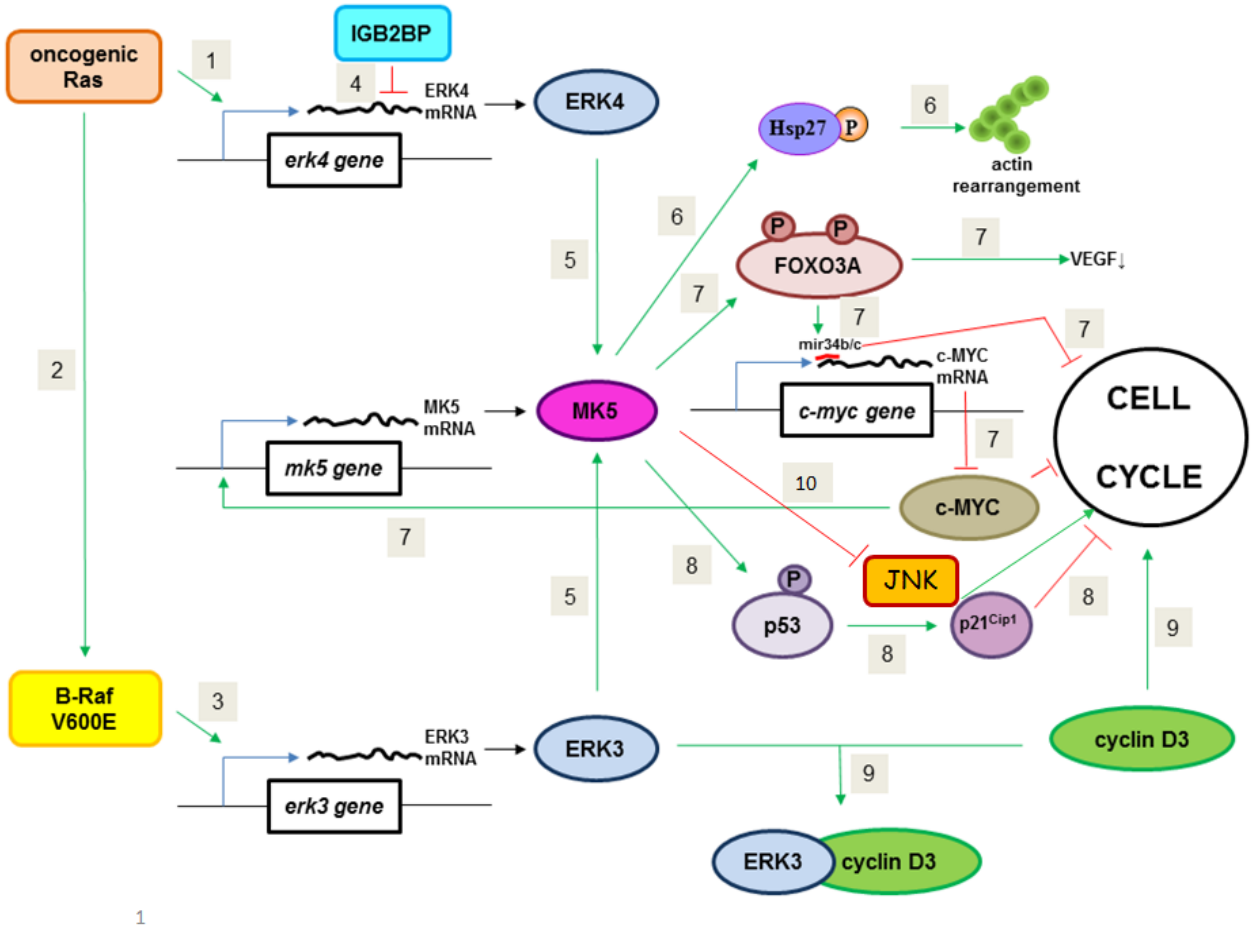


Figure 1.5: Molecular mechanism of the pro and anti-oncogenic properties ofERK3, ERK4 and MK5 (1,2) Oncogenic RAS increases ERK4 levels and activates RAF; (3)Activated RAF(or $BRAF^{V600E}$) stimulates transcription of *erk3* gene;(4)The RNA binding protein IGB2BP can inhibit translation of ERK4 mRNA; (5) ERK3 and ERK4 proteins can activate MK5;(6)Active MK5 can phosphorylate Hsp27,affecting actin remodelling and cell migration;(7) MK5 can also phosphorylate transcription factor FOXO3a, which trigger transcription of microRNA mir34b/c, therefore inhibits the cell cycle by preventing translation of c-*myc* mRNA and hence the production of c-MYC. C-MYC binds to the promoter of the *mk5* gene and enhances transcription of this gene. FOXO3a also reduces expression of VEGF and may thus hamper angiogenesis; (8) MK5-mediated cell cycle arrest via phosphorylation of p53 at Ser-37, stimulation of the transcriptional activity of p53, resulting in enhanced expression of p21Cip1;(9) ERK3 can sequester cyclin D3and may cause cell cycle arrest; (10) MK5 suppresses JNK activity in both normal and cancer hematopoietic cells. Deletion of MK5 in hematopoietic cells with activated ras accelerates development of the tumor through hyper-activation of JNK pathway [Kostenko et al. Mol Signal 2012]

Alternatively, the mechanism might be used when rapid cell division is required by switching off one of the loop components to allow accumulation of Myc protein. The MK5/PRAK-FoxO3a-miR34b/c negative regulation of Myc expression is lost in colorectal cancer (CRC), most probably by the downregulation of MK5/PRAK during tumor progression [Kress et al., 2011].

A recent study on osteosarcoma U2OS cells revealed a role of the ERK4/MK5 pathway in IGF2BP-induced tumor cell migration [Stöhrn et al., 2012].Insulin-like growth factor 2 mRNA-binding protein (IGF2BP) participates in cell polarity and migration and its overexpression was noticed in various human cancers. Overexpression of IGF2BP promoted velocity of cell migration by preventing translation of ERK4 mRNA, resulting in perturbed MK5 activation and MK5-mediated Hsp27 phosphorylation [**Figure 1.4 (4, 5, 6)].**

Another study of Yoshizuka showed that once the tumor is established, MK5 participates in tumor growth and progression by stimulating angiogenesis [Yoshizuka et al., 2012a]. Vascular endothelial growth factor (VEGF) binds to VEGF receptor 2 (VEGFR2) and activates $p38^{MAPK}$ pathway. Once activated, MK5 mediates endothelial cell migration via phosphorylation of focal adhesion kinase (FAK) and cytoskeletal reorganization. The exact mechanism for MK5-mediated FAK activation remains to be solved, but these studies illustrate a tumor promoting function (angiogenesis) of MK5.

It is also important to mention that FOXO3a was shown to repress expression of VEGF in breast cancer [Karadedou et al., 2012]. FOXO3also stimulates expression of miR34b/c which causes cell cycle G_1 arrest and reduces cell motility and invasion in melanoma cells [Mazar et. al., 2011]. By mediating FOXO3a phosphorylation, MK5 can either promote angiogenesis by increasing expression of VEGF or reduce invasiveness by upregulation of miR-34b.The MK5 dual potential is cell context dependent.

1.11 Therapeutic targets in melanoma

Efficient therapeutic strategies for melanomas with *N-RAS* mutations have not been realized. However, promising strategies for NRAS mutant melanomas include targeting membrane localization of NRAS, or inhibition of proteins mRNA with small interfering RNAs (siRNAs). Unfortunately the delivery of the small antisense oligonucleotide is challenging and less successful. Inhibitors that target signaling downstream of RAS such as MEK1/2 and /or ERK1/2 activity, or dual targeting of the ERK1/2 and PI3K/AKT pathways are under clinical evaluation [Fergal et al., 2012].

Many new drugs such as BRAF, KIT and MEK inhibitors are under evaluation in treating melanoma with *BRAF* mutations, but drug resistance is really a challenge. Vemurafenib

(PLX4032) was approved by Food and Drug Administration (FDA) in August 2011 for the treatment of unresectable melanoma [Flaherty et al., 2010]. The drug works only in patients with ^{V600E} *BRAF* mutation, pointing to dependency of BRAF-mutant melanomas on BRAF kinase activity. Unfortunately, patients acquired resistance to vemurafenib within few months after treatment started and the disease relapsed. Nazarian et al. showed that melanoma acquired resistance to vemurafenib is due to activation of RTK (PDGFβ)-dependent survival pathway or acquiring new mutation in *NRAS* that reactivates MAPK pathway [Nazarian et al., 2010]. The MDX-010 human monoclonal antibody (also known as ipilimumab) was also used in phase III clinical trial in patients with stage IV melanoma. Cytotoxic T lymphocyte- associated antigen 4 (CTLA4) receptor impairs the Cytotoxic T lymphocytes (CTLs) recognition of tumor cells. Ipilimumab blocks CTLA4 inhibitory signaling, allowing CTLA4 to activate the immune response [Flaherty et al., 2012]. Ipilimumab combined with peptide vaccine or dacarbazine chemotherapy improved overall survival compared with vaccine/dacarbazine alone [Hodi et al., 2011; Robert et al, 2011] and has been recently approved by the FDA for the treatment of metastatic melanoma.

New drug therapy against $BRAF^{V600E}$ is tested to overcome metastatic melanoma relapse after vemurafenib treatment. Inhibition of Aurora B kinase, a downstream target of BRAF/ERK pathway, could be a valuable target in metastatic melanoma resistant to vemurafenib or patients with wild-type BRAF. In a study of Bonet, Aurora B inhibition by AZD1152-HQOPA triggers cell cycle arrest, cellular senescence and cell death by mitotic catastrophe. Aurora kinases regulate chromosomes movement and segregation during mitosis and their overexpression in tumor cells causes unequal distribution of genetic material, therefore an abnormal number of chromosomes (aneuploidy) [Bonet et al., 2012].

Because MK5 can act as both tumor suppressor and tumor promoter, drugs should be designed in the near future to inhibit or stimulate MK5's activity in cancers, including melanoma. There are some selective MK5 inhibitors that have been identified, but they have not been tested in clinical trials [Kostenko et al., 2011b; Anwar et. al., 2011; Andrews et al., 2012].

1.12 Aims of the Master thesis

Melanoma is one of the most aggressive skin cancers and many studies are conducted to find suitable target therapies to overcome this disease. The main purpose of this thesis was to investigate the role of MK5 in melanoma cell lines with *BRAF a*nd *NRAS* mutations and to test if this protein kinase can be used as target therapy in melanoma. For this purpose following studies have been performed:

- use melanoma cell lines transiently/stably expressing MK5 to investigate the possible anti-tumorigenic effect of MK5 and its interference in cell proliferation (MTT and ELISA studies).
- verify the expression of p21 and p16 senescence biomarkers in the cells that express MK5
- evaluate senescence status of cells overexpressing MK5

2. Materials

Table 2.1 Kits used in this study

Kit	Manufacturer	Purpose
Cell proliferation ELISA, (chemiluminiscent)	Roche Applied Science	Cell proliferation assay
Cell Proliferation Kit I (MTT)	Roche Applied Science	Cell viability assay
Cellular Senescence Kit	Millipore	Cellular senescence assay
Luciferase kit	Promega	Luciferase assay
iScript™ cDNA Synthesis Kit	Bio-Rad	RT-PCR
NucleoBond® Xtra Midi	Macherey-Nagel (Duren, Germany)	Plasmid DNA purification, medium quantities
QIAmp DNA Mini Kit	Qiagen	DNA purification from cultured cells
NucleoSpin® RNA II	Macherey-Nagel (Duren, Germany)	Total RNA isolation

Table 2.2: Cell lines used in this study

Cell -line	Origin	Tissue	Cell type	Reference number	Purpose
A375	Human 54yo female	Skin	Malignant melanoma	CRL-1619	Transfection and proliferation studies
SK-MEL-2	Human 60yo male	Skin	Metastatic melanoma	HTB-68	Transfection and proliferation studies
WM266-4	Human	Skin	Metastatic Primary epithelioid tumor	CRL-1676	Transfection, proliferation and senescence studies

Table 2.3: Plasmids used in this study

Plasmid construct	Size of plasmid	Source	Properties	Purpose

p21-LUC	7.4 kbp	X.F. Wang; Duke University (Datto *et al*., 1995)	Amp^R	Transfection and Luciferase assay
pEGFP-C1	4.7 kbp	Clontech	Kan^R	Control for transfection western blot and other assays
pEGFP-MK5 L337A	6.1 kbp	Seternes *et al*. ; 2002	Kan^R	Transfection
pEGFP-MK5 T182A	6.1 kbp	Seternes *et al*. ; 2002	Kan^R	Transfection
pEGFP-MK5 WT	6.1 kbp	Seternes *et al*. ; 2002	Kan^R	Transfection

Table 2.4: Primers used in this study

Analysis	Primer	Sequence	Source
N-RAS	N-RAS exon2 fr1 F	3'-GATGTGGCTCGCCAATTAAC	Sigma-Aldrich
N-RAS	N-RAS exon2 fr1 R	3'-AAGTGGTTCTGGATTAGCTGGA	Sigma-Aldrich
N-RAS	N-RAS exon2 fr2 F	3'-TACAAACTGGTGGTGGTTGG	Sigma-Aldrich
N-RAS	N-RAS exon2 fr2 R	3'- CCGACAAGTGAGAGACAGGA	Sigma-Aldrich
N-RAS	N-RAS exon3 fr1 F	3'-TTGCATTCCCTGTGGTTTTT	Sigma-Aldrich
N-RAS	N-RAS exon3 fr1 R	3'-TTGGTCTCTCATGGCACTGT	Sigma-Aldrich
N-RAS	N-RAS exon3 fr2 F	3'-TGGTGAAACCTGTTTGTTGG	Sigma-Aldrich
N-RAS	N-RAS exon3 fr2 R	3'- CACAAAGATCATCCTTTCAGAGAA	Sigma-Aldrich
B-RAF	intron 14-2 F	5'-TGCTTGCTCTGATAAGGAAAATG	Sigma-Aldrich
B-RAF	exon 15-2 R	5'-TGTCTGGATCCATTTTGTGG	Sigma-Aldrich
$P21^{CIP1/WAF1}$	P21-F	5'-GGTGGCTATTTTGTCCTTGG-3'	Sigma-Aldrich
$P21^{CIP1/WAF1}$	P21-R	5'-ACAGGTCCACATGGTCTTCC-3'	Sigma-Aldrich
MK5	MK5-S115D F	5'-CTATTTCAGAGAATCGCACAGCACCGGCAC-3'	Eurogentec
MK5	MK5 - 433.R	5'-GCTAAAACTCTGCTAAGCATCCCTCAGGAGCTTGC-3'	Sigma-Aldrich

hmAPRT	APRT F (79-94)	5'-CCGCAGCTTCCCCGACTTCCC-3'	Sigma Aldrich
hmAPRT	APRT R (519-499)	5'-GCGAGGTCAGCTCCACCAGGC-3'	Sigma Aldrich

Table 2.5: Growth media used in this study

Growth media	Manufacturer/ Contents	Purpose
DMEM	Sigma-Aldrich. Standard Dulbecco's modified Eagle's medium	Melanoma cell culture (A375cells)
EMEM	Sigma –Aldrich/Lonza Eagle's minimal essential medium with sodium pyruvate and non-essential amino acids	Melanoma cell culture (SK-MEL-2,WM 266-4cells)
FBS	Gibco®. Heat inactivated Foetal Bovine Serum	Melanoma cell culture
LB (Luria-Bertani)	950 ml dH_2O, 10 g bactotryptone, 5 g Yeast extract, 10 g NaCl, NaOH to pH 7.0 (± 0.2 ml), appropriate antibiotics	Bacterial culture
Opti-MEM®	Gibco®. GlutaMAX™ I, 2.4 g/l sodium bicarbonate, HEPES, sodium pyruvate, hypoxanthine, thymidine, L-glutamine, trace elements, growth factors, 1.1 mg/l phenol red	Transfection of mammalian cell culture

Table 2.6: Buffers and solutions used in this study

Buffers and solutions	Manufacturer/Contents	Purpose
1xPBS(w/Ca^{+2}, w/Mg^{+2})	Biochrom, powder dissolved in dH_2O, pH=7,4	Melanoma cell culture,
1xTBS (Tris buffered saline)	50mM Tris, 150mMNaCl, p H=7,6	Western blot
NuPAGE LDS sample buffer (4X)	Invitrogen	Western blot
Sample buffer	NuPAGE LDS Sample buffer (4x). H2O, 1M DTT	Harvesting cells
NuPAGE 20x Running buffer	Invitrogen, working dilution 1:20	Western blot
TBST	TBS with 0,1% Tween 20	Western blot
10x Washing buffer	100 nM Tris HCl pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$ and dH_2O up to 1 l. Working dilution 1:10	Western blot
1x TE buffer (pH 8.0)	100 mM Tris/ 10 mM EDTA	Plasmid DNA purification/ DNA storage
CDP star buffer	10ml DEA+850mldH_2O, pH=9,5	Western blot
1x GelRed	Biotium (Hayward, CA, USA)	Agarose gel electrophoresis

50x TAE (Tris Acetat EDTA)	242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0), dH_2O up to 1 l. Working dilution 1:50.	Agarose gel electrophoresis
6x Loading buffer	0.25 % bromophenol blue, 40 % sucrose	Agarose gel electrophoresis
SeaKem LE agarose	Lonza	Agarose gel electrophoresis
JumpStart™ Taq Read Mix™	Sigma	PCR
BigDye Terminator v 3.1	Applied Biosystems	Sequencing
Trypsin/ EDTA	Lonza 0.25% trypsin inPBS and 0.05% Na_2-EDTA	Melanoma cell culture
96% Ethanol	Sigma-Aldrich	Plasmid DNA purification
Blocking buffer	150 ml PBS, 7.5 g low fat milk powder and 150 µl Tween 20	Western blot
Blotting buffer	5.8 g Tris base, 29 g glycine, 200 ml methanol and 800 ml dH_2O	Western blot
Isopropanol	Arcus	Plasmid DNA purification
MTT stock solution	5 mg/ml MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide; Sigma-Aldrich and Roche Applied Science) in PBS. Working dilution 1:20	Cell viability assay
Solubilization solution	Roche Applied Science	Cell viability assay
Stop solution	100 ml isopropanol, 330 µl 37 % HCl	Cell viability assay
BrdU	Roche Applied Science	Cell proliferation assay
FixDenat solution	Roche Applied Science	Cell proliferation assay
Washing buffer	Roche Applied Science	Cell proliferation assay
Substrate solution	Roche Applied Science	Cell proliferation assay
SA-β-Gal detection solution	Millipore/ solution A(10x), solution B (10x), X-gal(40x), PBS until 2ml	Cellular senescence assay
Forskolin (FSK) 10mM in DMSO		Stimulation of cells
Tropix® Lysis buffer	Promega	Luciferase assay

Table 2.7: Transfection reagents used in this study

Transfection reagents	**Manufacturer**	**Purpose**
Lipofectamine® 2000	Invitrogen	Transfection of melanoma cells
Metafectene ®Pro	Biontex	Transfection of melanoma cells

Table 2.8: Enzymes used in this study

Enzyme	Manufacturer	Purpose
Big Dye®Terminator v3.1	Applied Biosystems	Sequencing
ExoSAP-IT	USB®	Clean-up of PCR product

Table 2.9: Molecular markers used in this study

Molecular marker	Manufacturer	Purpose
1kb Plus DNA ladder	Invitrogen	Agarose gel electrophoresis
GelRed™	Biotium	Agarose gel electrophoresis
MagicMarker™ XP Western Standard	Invitrogen	Western blot
SeeBlue® Plus 2 Prestained Standard (1x)	Invitrogen	Western blot

Table 2.10: Antibodies used in this study

Antibody	Manufacturer	Dilution	Purpose
anti-GFP $IgG_1\kappa$ (Monoclonal mouse)	Roche	1:1000	Primary antibody for detection of GFP and GFP fusion proteins in western blot
PRAK (A-7) (Monoclonal mouse IgG_1)	Santa Cruz Biotechnology	1:1000	Primary antibody for detection of MK5 protein in western blot
Goat anti-rabbit Ig/AP (Polyclonal)	Southern biotech	1:2000	Secondary antibody in western blot
Rabbit anti-mouse Ig/AP (Polyclonal)	Dako	1:2000	Secondary antibody in western blot
anti-p16 (C-20):Sc-468 Polyclonal rabbit	Santa Cruz Biotechnology	1:1000	Primary antibody for detection of p16 protein in western blot
ERK-2	Santa Cruz	1:1000	Loading control

Table 2.11: Equipment used in this study

Equipment	Manufacturer	Purpose
AccuBlock Digital Dry Bath	Labnet	Heating block
Scepter automatic cell counter	Millipore	Counting cells
Centrifuge Avanti® J-26 XP	Beckman Coulter™	Centrifugation of ≥ 15 ml
G-Box	Syngene	UV-camera for gels

GeneAmp PCR System 9700	Applied Biosystem	Thermal cycler
Rotator SB3	Stuart	Tube rotator
Immobilon®-P Transfer Membrane pore size 0.45 µm	Millipore®	Western blot
Infros AG CH-4103 bottmingen	Tamro	Shaker
LAS-4000	ImageQuant™	Western blot Luminescent Image Analyzer
Luminometer	Luminoskan, Finland	Luciferase assay
Luminoscan Ascent	Luminoskan, Finland	Microplate Luminometer for cell proliferation assay and luciferase assay
VERSAMax microplate reader	E.Pederson&SØNN Molecular Devices	MTT assay
Biorupture Sonicator System	Diagenode S.A Belgium	Sonicate the samples for western blot
Flow cytometer	BD Bioscence	Selection of stable cell lines
Inverted microscope	Leica	Counting cells in senescence assay
Microfuge® 22R refrigerated centrifuge	Beckman Coulter™	Centrifugation of 1.5 ml Eppendorf tubes
Fluorescence microscope	Leica	Check transient and stable transfection with EGFP plasmid constructs
NuPAGE® 4-12 % Bis-Tris gel	Invitrogen	Western blot
Spectrophotometer ND-1000	Saveen Werner	Nucleic acid measurements
Sub Cell system	Bio-Rad	Agarose gel electrophoresis
TW8	Julabo	Water bath
Versamax multiplate reader	Molecular device	Spectrophotometer for MTT assay
Water jacketed incubator	Forma scientific	Humidified CO_2-incubator
XCell SureLock™ Mini-Cell	Invitrogen	SDS-PAGE and Western Blot
T-100 PCR machine	BioRAD	PCR
Spectrophotometer ND-1000	Saveen Werner	Nucleic acid measurements

Table 2.12: Antibiotics used in this study

Antibiotic	**Manufacturer**	**Purpose**
G418 Geneticin 64mg/ml	Calbochem	Establishing stable cell line
Kanamycin	Invitrogen	Bacterial culture
Penicillin (100 U/ml) Streptomycin (100 µl/ml)	Sigma-Aldrich	Melanoma cell culture

3. Methods

This part describes the methods that have been used in this study starting with the purification of nucleic acid, working with mammalian cells, PCR and sequencing. The flowchart from the **Figure 3.1** gives a short overview of the methods.

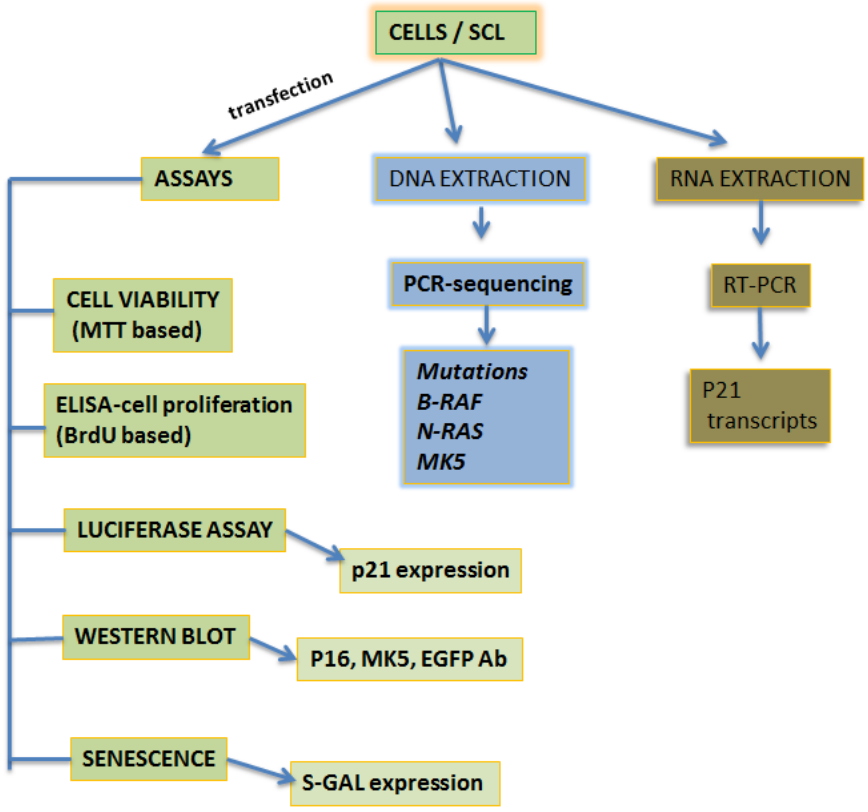


Figure 3.1: Flowchart with the methods used in this study. Melanoma cells were transiently or stable transfected with different plasmid DNA containing *mk5* gene then used in proliferation and senescence assays. The expression of p16 and senescence –associated β –galactosidase (S-GAL), markers for cell cycle arrest and senescence, respectively, was investigated by Luciferase assay, Western blot and Senescence assays. DNA isolated from cells was checked for mutations in *NRAS*, *BRAF* and *MK5* genes. The expression of p21, a marker of cell cycle arrest, was investigated by RT-PCR. (SCL- stable cell line).

3.1 Purification of nucleic acid

Plasmids are the most common vectors used in genetics and biotechnology labs.

A foreign DNA (a particular gene) can be inserted into a plasmid vector and expressed in competent bacterial cells in the presence of a selective antibiotic marker. Plasmids are also used for producing large amounts of protein from the inserted gene in appropriate host cells such as bacteria or yeast [Gupka, 2008].

To investigate the expression and the role of different proteins in melanoma cells, both DNA and RNA were isolated from cells and used for different experiments in this study.

3.1.1 Protocol for plasmid purification with Nucleobond®Xtra Midi Kit

Nucleobond®Xtra Midi Kit from Machery- Nagel (**Table 2.1)** was used to purify high-copy plasmids from Escherichia coli (E. coli), DH5 strain bacterial culture. The method consists on ionic interaction of positively charged silica-based membrane with negatively charged phosphate backbone of the plasmid DNA, binding of nucleic acid to the column, followed by the elution of pure DNA in alkaline conditions [user manual 2012 including references available at http://www.mnnet.com/Portals/8/attachments/Redakteure_Bio/Protocols/Plasmid%20DNA%20Purification/UM_pDNA_NuBoXtra.pdf].

Bacteria transformed with the plasmid of interest were grown overnight in 100ml LB containing the appropriate antibiotics (**Table 2.10**) at 37°C and 220 rpm. The cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4°C, then the supernatant was discarded and the pellet resuspended in 8 ml Resuspension buffer (RES) containing RNase to eliminate all the RNA. Cells were thereafter lysed through alkaline lysis with NaOH/SDS by adding 8 ml of lysis buffer (LYS) and inverting the tubes 4-6 times before incubation for 5min at RT. NaOH breaks the cell wall and disrupts the hydrogen bonding between DNA bases, converting dsDNA into ssDNA and SDS denatures most of the proteins in the cell, helping the separation of the proteins from the plasmid later in the process. During the incubation time, the column and the inserted column filter were equilibrated by applying 12 ml equilibration buffer (EQU) to the rim of the column filter and allowing the column to empty itself by gravity flow. The lysate was neutralized by adding 8 ml neutralization buffer (NEU) and mixing by gentle inversion of the tubes 10 to 15 times before loading onto the filter. Neutralization solution contains potassium acetate which decreases the alkalinity of the mixture, allowing the plasmid DNA to re-nature to dsDNA, while genomic DNA, SDS and denatured cellular proteins stick together and form a white precipitate.

The lysate was then poured on the column filter and loaded into the column. When column had emptied, the filter was removed and the column was washed with 5 ml equilibration buffer (EQU) and 5 ml of washing buffer (WASH) and plasmid DNA was eluted from the column by adding 5 ml elution buffer (ELU). The eluted DNA was precipitated by adding 3.5 ml isopropanol at room temperature. The DNA was pelleted by centrifugation at 15,000 x g for 30

minutes at 4°C, subsequently washed with 5 ml of 96% ethanol at room temperature. After a quick centrifugation at 15,000 x g for 5 minutes at 4°C, the ethanol was discarded and pelleted plasmid DNA was resuspended in 200 µl 1x TE buffer. The DNA concentration and purity was measured by Nanodrop.

3.1.2 Protocol for purification of DNA from cultured cells

QIAmp DNA mini kit from Qiagen (**Table 2.1**) was used to isolate DNA from melanoma cells. Large flasks (T175) were used for growing the cells until 90% confluency. Cells were then enzymatically detached by using trypsin, counted and up to $5x10^6$ cells were pelleted by centrifugation for 5 min at 200xg.The cell pellet was resuspended in an Eppendorf tube with 200µl PBS, then lysed by adding 200µl AL buffer and proteins denatured by adding 20µl proteinase K. Samples were mixed by pulse-vortexing for 15 sec. and incubated for 10 min at 56°C for an efficient lysis. In the next step, 200µl ethanol 96% was added to the samples, followed by a brief mix by pulse-vortexing for 15 sec. The lysate was transferred to a spin column in a 2ml collection tube and centrifuged for 1min at 6000xg.The spin column was washed with 500µl washing buffer AW1 and AW2, the filtrate discarded each time in a new 2ml collection tube by centrifugation for 1 min at 600xg and 20.000xg for 3 min, respectively. After removing the contaminants, DNA was eluted in 200µl elution buffer (AE), incubated for 5 min at RT then centrifuged at 6000xg for 1min.The concentration and the purity of the DNA were measured with Nanodrop. The samples were stored at -20°C until use.

3.1.3 Protocol for purification of total RNA

To isolate RNA from mammalian cells the Nucleospin®RNAII kit from Machery-Nagel was used [user manual 2011 and references available at http://www.mn-net.com/Portals/8/attachments/Redakteure_Bio/Protocols/RNA%20and%20mRNA/UM_TotalRNA.pdf].

The protocol allows the purification of up to 70µg of total RNA from up to $5x10^6$ eukaryotic cells. All steps were performed at room temperature and all centrifugation steps were performed at 11,000xg. The cells of interest were grown in T125cm^2 flask, collected in a 15ml tube by centrifugation and lysed by addition of 350µ of RA1 buffer and 3,5µl 1M DTT. The RA1 buffer contains large amounts of chaotropic ions, thus preventing the degradation of RNA by

inactivating RNases and creating appropriate binding conditions for adsorption of RNA to the silica membrane. The lysate was filtrated by centrifugation for 1 minute. After the removal of the filter, 350µl of 70% ethanol was added for adjusting RNA binding conditions. The lysate was loaded into a column and centrifuged for 30 seconds, the silica membrane desalted with 350µl MDB (Membrane desalting buffer). Salt removal improves the digestion of contaminating DNA by rDNase solution (10µlrDNase and 90µlReacton Buffer for rDNAse) which is directly applied to the silica membrane followed by a 15minutes incubation time. Salt, metabolites, macromolecular cellular components were removed from the silica membrane with 2 different washing buffers (200µRA2 ,600 and 250µl RA3buffer) in 3 washing steps, each step followed by centrifugation for 30 seconds (2 minutes in the last step). Pure RNA was finally eluted under low ionic strength conditions with RNase-free water. The concentration and quality of total RNA was determined by NanoDrop and immediately placed at -70^{o}C to avoid its degradation.

3.2 Evaluation of nucleic acid

To determine the concentration and the purity of nucleic acid, UV-spectroscopy method was used. In case of troubleshooting the nucleic acid could be checked on agarose gel electrophoresis for integrity and purity. As described by the Beer –Lambert law, when UV light passes through a sample containing light absorbing molecules such as DNA or RNA, there is a linear relationship between light absorbance and concentration of nucleic acids. The purity of the samples can be assessed by evaluating the ratio of absorbed light between different wavelengths. The mean absorbance peak for nucleic acids is at 260nm .The ratio of absorbance at 260 nm versus 280nm is used to evaluate DNA contamination with proteins. In general, pure DNA and RNA display an A_{260}/A_{280} ratio of 1.8 and 2 and an A_{260}/A_{230} ratio of 1.8 and 2, respectively .If the ratio is lower there is possibility of contamination with proteins, salts and fatty acids [Kieleczawa J, 2006]. In this study Nanodrop was used to measure the nucleic acid concentration.

3.3 Mammalian cell culture techniques

3.3.1 Protocol for sub-culturing of cells

For optimal growth conditions of cell cultures, it is important to maintain and use the cells in the log phase of growth. Adherent cells grow *in vitro* either until they cover the whole surface or consume all nutrients from the medium. To keep cells viable and healthy they should be "splited" or sub-cultured before they reach the stationary growth phase. In this study the cells were sub-cultured by enzymatic treatment with trypsin, resuspended in medium containing FBS and a part of the cells were transferred in a new culture flask [Davey et al., 2004]. Briefly, the medium was aspirated and cells were washed once with appropriate amount of PBS (**Table 2.6)**. After aspirating the PBS, cells were detached from the bottom of the flask by adding a small amount of trypsin followed by 1-5 minutes incubation in the humidified CO_2 incubator at 37°C.Trypsin was inactivated by adding medium containing FBS. Cells were resuspended in the medium by gentle pipetting up and down. Approximately 10% of the cell suspension was transferred in a new flask with complete fresh medium. A split ratio of 1:10 is commonly used, but if required the ration was changed to 1:5 or 1:15.The appropriate volumes of reagents used for splitting the cells are shown in **Table 3.1**

Table 3.1: Regents used for sub- culturing of cells

Reagents	**Medium culture flask**	**Large culture flask**
Growth medium	Up to a total volume of 15 ml	Up to a total volume of 35 ml
FBS	10 %	10 %
PBS	5 ml	10 ml
Trypsin/EDTA	1 ml	2 ml
Growth medium for resuspension	4 ml	9 ml

3.3.2 Protocol for seeding out cells

In this study, cells were seeded out in different concentration depending on the type of plate and experiment. A more detailed description is given in **Table 3.2.**

After the cells of interest were resupended as described in the sub-culturing protocol, a dilution of cell suspension was prepared and a sample of this dilution was aspirated into the Scepter sensor of Scepter Cell Counter instrument (**Table 2.11**). The instrument displays a histogram with the size or volume distribution of cell population, average cell size and cell concentration /ml. The concentration of cells is then multiplied with the dilution factor in order to obtain the total number of cells/ml. To calculate the number of cells needed for all wells, the number of cells wanted in each well was multiplied by the number of wells and divided by the concentration of the cell suspension. The cell suspension was diluted in growth medium and the appropriate amount of diluted cells suspension was applied to the wells.

Table 3.2: The number of cells and the volume of media used in different applications

Type of plate	**Number of cells/well**	**Volume/well**	**Application**
96well	5000-10.000	200µl	Cell proliferation (ELISA)
24well	$2x10^4$ - $8x10^4$	500 µl	Cell viability (MTT)
12well	$8x10^4$ - $1x10^5$	1000µl	Luciferase assay
6well	$1x10^5$ - $3x10^5$	2000 µl	WB, Cellular senescence assay
10cm	$1x10^6$	8ml	Transfection for establishing stable cell lines

3.3.3. Harvesting cells

A certain time after different procedures on cells such as seeding out, transfection, serum starvation, stimulation or inhibition, media was removed and cells were collected in different lysis buffers and used for specific experiments.

3.3.3.1Protocol for harvesting the cells for protein analysis

Stable cell lines expressing different MK5 variants were first seeded out in 6-well-plate as indicated in in the **Table 3.3** .The day after seeding, two experimental conditions were chosen to

monitor the expression of p16 protein in the cells: 1). cells were harvest the next day after seeding or; 2). cells were serum starved for 18hrs then grown in complete medium with serum for up to 10hrs, and subsequently harvested for western blot.

To harvest the cells, media was first aspirated from wells, followed by a washing step with warm PBS. Lysis buffer was prepared as described in the **Table 3.4**. Eighty µl of buffer was added to each well and cells were collected with cell scraper. The lysed cells were transferred to Eppendorf tubes. The lysed cells were denatured at 70°C for 10 min and sonicated 15times with 10sec on and 10 sec off at 4 ^{0}C. Sonication was performed to shear the DNA. It makes the samples less viscous and easier to load on the polyacrylamide gel.

Table 3.4: Contents of the lysis buffer used for Western Blot

Reagents	**Amount for one well**
4 x LDS buffer	20 µl
dH_20	52 µl
1 M DTT	8µl
Total volume	80 µl

3.3.3.2 Procedure for harvesting the cells for DNA/RNA purification

Cells were grown in large media culture (T175) until were 80% confluent then collected in 15 ml tubes after the cells had ben trypsinized and resuspended. An amount of maxim $5x10^6$ cells were used for the extraction and purification of DNA /RNA. The cells suspension was centrifuged at 1500g for 5min and the cell pellet was homogenized in 250µl PBS for DNA extraction and 350µl RA1 buffer for RNA extraction.

3.3.3.3Protocol for harvesting the cells for Luciferase assay

Cells were seeded in 6-wel-plates and transfected with p21-LUC plasmid (**Table 2.3)** the next day. Twenty four hours after transfection medium was removed from the wells, cells washed once with PBS then harvested in 100µl tropix lysis buffer and transferred in Eppendorf tubes. The buffer with the cells was centrifuged for 10 min at 13,000g and 20µl supernatant was used in luciferase assay.

3.4 Transfection of mammalian cells

In *transient transfection* genetic material is introduced into the cell and temporarily expressed and finally diluted through mitosis or degraded. This method is used for fast analysis of gene and small scale protein production.

To study the role of MK5 in proliferation of melanoma cells, plasmid DNA expressing different variants of MK5 have been transferred into cells by transfection. Two different liposome-mediated transfection methods have been used in this study. A liposome- mediated transfection involves the mixing of DNA with lipids to produce liposome which allows the cellular membrane for the uptake of DNA into the cell by endocytosis [Mülhardt, 2007].

3.4.1 Lipofectamine^{TM}2000 protocol

The day before transfection the cells were seeded out as described in the seeding protocol. The following day, at 70-90% confluency, cells were used for transfection. Lipofectamine^{TM}2000 (**Table 2.7)** and DNA were diluted in OptiMem as indicated in **Table 3.5**. The ratio between DNA and Lipofectamine was 1:2.5 or 1:3. The diluted Lipofectamine^{TM}2000 was incubated for 5min then combined and incubated for 20 min with diluted DNA. All steps were performed at room temperature. The medium was replaced with fresh medium and the combined Lipofectamine/DNA transfection mixture was added to the well. The cells were incubated at 37^0C in humidified CO_2 incubator. After 4hours the medium was removed and replaced with fresh growth medium. The plates were placed back into the incubator and left until the next day.

Table 3.5: Amounts of reagents for transfection with Lipofectamine ^{TM}2000 and Metafectene used in different studies (12well-plate for Luciferase assays, 24-well plate for cell viability MTT based assays, 96-well plate for ELISA cell proliferation assays, 10 cm plate for stable transfection).

Culture plate	12-well plate	24-well plate	96-well plate	10cm plate
DNA amount(µg)	0.5	0.25	0.1	6-8
Lipofectamine^{TM}2000 or Metafectene amount(µl)	1.25-1.5	0.65- 0.75	0.3	15-24
OptiMEM for dilution of DNA/Lipofectamine (µl)	100	50	25	2000

OptiMEM for dilution of DNA/Metafectene (µl)	50	50	30-50	700

3.4.2 Metafectene ®Pro protocol

The cells were seeded in an optimal concentration as describe in the seeding protocol. Metafectene ®Pro (Table 2.7) and DNA were diluted in OptiMem as indicated in the Table 3.5. The ratio between DNA and Metafectene was 1:3.After 5 min incubation, the diluted metafectene was combined with diluted DNA, followed by an additional 20 min incubation. All steps were performed at room temperature. The cell medium was replaced with fresh medium and the combined Metafectene/DNA transfection mixture was added to the well. The cells were incubated at 37^0C in humidified CO_2 incubator until next day.

3.5 Establishing stable cell lines

Stable cell lines consist in the long term expression of a gene of interest, sustained by the presence of an antibiotic selective marker. The main steps include the introduction of the gene into the cell by transfection, subsequently into the nucleus, and finally the integration of the gene into the chromosomal DNA. Since the integration into the host chromosomes is a rare event, a selection marker, such as an antibiotic resistance gene, can be co-expressed with the gene of interest in the same plasmid. Alternatively, the plasmid encoding the selective marker can be co-transfected with the plasmid containing the gene of interest. The selection of stable transfected cells is based on resistance to antibiotics. After gene transfer, the cells were cultivated in medium containing the selective antibiotic. In our case, G418 Geneticin was used to select stably transfected WM266-4 melanoma cells expressing different MK5 variants.
Another two melanoma cell lines, A375 and SKMEL-2, were established by two other colleagues and used in this study.
[http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_TechREF_Generation_of_Stable_Cell_Lines_low_res.pdf]

3.5.1 G418 screening

The G418 Geneticin (**Table 2.12**) is an aminoglycoside antibiotic, related to Gentamicin that interferes with protein synthesis in eukaryotic cells [http://products.invitrogen.com/ivgn/product/10131027]

The gene of interest (in this case *MK5* gene) is cloned into the EGFP-C1 vector which encodes kanamycin resistance gene for selection in bacteria, and neomycin for selection in mammalian cells. The product of the resistance genes (neo^r/kan^r) inactivates G418 by phosphorylation, therefore G418 can be used as selective agent for those cells that have incorporated the gene.

The concentration of selective agent varies from cell line to cell line. Thus, several G418 concentrations have been tested for WM266-4 melanoma cells to identify the maximum concentration that can be used to select only stable-transfected cells. These are the G418 concentrations used in the screening: 200, 400, 600, 800, 1000, 1200 µg/ml. The cells were screened for 6-8 days and the medium containing G418 was replaced every two days. After G418 screening, cells were transfected (as indicated in the **Table 3.5**) in 10cm plates. The day after transfection the medium was changed with fresh medium containing 200µg/ml G418. At 90% confluency, cells were transferred into medium, then into large flasks. At this stage there was a mix population of untransfected cells and stable cells, therefore cells were sorted by flow cytometry (FACS) to select only cells expressing EGFP-MK5 constructs.

3.6 Fluorescence -Activated Cell Sorting (FACS)

The cell population if interest was sorted by flow cytometry for further studies.. In this case fluorescent green cells, with a specific fluorescent intensity, were selected from non-fluorescent cells.

The flow cytometer (**Table 2.11)** has the fluidics system which transports particles in a stream of fluid to the laser beam for interrogation then particles pass by an electronic detection. Only one particle should pass through the center of laser beam in order to have an optimal illumination and to collect relevant information.

www.med.umich.edu/flowcytometry/initialtraining/lessons/.../index.ht

Cells were detached from the bottom of culture flask by trypsinization and cell pellets were collected by centrifugation for 5min at 1500g. Suspension with single cells is needed for FACS sorting. Therefore, the cell pellets were resupended in 1-2 ml media, transferred into a conical tube containing a filter in the top through which cells pass as single cells. Another conical tube was necessary for collecting the cells during sorting. For each experiment non-transfected cells were used for auto-fluorescence control. After the preparation of cells until the end of sorting, cells were kept on ice to reduce their metabolic activity. After sorting, the cell pellets were collected by centrifugation for 5min at 1500xg then resuspended in fresh medium. The choice of culture flask depends on the number of sorted green cells. Knowing that many cells would die after this procedure, additional 5% FBS was added to the growth media to help the cells to recover. The cell culture flask was incubated at 37°C in a humidified CO_2 incubator until the next day, whereafter the cells were checked for viability and the medium was replaced with fresh complete medium and G418. The FACS sorting was performed between 2-4 times for each cell line.

3.7 Cell viability (MTT based) assay

Cell viability is defined as the number of healthy cells in a sample, without distinguishing between actively diving cells or quiescent cells. Morphology and metabolic activity could be used as indicators for cell viability from [Roche-applied- science manual].
MTT assay (Table 2.1) is a quantitative measurement of metabolic activity of the cells, which consists in cleavage of yellow tetrazolium salt MTT (3-[4, 5-dimethylthiazol-2yl]-2,5 –dephenyl-tetrazolium bromide) to purple formazan crystals by mitochondrial succinate- tetrazolium reductase enzymatic system in the metabolically active cells[Hughes D, 2003]. The pyridine nucleotide factors NADH and NADPH are involved in this cellular reduction. The assay can be used not only for determination of cell viability and factor-mediated toxicity but also for cell activation and proliferation. Two types of kits were used for MTT assay: MTT solution from Sigma and MTT kit from Roche –Applied Science.

3.7.1. Protocol for MTT assay (Sigma)

Preparation of solutions:

- MTT stock solution of 5mg/ml was prepared in PBS, sterile filtered and covered with aluminium foil to preserve it from light
- Stop solution: 96.7% Isopropanol/3.3% HCl. To prepare 100 ml: mix100ml isopropanol with 330µl of 37% HCl.

The stable cell lines were seeded in24 -well-plates at optimal density (as indicated in seeding protocol).Incubation of cell culture was for at least 24hrs to allow cell propagation. It is highly important that the assays are carried out before cells reach full confluency. At the time of the assay, 1:10 (or 1:20) dilution of stock MTT solution in new complete growing medium (pre-warmed) was prepared. The medium from the wells was discarded and changed to the new complete growing medium containing MTT (use 250µl medium/well and 25µl MTT stock solution). The plate was covered with aluminium folium and incubated for 2 hours at 37ºC. During the incubation purple formazan crystals are formed. These salt crystals are insoluble in aqueous solution, but may be solubilized by adding solubilization solution. After incubation, 250 µL of stop/dissolving solution (96.7% Isopropanol/3.3% HCl) was added to each well, followed by incubation for 1 hour at room temperature in a rocker plate. At half time of this incubation the content in wells was pipetted up and down with a micropipette to dissolve the formazan crystals that might have formed. From each well 3x 100µl was transferred into wells from 96- well plates (Nunc cell culture plates). The absorbance was measured with VersaMax microplate reader (**Table 2.11**) at 570 nm with the subtraction of the background at 650 nm. The SoftMax Pro 5.4.1 software program was used to read to results.

3.7.2 Protocol for MTT assay (Roche Applied Science)

The principle is identical, but the protocol differs in in this sense that the MTT solution was incubated for 4 hours with the cells and SDS buffer *(10% SDS in 0.01M HCl)* instead of stop solution was used for solubilisation of the formazan crystals. SDS buffer was added for over -night incubation at 37ºC. The plates, volumes and dilutions were identical in both protocols. The absorbance for background was read at 660-690 nm.

3.8 Cell proliferation ELISA, BrdU (chemiluminescent) assay protocol

Cell proliferation is defined as the number of cells that are dividing in the culture and therefore the synthesis of new DNA in the cells could be a marker for measuring cell proliferation.

For measuring the proliferation of melanoma cells, the Cell proliferation ELISA, BrdU(chemiluminescent) kit (Table 2.1) was used. This immunoassay is based on the measurement of BrdU (5-bromo-2'-deoxiurydine) incorporation during DNA synthesis in proliferating cells [from user manual http://www.roche-applied-science.com/PROD_INF/MANUALS/CELL_MAN/apoptosis_074.pdf].

Cells grown in black 96-well MP (tissue culture grade, clear bottom) were either transiently or stable transfected with MK5 encoding plasmids, or stimulated with FSK. For the proliferation assay, cells were first labeled by the addition of BrdU and kept in a humidified atmosphere at 37^0C. During the 20-24hrs of labeling period, BrdU is incorporated in place of thymidine into the DNA of proliferating cells. After removing the labeling medium by tapping off the plate, cells were fixed, and genomic DNA denatured in one step by adding FixDenat solution. The denaturation of DNA is necessary to expose the incorporated BrdU for detection by the antibody. After removing FixDenat by flicking off and tapping, the peroxidase –conjugated anti-BrdU antibody (anti-BrdU-POD) was added, in order to locate and bind to the BrdU incorporated into the newly synthesized cellular DNA. To reduce the background and increase precision, cells were washed three times with washing solution. The immune complexes were detected by the subsequent substrate reaction. After sealing the bottom of the plate with black paper, the substrate was incubated by shaking the cells for 3-5 min. In the presence of hydrogen peroxide, POD catalyzes the oxidation of luminol/4-iodophenol substrate, generating a reaction product in excited state that decays to its ground state by emitting light. The generated signal (photons) is quantified by measuring the light emission using a scanning multi-well luminometer (luminescence ELISA reader).

The blank control was performed in each experimental setup and the relative light units (rlu) values obtained had to be subtracted from all other values. The background control (without BrdU) was made only once with the respective cell type and rlu values should not exceed more

than 2% of the respective value in the presence of BrdU. The reagents and reaction conditions are described in the Table 3.6

Table 3.6: Reaction conditions and reagents used for ELISA assay

Reagents	Volume/ well	Incubation time	Temperature (^{0}C)
BrdU	10µM final conc in 100µl	20-24hrs	37
FixDenat	200 µl	30min	RT
Anti-BrdU-POD	100µ	90min	RT
Washing Buffer	3x 300µl	5min	RT
Substrate solution	100µl	3min	RT

3.9 Protocol for Luciferase assay

The *luciferase* gene of the firefly *Photinus pyralis* is a commonly used reporter gene to monitor the strength of a promoter of interest. In this case the *luciferase* reporter gene was placed under control of the p21 promoter. The *luciferase gene* can be inserted into organisms or transfected into all type of cells.The strength of the promoter can be examined by measuring the amount of luciferase produced: high luciferase values indicate strong promoter activity, while low luciferase activity suggests weak promoter activity. This enzyme uses ATP to oxidize its substrate luciferin to oxyluciferin (**Figure 3.2**). This reaction emits light in the blue range of the visible spectrum, 440-479 nm. The amount of light emitted reflects the amount of luciferin oxidized, therefore the strength of the promoter [www.promega.com]. A special instrument (luminometer) allows the fast measurement of the amount of light emitted.

For the luciferase assay, 1x10^{5}cells per well were seeded in 12-well plates as described in seeding protocol (3.3.2). The next day cells were transfected (as in 3.4.1) with the luciferase reporter plasmid containing the luciferase gene under the control of the p21 promoter. The day after transfection, the medium was removed from the wells and cells were washed with 2 ml PBS. Subsequently, the cells were harvest in 100 µl Tropix lysis buffer (**Table 2.6**) containing 0.5 mM DTT as indicated in the harvesting protocol 3.3.3.3. From each sample, 20µl supernatant

was transferred to a 96-well plate (white, flat bottom) together with two blank samples containing 20 µl lysis buffer. After the addition of 100 µl luciferase buffer, the samples were measured in a luminometer.

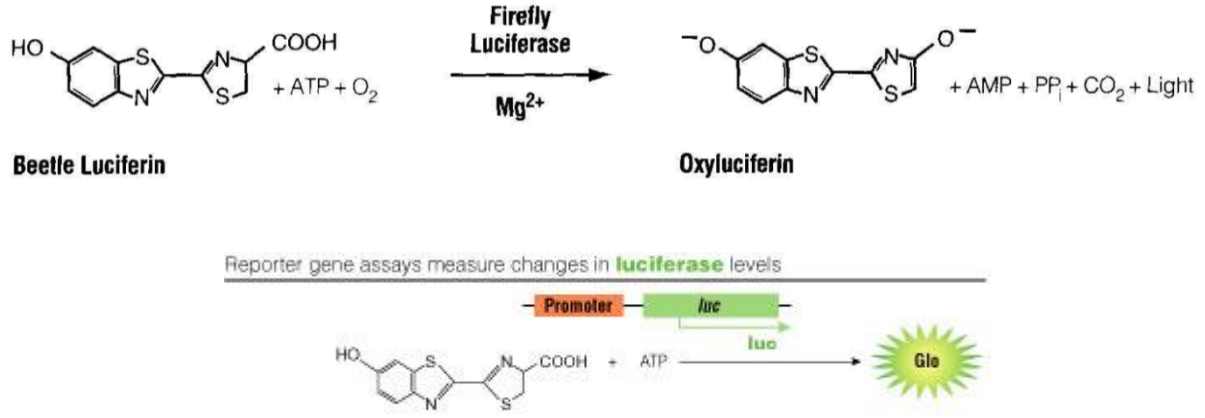


Figure 3.2: Luciferase oxidizes luciferin in the presence of ATP. This reaction is accompanied by the release of light. Luciferase is encoded by the reporter plasmid containing the luciferase gene under control of a promoter (in our case p21 promoter) www.promega.com

3.10 Cellular senescence

Senescence plays an important role in regulating cellular lifespan both in vitro and in vivo [Levine F, 2005]. Senescent cells do not proliferate, but are still metabolically active. Furthermore, changes of cell form (increase in size, flat morphology) and cell function (gene expression) are related to aging. Different studies suggest that senescence represents a tumor suppressor mechanism and its induction may interfere with the development of cancer [Dimri et al, 2005].

3.10.1 Protocol for Cellular senescence assay

The principle of this assay is based on detection of senescence-associated β-galactosidase (SA-β-gal). This enzyme is present only in senescent cells, not in pre-senescent, quiescent or proliferating cells. SA-β-gal catalyzes the hydrolysis of X-gal, which results in accumulation of blue color in senescent cells.

The protocol was adapted for cells grown in 6- well plate. The cells were grown for 1-2 days before the assay was performed. The medium was aspirated from the wells, cells were washed

once with 2ml 1xPBS and cells with 1x Fixing Solution (1ml per well).After 15minutes incubation at room temperature, the fixing solution was aspirated and the cells were washed twice with 1xPBS and 2ml of freshly prepared 1xSA-β-gal Detection Solution (as indicated in **Table 3.7**) was then added. Cells were incubated at 37^0C, without CO_2 and protected from the light, for at least 4hours or left overnight. At the end of incubation, cells were washed twice with 1xPBS and the blue stained cell counted under phase contrast or light microscopy.

Table 3.7: Preparation of SA-β-gal Detection Solution

Reagents	**1 well of a 6-well plate**
Staining solution A (10X)	200µl
Staining solution B (10X)	200µl
X-Gal	50µl
PBS	1.55ml
Total	2ml

3.11 Protocol for serum starvation and FSK stimulation

In case of cell viability and cell proliferation assays, cells were serum starved before stimulation with forskolin (FSK). Two variants were used in this study: serum starvation (1% FBS) for 18-24 hours before the cells were grown in either medium with 2% FBS or medium containing 10% FBS. Cells were stimulated with 10µM (FSK) for 1-3 hours or over- night.

In ELISA assay, the growth of cells in media containing different FBS concentration started at the same time with BrdU labeling of the cells for 20-24 hours or for 3hours after the over- night labeling.

3.12 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a very common method used for separation of proteins in an electric field, having polyacrylamide as support medium and SDS for denaturing of proteins. SDS is an anionic detergent that disrupts the tertiary structure of the proteins and when denatured proteins bind to SDS they become negatively charged. The denaturation takes also place at high temperature (70ºC) and in the

presence of DTT (dithiothreitol) which reduces disulfide bonds of proteins [S-S to SH SH], preventing intra-molecular and intermolecular sulfide bond forming between cysteine residues of the protein. The pore size and the concentration of the gel can be constant or graded and will cause molecules to migrate with different speed through the gel depending on their molecular mass [Sambrook & Russell, 2001].

3.12.1 Protocol for SDS-PAGE

For the SDS-PAGE, the precast NuPage® Novex 4-12 % Bis-Tris Minigel from Invitrogen was used (**Table 2.7**). The cell lysate was SDS- denatured and sonicated before loading into the wells of the gel. The gel was placed in the XCell SureLock™ Mini-Cell, submersed in running buffer (MES SDS) and 10 µl of each sample was loaded. Two µl of markers of known molecular mass such as SeeBlue® (**Table 2.9**) and MagicMarker™ (**Table 2.9**) were applied on the gel to compare and estimate the mass (kDa) of denatured proteins. The gel was run for 50 minutes at 200 V and 70 mA in the NuPage® gel-program.

3.13. Western Blotting

In Western blotting or immunoblotting the proteins are first separated by SDS polyacrylamide gel electrophoresis. The proteins are immobilized on a nylon or nitrocelulose membrane by electrophoretic transfer. The proteins of interest are targeted by incubating the membrane with specific primary antibodies directed against the target protein. The target protein: primary antibody complex is detected by secondary antibodies conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase. The secondary antibody is directed against the constant region of the primary antibody. The enzyme- labeled antibody is used to cleave a chemiluminescent substrate and the amount of luminescence that is produced in reaction is equivalent with the amount of target protein detected from the protein –primary antibody – secondary antibody complex [Sambrook & Russell, 2001].

3.13.1 Protocol for Western Blotting

For the blotting procedure, the Immobilin® transfer membrane (**Table 2.11**) was washed for 3 s in methanol, 10 s in dH_2O and 5 min in blotting buffer and the blotting pads and Whatman filter papers were soaked in blotting buffer. All the steps were performed at room

temperature unless stated otherwise. After gel electrophoresis, the cassette was emptied, the gel and the membrane were sandwiched along with filter papers, sponge pads and buffer in an another cassette (as following: the cathode core on the bottom, three blotting pads, followed by twoWhatman filter papers, the gel, the membrane, another two Whatman filter papers and three blotting pads, the anode core placed on top. The sandwich was placed in the XCell SureLock™ Mini-Cell. The Mini-Cell inner chamber was filled with blotting buffer and the outer chamber with cold water. The blotting was performed for one hour at 30 V and 160 mA. The proteins are transferred to the membrane based on hydrophobic and charged interactions between the membrane and the proteins.

After blotting, the membrane was washed for 10 min in TBS (**Table 2.6)** before incubating the membrane for 1 hour on the rocker plate with blocking buffer containing non-fat dry milk protein in TBS with 0, 1% Tween 20 detergent. The milk protein from this solution will attach to all the places on the membrane except where the target protein is, hence preventing unspecific binding of the primary antibody. Thus the primary antibody can be used to recognize and bind to the protein of interest. The membrane was transferred to a 50ml Falcon tube containing 3 ml blocking buffer and the primary antibody (Table 2.10) and incubated overnight at 4°C on a rotating wheel. After incubation, the blocking buffer with the primary antibody was removed and the membrane was washed 3 times 5 min with TBST, then the membrane was incubated with 3 ml blocking buffer and the secondary antibody (Table 2.10) for 1 hour on the rotating wheel. After two washing steps with TBST and Washing buffer (2x5min each step), 4ml CDP star buffer (Table 2.6) with the CDP star substrate (1:1000) was added to the membrane and incubated for 5 min. The membrane was carefully placed in a plastic bag and analysed on the ImageQuant™ LAS 4000.

3.14 PCR

Polymerase chain reaction (PCR) is a molecular biology technique used to amplify DNA sequences. This is achieved by using oligonucleotide primers that will bind to complementary strands of target DNA via base pairing.

The PCR amplification is done by thermal cycling, each cycle with three main steps: denaturation, annealing and extension .The denaturation step takes place at temperatures higher

than 90ºC when hydrogen bonds between DNA strands are broken and double-stranded DNA is denatured to single stranded DNA (ssDNA). In the annealing step the temperature is lowered (usually to 50-60ºC) so that the oligonucleotide primers can bind to each complementary single strand of DNA (ssDNA). The annealing temperature is determined by the length and base composition of the primers. In the extension step (72ºC) thermostable and proofreading DNA polymerase enzyme adds nucleotides to 3' end of the oligonucleotide primers and thus synthesizes a DNA strand. The target DNA is exponentially amplified in the reaction (2^n, where n is the number of cycles)[http://www.web-books.com/MoBio/Free/Ch9E.htm].

In this study PCR was used for screening of mutations in the melanoma cell lines, using primers (Table 2.4) directed against *B-RAF, N-RAS or MK5* gene sequences.

3.14.1 Standard PCR protocol

In this study, a ready mix including thermo stable DNA polymerase (Taq DNA polymerase), nucleotides and buffers were used. All reagents were kept on ice. The reaction mix was made as described in **Table 3.8** and distributed in PCR tubes. The volume of DNA used in reaction varies depending on its concentration. The PCR tubes were placed in a PCR machine and incubated in the program described in **Table 3.9.**

Table 3.8: Reaction mix for standard PCR

Reagents	**Amount per reaction**
Jumpstart™ Taq Ready Mix™	15 µl
Forward primer (10µM) or (100ng/µl)	1 µl or 1.5µl
Reverse primer (10µM) or (100ng/µl)	1 µl or 1.5µl
Template DNA (500ng)	Up to 7 µl
dH_2O	Up to total volume of 30 µl

Table 3.9: Thermal cycling program used for standard PCR

Number of cycles	**Temperature (°C)**	**Time**
1	96	5 min
30	96	20 sec
	58	20 sec
	72	45 sec

1	72	6min
1	4	∞

3.14.2 Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method to analyze the expression of genes, to determine the presence or absence of transcripts and for producing cDNA for cloning. In the first step RNA strand is reverse transcribed to its complementary DNA (or cDNA) by random hexamer primers and reverse transcriptase, followed by amplification of resulted DNA using polymerase chain reaction. OligoT primers can also be used, but will only convert polyA RNA (i.e. mRNA) into cDNA [Sambrook & Russell, 2001].

The iScript cDNA synthesis kit (**Table 2.1**) from Bio-Rad was used for the synthesis of cDNA. The kit optimized for the production of targets < 1kb in length. The components were mixed as indicated in **Table 3.10.**

Table 3.10: Reaction mix for RT-PCR

Reagents	**Amount per reaction**
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
RNA template	1µg
Nuclease-free water	Up to a Total volume of 20 µl

The complete reaction mix was incubated for 5minutes at 25ºC, 30 minutes at 42ºC, 5 minutes at 85ºC and finally hold at 4ºC.The presence of transcript of interest was determined by cDNA amplification using specific primers during PCR. The reaction mix was made as indicated in the Table 3.11 and run as indicated in **Table 3.12.**

Table 3.11: Reaction mix for PCR

Reagents	**Amount per reaction**	**Amount in ctr**
Jumpstart^{TM}Taq ReadyMix^{TM}	15 µl	15 µl
Forward primer(100ng/µl)	1.5 µl	1.5 µl

Reverse primer(100ng/µl)	1.5 µl	1.5 µl
dH_2O	10 µl	12 µl
cDNA	2µl	-
Total	30 µl	30 µl

Table 3.12: Thermal cycle used for cDNA amplification

Number of cycles	Temperature (°C)	Time
1	94	5 min
35	94	30 sec
	60	30 sec
	72	30sec
1	72	7 min
1	4	∞

The post-PCR samples were analyzed on 2% agarose gel by agarose gel electrophoresis. The PCR-products were visualized using Gel Red and the image was captured with G-Box (Syngene) instrument (**Table 2.11**) by using Gene Snap Acquisition Image program.

3.15 ExoSAP-IT treatment

After PCR amplification, the samples are cleaned –up with ExoSAP-IT reagent to remove un-comsumed dNTPs or remaining primers that might interfere with downstream applications such as DNA sequencing. ExoSAP-IT (**Table 2.8**) is a combination of two hydrolytic enzymes in a special buffer that act upon the PCR product: Exonuclease I removes residual single-stranded primers and extraneous single- stranded DNA and Shrimp Alkaline Phosphatase Recombinant (rSAP) removes the remaining dNTPs [http://www.affymetrix.com].

3.15.1 ExoSAP-IT protocol

Four µl of ExoSAP-IT was mixed with 10 µl post-PCR product. The treatment was performed in a thermal cycler with the program described in **Table 3.13**

Table 3.13: Program in thermal cycler for ExoSAP-IT treatment

Number of cycles	Temperature (^{0}C)	Time
1	37	15min
1	80	15min
1	4	∞

3.16 DNA Sequencing

In this study DNA from melanoma cells (**Table 2.2**) was verified by sequencing for mutations in the *B-RAF, N-RAS* and *MK5* genes.

A mixture of DNA template, primer, the four dNTPs, the four fluorescently labeled ddNTPs, buffer and DNA polymerase is added in each reaction tube for PCR- based sequencing, also called' dye –terminator sequencing'.

In principle, the modified ddNTPs are lacking the 3'-OH group required for phoshodiester bond formation between two nucleotides, thus terminating the elongation of DNA strand. Because these ddNTPs are incorporated randomly, DNA fragments with different length will be obtained. All ddNTP fragments will contain a dye at 3'end.The color of each dye is detected (by a laser), identified (by light sensor) and the corresponding nucleotide and its position recorded by a computer. It is a very convenient and rapid method, largely used in research and diagnostics [Graham CA & Hill JM. 2001].

3.16.1 Sequencing protocol

The sequencing reaction is performed with the reagents described in **Table 3.14** and using the conditions described in **Table 3.15**.The Big Dye 3.1 reagent (**Table 2.9**) contains a ready mix of DNA-polymerase, dNTPs and fluorescence-labeled dNTPs. The sequencing reactions were analyzed by the sequencing facility at UNN using a 313xl Genetic analyzer from Applied Biosystems/Hitachi. For DNA sequences with more than 600bp the volume of Big Dye 3.1 was doubled. The results were compared with nucleotide sequences template from PubMed using the Blast program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3.14: Sequencing reaction mix

Reagent	**Amount per reaction**	
Big Dye 3.1	0.5 µl	1 µl
5x sequencing buffer	3.5 µl	3.5 µl
dH_2O	14 µl	13.5 µl
Mastermix per sample	18	18
PCR product	1	1
Primer 3.2 pmol/µl	1	1
Total sequencing reaction	20µl	20 µl

Table 3.15: PCR program for sequencing

Number of cycles	**Temperature (^{0}C)**	**Time**
30	96	30sec
	50	5sec
	60	4min
1	4	∞

3.17 Protocol for agarose gel electrophoresis

Agarose gel electrophoresis is a method used to separate DNA fragments by their size. In response to an electrical current, DNA will migrate through an agarose matrix. The relationship of size to migration rate of DNA fragments is linear, except the very large fragments. The larger fragments migrate slower on the gel than the small DNA fragments. The concentration of the matrix can be also adjusted depending on the DNA size.
[http://faculty.plattsburgh.edu/donald.slish/electrophoresis.html]
The agarose gel electrophoresis was performed as follows. A 2 % agarose gel was prepared by adding 2 g of agar to 100 ml 1x TAE buffer (Table 2.6), then heated in the microwave for approximately 1 min and subsequently cooled down to 60°C. Thereafter 10µl GelRed fluorescent dye (Table 2.6) was used to stain the gel for visualization of DNA. The gel was poured into a gel-mold, containing a comb in order to form the wells. The gel was left to solidify for approximately 20 min. The samples were prepared by mixing 2µl cDNA, 8 µl dH_2O and 2µl of

6x loading buffer. The gel was transferred to an electrophoresis chamber with 1x TAE buffer. The samples and 6 µl of the 1 kb^+ molecular marker were loaded onto the gel. Finally, the gel was run for 1hour at 90 V and 400mA. The negatively charged DNA will migrate towards the positively charged electrode. The DNA was visualized by UV-light (302 nm) and results were captured using the G-Box from Syngene.

In this study the agarose gel electrophoresis was used to check the expression of p21 transcripts in melanoma cell lines.

3.18 Statistical analysis

The Student's t-test was employed to determine the statistical differences in proliferation between different cell lines.

4. Results and discussions

4.1Establishing stable cell lines

4.1.1Titration of G418 selective marker in melanoma cells

The main purpose for establishing stable cell lines was to incorporate the coding sequence of and to constitutively express different MK5 variants in melanoma cells and to seek for interference of MK5 with RAS/RAF –induced proliferation/senescence. For this purpose, cells were transiently transfected with plasmid constructs encoding EGFP and EGFP-MK5 in the presence of G418 (Geneticin) as a selective marker. This drug was chosen because the plasmids contain the neomycine resistance gene. The concentration of G418 to which cells become resistant to, varies from cell line to cell line. G418 concentrations varying from 0, 200, 300, 400, 600, 800 and 1000µg/ml were tested on WM266-4 melanoma cells to establish the minimal concentration that was toxic for the cells.The results of the G418 titration in WM266-4 melanoma cells are presented below in the **Table 4.1** and **Figure 4.1.**

Table 4.1:G418 titration in WM266-4 melanoma cells, using concentrations ranging from 0 to 1000µg/ml .Cells were grown in 6-well plate in complete medium with different G418 concentrations and observed during 6 days.

Conc ug/ml	24h	48h	72h	**120h**	164h
0 (control)	Normal aspect few dead cells	normal		Confluent and normal aspect	Confluent and many dead cells
200		Fewer cells than in control		Less than 20% of cells survived	Few living cells
400		Less cells than with 200µg/ml conc		1-2% living cells, mostly dead cells	No living cells
600	Some dead cells	More dead cells than alive		No living cells, discard the plate	
800					
1000					

G418 was used for establishing stable cell lines from 3 types of melanoma cells. Since A375 and SKMEL-2 cells were established by other colleagues, only the working concentration is indicated in the **Table 4.2**.

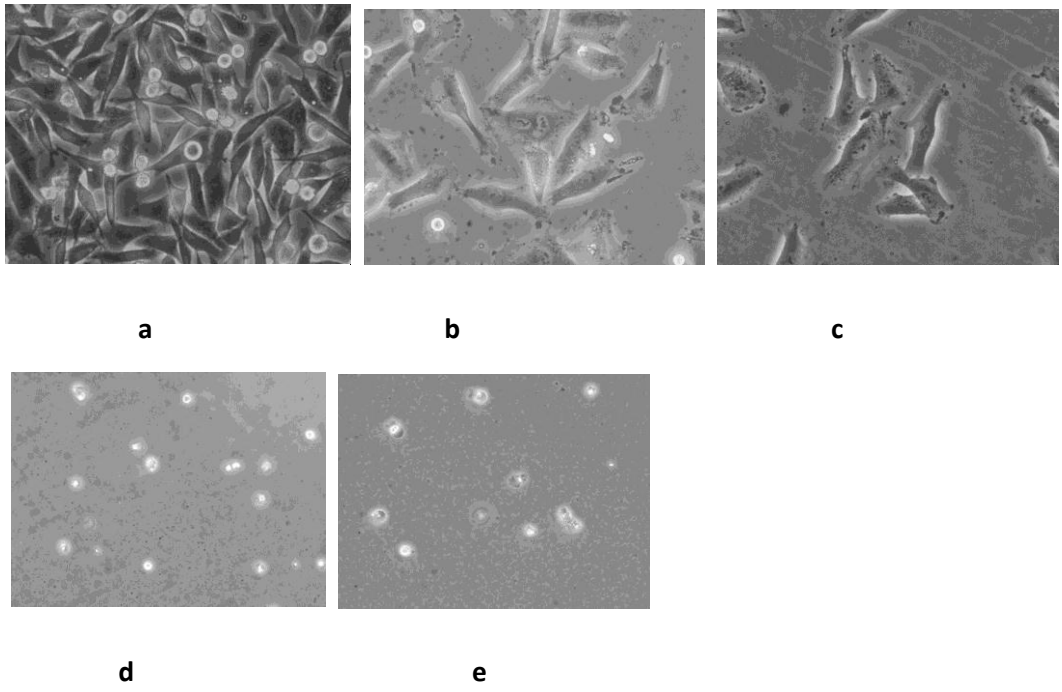


Figure 4.1: WM266-4 melanoma cells grown for 6 days in different concentration of Geneticin (G418) selective marker: a). no marker; b). 200µg/ml G418; c). 300µg/mlG418; d). 400µg/mlG418; e). 600µg/mlG418. The pictures were taken with normal light on fluorescence microscope.

Table 4.2: G418 working concentration in melanoma cell lines

Name of melanoma cell line	G418 µg/ml (working concentration)
A375	1200
SKMEL-2	400
WM266-4	200

4.1.2 Expression of MK5 in melanoma cells

After establishing melanoma cells expressing different MK5variants, the equal expression of these variants was determined by Western blot. The following MK5 variants were used in this study: MK5 wild-type (wt or WT), kinase dead MK5-T182A mutant (in which Threonine residue 182, situated in the activation loop, is replaced with non-phosphorytable Alanine residue), and constitutive active MK5-L337A mutant (in which Leucine has been replaced with Alanine in

NES site, thus impairing nuclear export, but also causing a constitutive activation of MK5) [Seternes et al., 2002].

In A375 cells, the western blot with PRAK (which also recognize MK5) and GFP specific antibodies indicates that EGFP-MK5 fusion protein is expressed in those cells that have been transfected with the plasmid encoding EGFP-MK5 (**Figure 4.2**, lanes 3, 4, 5), but not in untransfected cells or cells expressing only EGFP-C1 (lanes 1, and 2). To ensure equal loading, the membrane was stripped and reincubated with antibodies against ERK2. This protein is ubiquitously expressed and used as a loading control in western blotting [Dittmer and Dittmer, 2006]. Although there is unequal level of expression of the MK5 variants in the different stable cell lines, the results show that establishing stable cell lines was successful.

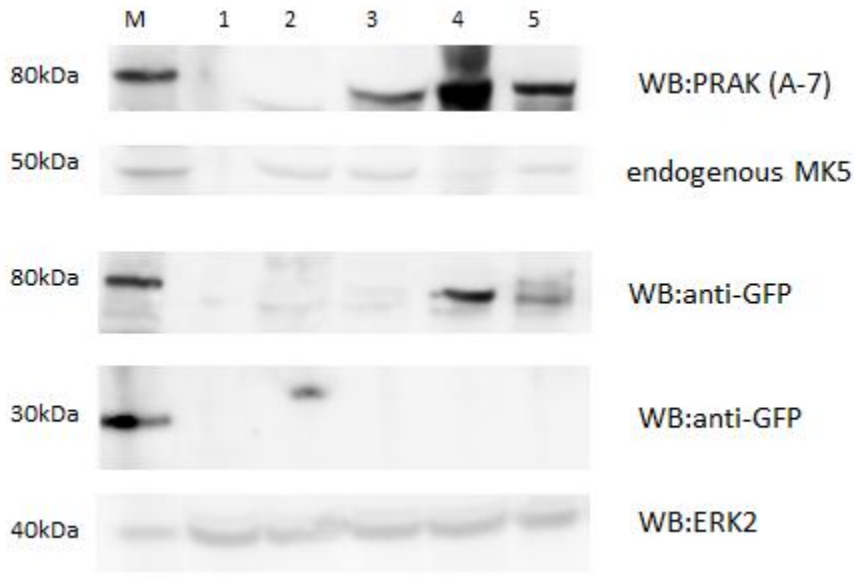


Figure 4.2: Western blot in A375 SCLs - expression of MK5 proteins. Lanes:M-molecular weight marker, (1) untransfected cells, 2-5 stable cell lines expressing: (2) EGFP-C1, (3) EGFP-MK5 WT, (4) EGFP-MK5 T182A, (5) EGFP-MK5 L337A

In WM266-4 cells, there is again clear, but variable expression levels of the different EGFP-MK5 fusion proteins, with higher expression of EGFP-MK5 WT and the L337A mutant compared to the EGFP-MK5-T182A mutant (**Figure 4.3,** lanes 2, 3 and 4; WB :PRAK). There are several unspecific bands < 30kDa, probably due to unspecific binding of GFP-antibody (**Figure 4.3**, WB: anti-GFP).

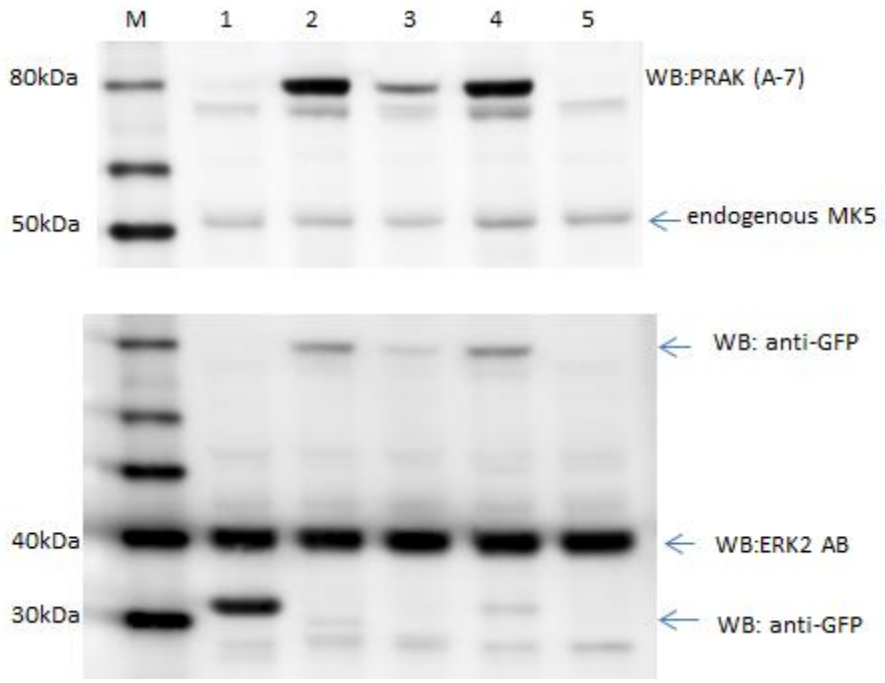


Figure 4.3: WB with WM 266-4 SCLs expression of MK5. Lanes: M-molecular weight marker; 1-4 stable cell lines expressing (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A; (5) cell lysate

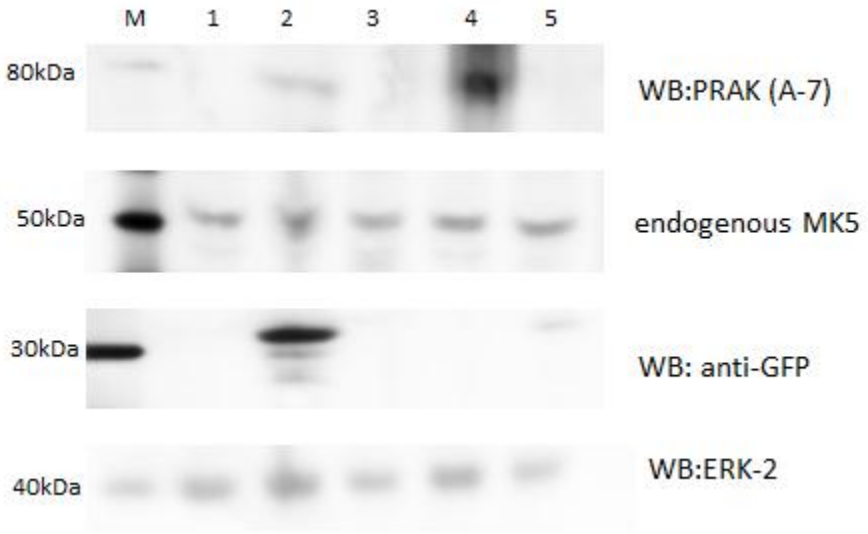


Figure 4.4: Western blot in SKMEL SCLs - expression of MK5; Lanes: M-molecular weight marker, (1) untransfected cells, 2-5 stable cell lines expressing: (2) EGFP-C1, (3) EGFP-MK5 WT, (4) EGFP-MK5 T182A, (5) EGFP-MK5 L337A

In case of SKMEL-2 cells, high expression of EGFP-MK5-T182A mutant, but not of the other variants was observed **(Figure 4.4** lanes 3 to 5, WB: PRAK). Furthermore, the blot against PRAK antibody shows a very week band for MK5 in the sample of stable cells containing only EGFP-C1, although the blot against anti-GFP (**Figure 4.4,** lane 2; WB: anti-GFP) clearly indicates the sample as being EGFP-C1 and not EGFP-MK5 fusion protein. One explanation could be that the quality of the cell lysate was poor. As a rule, after FACS sorting to single out cells that express EGFP or EGFP-MK5 fusion protein, cells were also analyzed under fluorescence microscope. As general observation, cells expressing EGFP-C1 displayed very intense green fluorescence in both the cytoplasmic and nucleic compartments, whereas the EGFP- MK5 variants were less intense green. It is possible that mRNAs for EGFP-MK5 fusion are less efficiently translated than EGFP mRNA or that fusion proteins are less stable. Alternatively, the fusion proteins may fold differently from EGFP and this conformation may affect the fluorescence of EGFP.

4.2. Experiments on A375 melanoma cells

The use of **xCELLigence system (**Roche Applied Science**)** for cell proliferation measurements was considered the ideal and initial plan in this study. This method is based on measurement of electrical impedance to analyze cells in real time, allowing the monitoring of cell viability, number (proliferation), morphology, cytotoxicity, attachment, migration and invasion potential [www.roche-applied-science.com]. Several experimental approaches have been tested such as different cell concentration varied from 5000 cells /well to 15000 cells/well in E-plates, untransfected versus transfected cells, uncoated versus fibronectin-coated slides, different cell types such as A375 and WM266-4 cells. Although there are successful studies in the literature with A375and SK-MEL-5 melanoma cells that monitor cell adhesion, proliferation and migration using the xCELLigence system [Bosserhoff et al.,2011,Chung et al., 2011], in this study no proliferation curve was observed in any of the performed experiments. This may be due to some troubleshooting of experimental settings. An example of typical results that have been obtained by impedance measurement with xCELligence system is enclosed in the **Appendix, Supplementary Figures 1-4.**

4.2.1 Cell proliferation

The influence of MK5 on cell proliferation was investigated in A375 melanoma cells transiently and stably transfected with different MK5 mutants such as EGFP-MK5wt, EGFP-MK5 T182A and EGFP-MK5-L337A. Untransfected cells or cells transfected with EGFP-C1 were used as control in the experiments.

The main idea was to establish stable cell lines that express different MK5 variants and then use these stable cell lines in the proliferation /senescence experiments. The time needed for the selection of the stable cell lines (A375, SKMEL-2 and WM266- 4) varied between one and four months .In the meantime, cells were transiently transfected with MK5 plasmids and used in the experiments.

Two different techniques have been applied in order to investigate the effect of MK5 on proliferation of melanoma cells. Cell viability (MTT based) assay offers an indirect measurement of the metabolic activity of the cells, independent of cell division, whereas ELISA assay measures the DNA synthesis (BrdU incorporation) and is a more reliable method (Roche Applied Science –user manual).

Different experimental procedures had high impact on the results. A375 melanoma cells have acquired the ^{V600E}BRAF mutation and their proliferation rate is higher than in normal melanocytes. In the MTT based assay, as indicated in **Figure 4.5**, *transient overexpression of* MK5 induced a general increase of cell viability, especially when overexpressing MK5-L337A, which gives 1.6 to two fold increase of cell viability compare with the controls, i.e. cells transfected with EGFP-C1vector and untransfected cells, respectively. There is also a slightly increase of cell viability in the presence of the other MK5 mutants.

In contrast to transiently transfected cells, *stable expression* of MK5-L337A in A375 melanoma cells reduced cell viability to 1.1 to 1.5 folds when cell were grown in 2% FBS and 10% FBS, respectively. In another words, there is a reduction of cell viability up to 30% when MK5-L337A is stably expressed in the cells. The results show that MK5-L337A, but not the other variants reduces cell viability of A375 melanoma cells and suggests that the kinase activity of the protein is required **(Figure 4.6).**

The differences in results obtained in experiments with cells transiently and stably expressing MK5, could be due to different experimental approaches. With an efficiency of 50 to 70% of transient transfection, a mixed population of untransfected cells and cells expressing MK5 could mask the real effect of MK5 on cell viability.

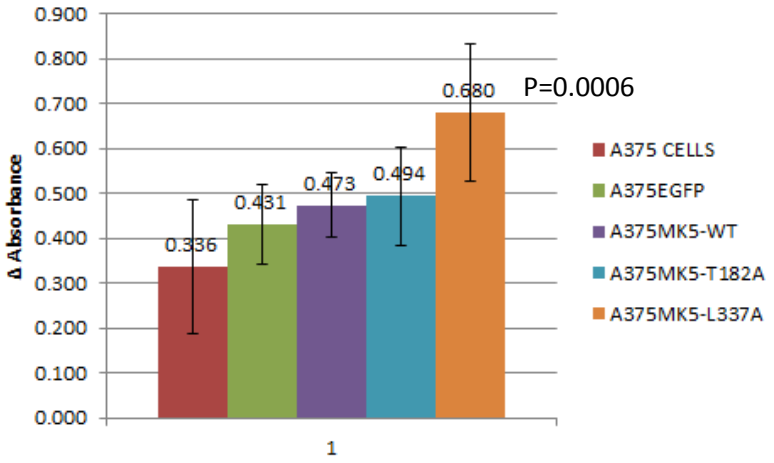


Figure 4.5: MTT assay on A375 cells stably transfected with different MK5 plasmids and forty eight hours after transfection the cells were used in MTT assay. Untransfected cells were used as control for the cells transfected with the negative control EGFP-C1 plasmid. Cells were grown in complete medium containing 10% serum. The absorbance measurement corresponds to the number of viable cells in the experiment. Each bar represents the average of 3 parallels and the standard deviation is indicated.

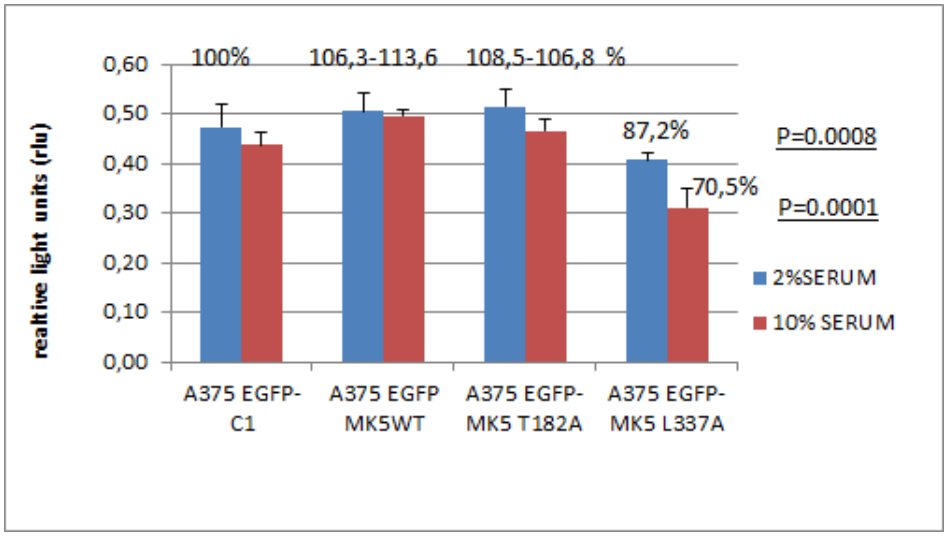


Figure 4.6: MTT assay on A375 stably expressing several MK5 variants. Cells were seeded out in 12-well-plate in complete medium. The next day cells were serum starved (1% serum) for 18-24 hours in order to synchronize cell

cycle progression. After starvation, cells were grown in two different serum concentrations (2% FBS and 10% FBS) with overnight incubation followed by MTT assay.

In ELISA experiments performed on A375-MK5 stable cell lines, the pattern is in a way similar to that obtained in MTT assays. There is a slightly increase in cell proliferation after 24hours of growing in medium containing 10% serum (15% to 17% increase in proliferation for cells expressing MK5- L337A and MK5-T182A mutants, respectively compared to untransfected cells), followed by a slightly decrease of cell proliferation in all MK5 mutants case (4.2 to 7%), difference that is not statistically significant (P=0.578) (**Figure 4.7).** It seems that in this case, constitutively activation of MK5 or its inactivation in cells has almost the same impact on cell proliferation, which is contrary to our hypothesis that active MK5 inhibits cell proliferation.

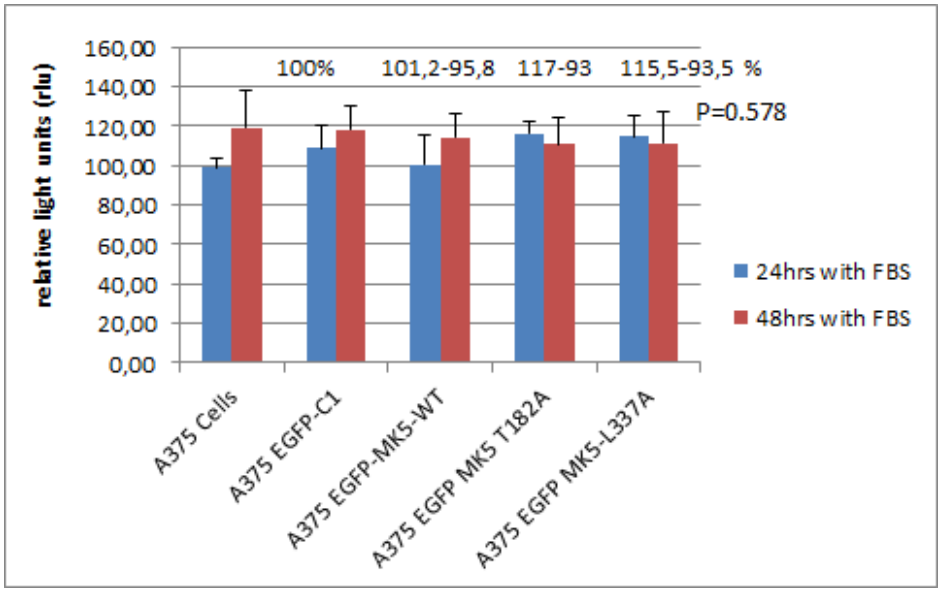


Figure 4.7: ELISA assay on A375 stable cell lines. The next day after seeding, cells were serum starved (in 1% FBS) for 18 hours and subsequently grown in medium containing 10% serum for 24h and 48h with the BrdU labeling in the last 24h of growth. BrdU incorporation, expressed as relative light units (rlu) was measured with a luminometer.

4.2.2 Cellular senescence

Cells undergo a finite number of divisions, a process called senescence. Senescence can be induced as response to aging, DNA damage, cellular and oxidative stresses.

The expression of $p21^{Cip/Waf1}$ and $p16^{INK4a}$ cyclin-dependent kinase inhibitors in the cells can be used as senescence markers. The p21 levels are increased in early senescence and decreased after senescence is achieved. Senescent-cell cycle arrest occurs before accumulation of p16, suggesting that p21 may be sufficient only to initiate this event. Thus upregulation of p16 is important to maintain senescence cell cycle arrest. In the late stage of senescence p16 levels are

high and accumulates in parallel with the expression of senescence-associated β-Galactosidase activity [Stein et. al., 1999]. Moreover, p16 rather than p21, is more frequently mutated in human cancer cells [Hirama and Koeffler, 1995].

It was suggested that MK5 mediates senescence by stimulating p53 transcriptional activity through phosphorylation at Ser-37, which in turn induces the expression of $p21^{Cip/Waf1}$ and leading to cell cycle arrest [Sun et al., 2007].

The effect of MK5 on p21 promoter activity was investigated in A375 melanoma cells by using Luciferase reporter assay. A375 stably expressing different MK5 mutants (wild-type, dominant inactive and constitutively active mutants) were transiently transfected with p21-Luc plasmid. This plasmid contains the luciferase gene under control of p21 promoter. As seen in the **Figure 4.8**, only MK5-L337A mutant was able to induce a higher p21promoter activity compared to the control, which correlates with the initiation of cell cycle arrest. The other MK5 variants actually reduced p21 promoter activity and may therefore have stimulatory effect on cell proliferation.. An ideal experiment should also include cells expressing only p21-Luc vector because as seen in the other experiments, EGFP –C1vector may interfere with cellular processes, therefore is debatable whether EGFP is the right control in proliferation/senescence assays. The student T-test indicated that the difference in luciferase activity (and hence p21 promoter activity) between the cells expressing EGFP-C1 and MK5-WT/MK5-T182A is very significant (P=0.0008 and P=0.0006, respectively), whereas the difference between the cells expressing EGFP-C1 and MK5-L337A is considered significant (P=0.0019) in the 95% confidential interval.

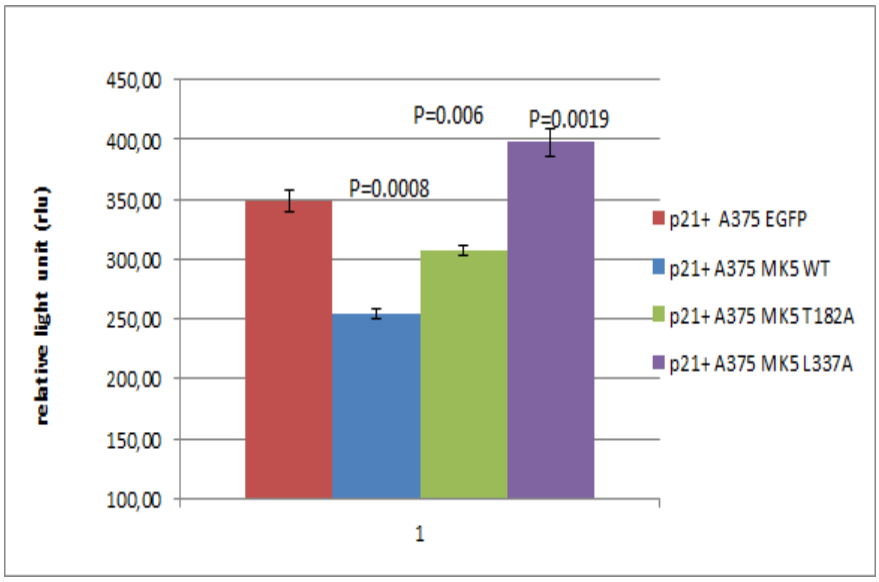
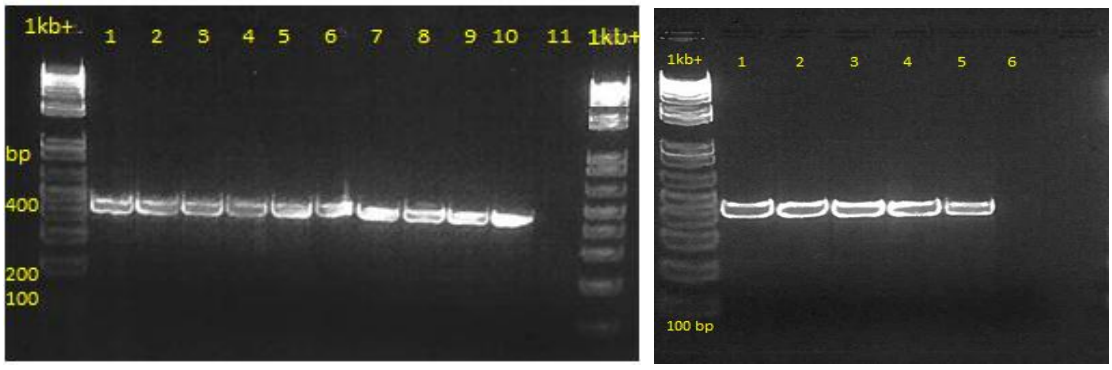


Figure 4.8: Luciferase assay of lysates from A375 SCL: cells that were transiently transfected with 1µg p21-Luc plasmid and either co-transfected with EGFP or EGFP-MK5 expressing plasmids. The day after transfection cell were lysed and used in the Luciferase assay. Each bar represents the average of three independent parallels. The standard deviation is shown and the t-test was used to calculate whether the differences observed with cells not expressing EGFP or EGFP-MK5 were significant.

Another approach for investigating senescence in melanoma cells was detection of the relative transcript levels of the p21 (*CDNK2A)* gene. RNA from A375, SKMEL-2 and WM266-4 melanoma cell lines was extracted and reverse-transcribed into cDNA which thereafter was used as template in PCR amplification with specific primers for the p21 gene.
First, the quality of cDNA was tested by PCR amplification of the adenine phosphoribosyl transferase (APRT) housekeeping gene using with APRT specific primers (**Figure 4.9)**. The APRT transcripts are widely expressed in cells. As shown in the **Figure 4.9**, APRT transcripts were expressed in all the samples, indicating that the generation of cDNA was successful and this cDNA could be further used in the experiments.

Next, cDNA was amplified with p21 specific primers. So far, five different p21 transcripts, all which might depend on p53 for their basal expression in the absence of stress, have been described [Gartel et al., 2005].The primers used in RT-PCR will amplify only p21transcript variant 2 (NM_0007867) which will give a 380bp fragment size and p21transcript variant 5 (NM_00120777) which will give a 421bp fragment size.



A **B**

Figure 4.9: Agarose gel electrophoresis of PCR products obtained after amplification of cDNA from melanoma cells with APRT primers. The size of APRT transcripts is 420bp. **Panel A:** The lanes1-4 contain cDNA fromA375 SCL expressing: (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A. Lane (5) - untransfected A375cells, lane (6)- untransfected SKMEL-2 cells.The lanes7-10 contain cDNA from SKMEL-2

SCL expressing:(7) EGFP-C1, (8) EGFP-MK5 T182A, (9) EGFP-MK5 L337A, (10) EGFP-MK5 WT. Lane (11) – negative control sample without cDNA. Panel B: The lanes1-4 contain amplified cDNA (with APRT primers) fromWM 266-4 SCL expressing: (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A; lane (5) untransfected WM266-4 cells; lane (6) negative control sample without cDNA;

Because the amplified cDNAs from all melanoma cell lines were tested together on agarose gel electrophoresis, all the results will be presented in this section.

p21 transcripts were present only in A375 and WM266-4 melanoma cells (**Figure 4.10** lanes 1-5 for A375 cells; **Figure 4.10** lane 13 for p21 expression in untransfected WM266-4 cells and **Figure 4.11** lanes 6-9 for p21 expression in WM266-4 SCLs).The p21 transcripts were absent in SKMEL-2 melanoma cells(**Figure 4.10** lanes 6-10 and **Figure 4.10** lanes 1-5).

Figure 4.10: Agarose gel electrophoresis of cDNA from melanoma cells amplified with p21 primers. Lanes 1-4 contain cDNA fromA375 SCL expressing: (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4)EGFP-MK5 L337A. Lane (5) - untransfected A375 cells. Lanes 6-9 contain cDNA from SKMEL-2 SCL expressing: (6) EGFP-C1, (7) EGFP-MK5 WT, (8) EGFP-MK5 T182A, (9) EGFP-MK5 L337A. Lane (10) - untransfected SKMEL-2 cells; lane (11) negative control sample without cDNA; lane (12) amplified cDNA (with APRT primers) from WM 266-4, lane (13) amplified cDNA (with p21 primers) from WM 266-4 cells.

Figure 4.11: Agarose gel electrophoresis of PCR amplicons obtained after amplification of cDNA from melanoma cells amplified with p21 primers. Lane (1) contains cDNA from untransfected SKMEL-2 cells, lanes 2-5 contain cDNA from SKMEL-2 SCL expressing: (2) EGFP-C1, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A, (5) EGFP-MK5 WT. Lanes 6-9 contain cDNA from WM 266-4 SCL expressing: (6) EGFP-C1, (7) EGFP-MK5 WT, (8) EGFP-MK5 T182A, (9) EGFP-MK5 L337A; lane (10) contains cDNA from untransfected WM266-4 cells; lane (11) negative control sample without cDNA.

The findings of Sun and colleagues (2007) that MK5 mediates senescence through increased expression of p21[Sun et al., 2007], are in accordance with the results obtained in A375 and WM266-4 melanoma cell lines, although the expression of p21 is observed not only in cells stably transfected with different MK5 mutants, but also in untransfected melanoma cells. In our case, it seems that overexpression of MK5 does not change the levels of p21 transcripts. Furthermore, it is possible that RT-PCR method is not sensitive enough to detect low levels of or changes in the relative amount of p21transcript in SKMEL-2 cells. Real time quantitative PCR would have been a better approach to detect and measure p21 transcript levels. Time restrictions prevented performing doing these experiments.

The cellular senescence was also investigated in established A375 cell lines using western blot against p16 ^{INK4a} tumor suppressor protein , which as p21$^{Cip1/Waf1\ that}$ inhibits cyclin-dependent kinases (CDKs) and induces G1-cell cycle arrest [Shapiro et al., 2000;Rayes et al., 2012]. A typical rapidly proliferating human cell culture uses 24 hours to complete a cell cycle with G1 phase completed in 11 hours [Cooper GB, 2000]. Since p16 protein is not continuously expressed in the cells two experimental approaches were used.

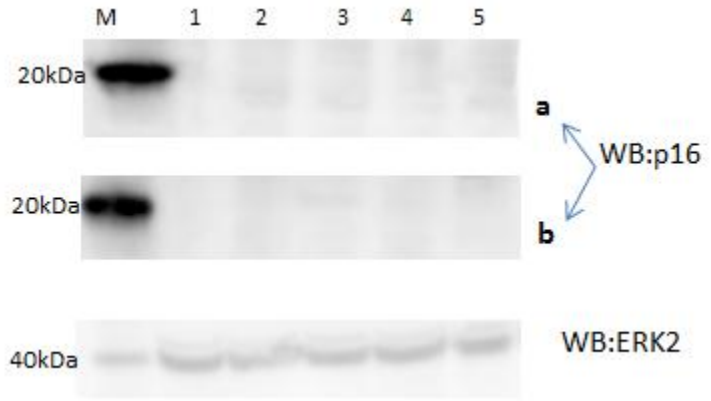


Figure 4.12: **Expression of p16 protein in A375 SCLs** examined by western blot**. Panel a:** Cells harvested within24hrs after seeding**. Panel b:** Cells were first serum starved (1% FBS) for 24hrs, then grown in complete medium with 10% serum for maximum 10hours thereafter harvested. Samples: M-molecular weight marker, (1)

untransfected cells, 2-5 stable cell lines expressing: (2) EGFP-C1, (3) EGFP-MK5 WT, (4) EGFP-MK5 T182A, (5) EGFP-MK5 L337A. Bottom panel: equal loading was verified by examining the levels of ERK2.

In the first approach the cells were grown for 24hours in complete medium before use in western blot, whereas in the second approach cells were synchronized for cell cycle timing by serum starvation (1% serum) for 20-24 hours, then subsequently grown in complete medium with 10% serum between 7 and10 hours before use. No p16 ^{INK4a} expression was found in any of the A375 stable cell lines, indicating that no senescence occurred (**Figure 4.12).**

4.3 Experiments on WM266-4 melanoma cells

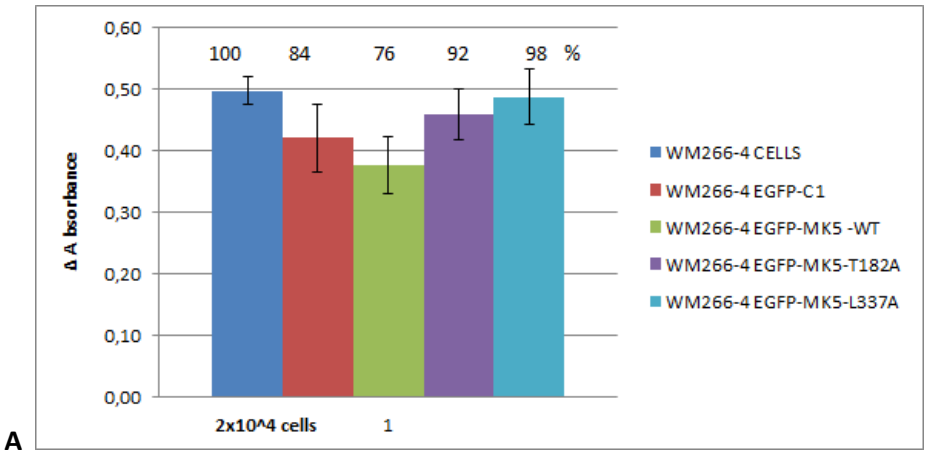
4.3.1 Cell proliferation

The anti-proliferative role of MK5 in WM-266-4 cells was investigated in several experimental approaches such as MTT, ELISA assays and by stimulating the cells with forskolin, an indirect activator of MK5 [Gerits et al., 2007]. The lack of a specific MK5 inhibitor urged us to use forskolin.

In MTT assays WM266-4 melanoma cells were used 24 hours after seeding (stable cell lines) or after transient transfection with EGFP-C1 and EGF-MK5 mutants. Cell viability/ proliferation measured by MTT assay was very much dependent on the number of cells analyzed and their state of confluency. At low cell density ($2x10^4$ cells/well) a slight decrease of cell proliferation was monitor, especially when expressing EGFP-MK5-WT in the cells **(Figure 4.13, panel A**), whereas cell proliferation almost reaches the "plateau phase" when double density of cells ($4x10^4$ cells/well) was used in the experiments (**Figure 4.13, panel B).**

Same "plateau-phase" was obtained in the experiments with WM266-4 cells transiently transfected with EGFP-C1 and EGF-MK5 mutants (results not shown). A high number of cells used in experiments or cells that are 80% confluent when performed the MTT assay makes it difficult to rely on the results or the method. This problem may be overcome by using a very low concentration of cells and avoiding cells to reach more than 70% confluency. This solution might not be suitable for cell lines that give low transfection efficiency in general, because too few cells will be transfected and the method may not be sensitive to measure a possible effect of MK5 on proliferation. The MTT results obtained in WM266-4 cells neither clearly show an anti-proliferative role of MK5, nor totally exclude this hypothesis.

Another approach to investigate the anti-proliferative role of MK5 in melanoma cells was stimulation of cells with forskolin (FSK). The FSK increases intracellular cAMP levels and activates PKA signaling pathway which triggers phosphorylation, activation and nuclear export of MK5 [Gerits et.al, 2007]. Cell exposed to FSK only transiently increase MK5 mRNA transcript levels, with unaltered MK5 protein levels [Gerits et.al, 2007].



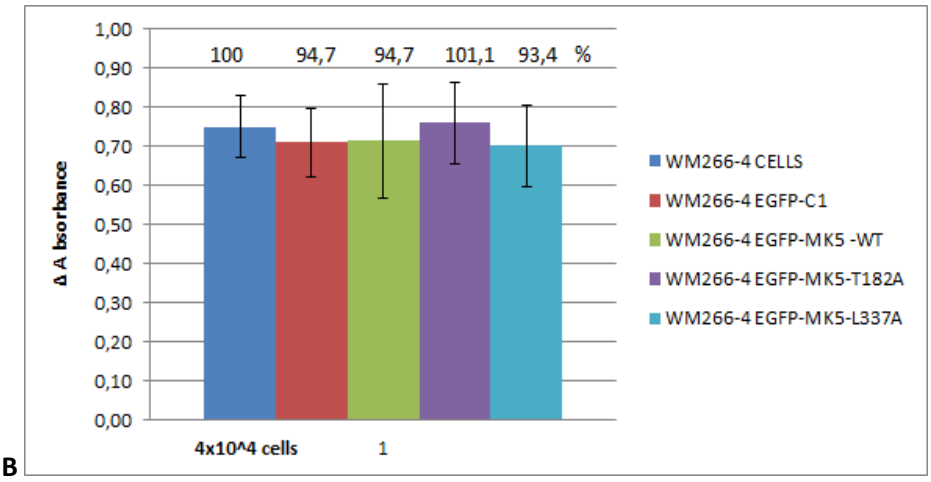


Figure 4.13: MTT assay on WM266-4 melanoma stable cell lines. Panels A and B – the experiment started with $2x10^4$ cells and $4x10^4$ cells. After growing in complete medium with 10% serum for 24 hours, cells were used for MTT assay when confluency did not reach more than 70-90%.

Transiently activation of endogenous MK5 by FSK stimulation of cells for 3 hours actually increased cell proliferation **(Figure 4.14)**, which is in contrast to results from ELISA assays, where overnight FSK stimulation slightly reduced proliferation rate (**Figure 4.15**). The FSK stimulation of cells represents an indirect investigation of MK5 role in cell proliferation because

not only MK5 but also other proteins interact with PKA in the cAMP/PKAsignaling pathway. Extensive cross-talk between the PKA and other MAP kinase pathways have been demonstrated so that a possible effect of forskolin on cell proliferation can be mediated by other MAP kinases than MK5 [Gerits et al., 2008].

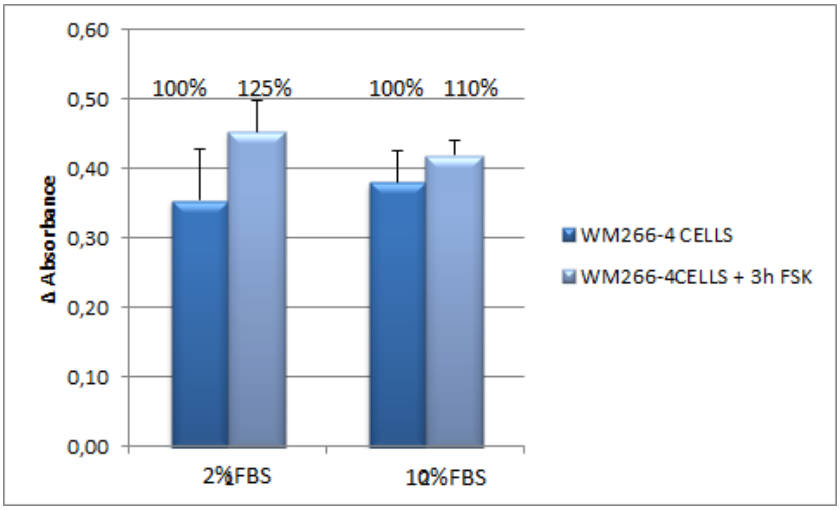


Figure 4.14: MTT assay on WM266-4 cells; first the cells were serum starved (1% FBS) for 18hours then stimulated with 10µM FSK for 3 hours and subsequently used for MT assay.

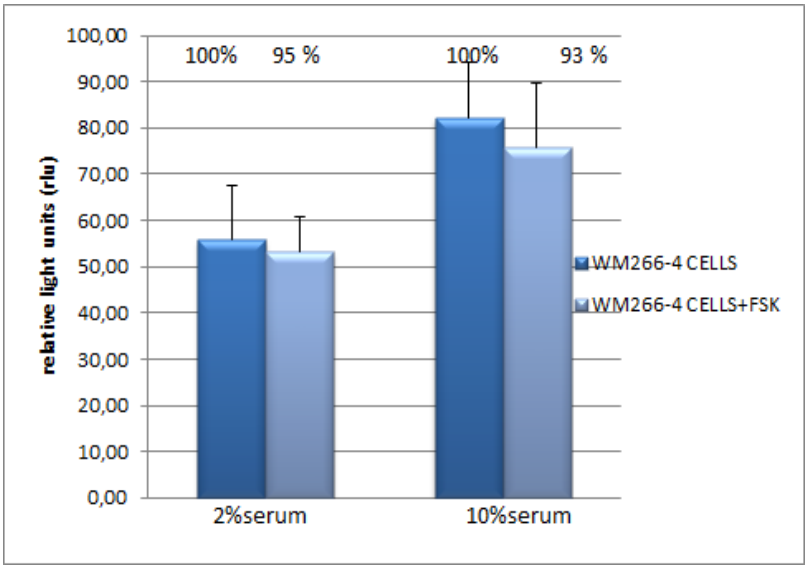


Figure 4.15 : ELISA assay on WM266-4 cells; first the cells were serum starved (1% FBS) for 18hrs then stimulated with 10µM FSK in the same time with BrdU labeling for 20hours and subsequently used for ELISA assay.

4.3.2 Cellular senescence

p16^{INK4a} is important in control of G1/S progression of melanocytes and therefore disruption of this pathway can lead to melanoma by preventing senescence. This pathway is inactivated in most of melanoma cell lines [Bennettet et al., 2003].

The cellular senescence was investigated using western blot with antibodies against p16 ^{INK4a} tumor suppressor protein. The p16 protein is overexpressed in cervical cancer because of inactivation of retinoblastoma protein by E7 viral protein of human papillomavirus (HPV) [Sano et. al., 1998] and therefore lysate from HeLa cells can be used as control for the p16 antibody. Similar to the other stable cell lines two variants were used to investigate the expression of p16 in WM266- 4 cells (**Figure 4.16**, panel a and b).The loading control was previously presented in the **Figure 4.3.** The results showed that p16 was weakly expressed in all WM266-4 stable cell lines and untransfected cells, indicating signs of senescence initiation (**Figure 4.16,** panel a, lanes 1-6). Overexpressing MK5 did not seem to influence p16 levels as the levels in cells expressing MK5 were comparable with the stable cell line expressing EGFP.

Detection of senescence-associated β –galactosidase (SA- β –gal) activity was also used as a biomarker to identify senescent cells.WM266-4 stable cells were seeded out in 6 -well plate and grown for two days before staining with SA-β-gal detection solution for overnight at 37°C.

Figure 4.16: WB with WM 266-4 SCLs expression of p16 proteins. **Panel a:** Cells harvested within 24hrs after growing. **Panel b:** Cells were first serum starved (1% FBS) for 18hrs, then grown in complete medium with 10% serum for 30hours then harvested. Lanes: M-molecular weight marker; 1-4 stable cell lines expressing (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A; (5) cell lysate,(6) lysate from HeLa cells used as positive control for the expression of p16 protein;

The blue cells were counted under light microscopy. These results indicate a higher presence of senescent cells when MK5-L337A is expressed in the cells compared to the control and other MK5variants, suggesting a possible tumor suppressive role of MK5 in this type of cells.

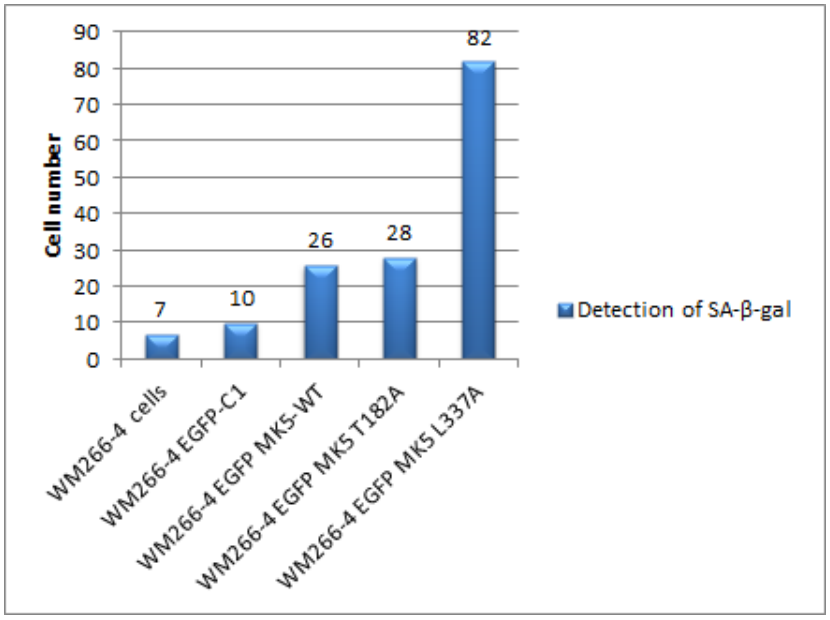


Figure 4.17: Detection of SA-β-gal in WM266-4 stable cell lines. The results are the mean of two experiments. In each experiment only the intense blue cells were counted from an average of 200 -250 cells from different microscopic fields in 6-well plate.

The senescent cells become flatter and bigger, with changed chromatin structure and gene expression [Ha et. al., 2008]. The same morphological characteristics were observed in melanoma cells expressing MK5-L336A mutant, but not in the other variants.
Due to limited time, the cellular senescence assay was not performed in the other melanoma cell types, but it will be interesting to see if the same pattern is followed.

4.4 Experiments on SKMEL-2 melanoma cells

4.4.1 Cell proliferation

Similar experimental approaches were applied for SK-MEL-2 cells including MTT, ELISA and FSK stimulation of cells. MTT assays were performed in both transiently and stably transfected SK-MEL-2 cells. First the cells were serum starved (1% FBS) for 18hours and subsequently grew in 2% or 10% FBS for 24 and 48 hours. There was a tendency for reduction of proliferation rate in time when cells expressed MK5, but interestingly cells grown in medium containing either 2% FBS or 10% FBS had a reduced proliferation rate in the first 24 hours which then increased after another 24 hours (**Figure 4.18**, panels A and B). In cells grown in 2% FBS proliferation rate was reduced to 36.5% (after 24hours) and 48% (after 48hours) when

MK5-WT was expressed**,** or from 56% (after 24hours) to 83% (after 48hours) when MK5-L337A was expressed (**Figure 4.18**, panels A versus panel B).

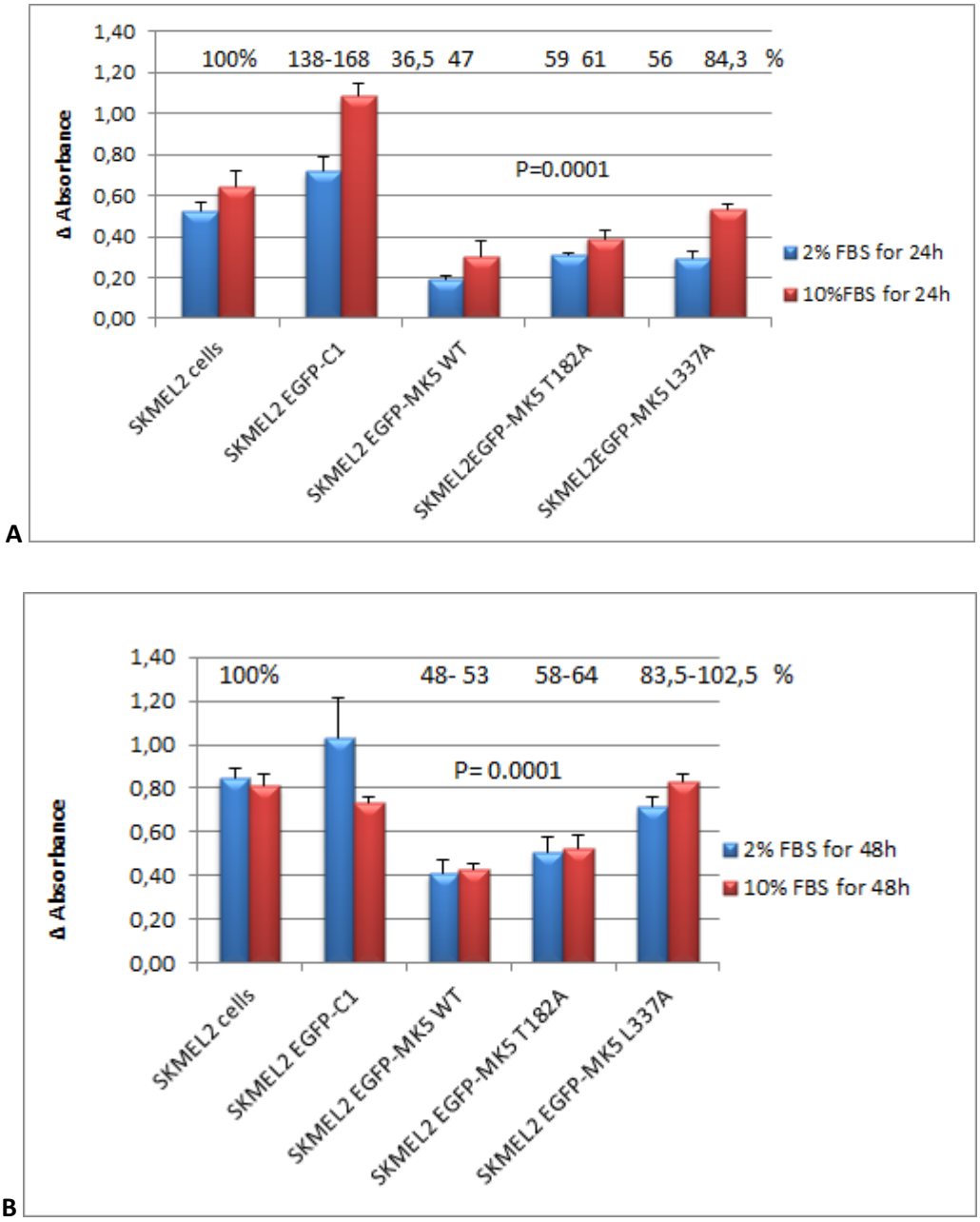


Figure 4.18: **panel A: MTT** assay on SKMEL2 SCLs; The day after seeding, the cells were serum starved (1% FBS) for 18hrs, subsequently grown in medium containing 2% or 10% serum for 24hrs and then were used for MTT assay**; panel B**- idem panel A only that cells were grown for 48 h in medium containing 2% or 10% serum after starvation.

A similar pattern was followed in the cells grown in 10% FBS, with almost 50% reduction of proliferation rate when cells express MK5-WT (**Figure 4.18**, panel B) or a change in proliferation rate from 83% (**Figure 4.18**, panels A) to 102%, when cells express MK5-L337A (**Figure 4.18**, panel B).

In this case and, as previously specified, MTT assays depend on experimental approaches. The values for EGFP-C1 samples were very high so only untransfected cell were considered as control in the experiment. The two tail student T-test indicates extremely significant difference (P=0.001) or very significant difference (P=0.0016) in 95% confidential interval when proliferation of MK5 variants expressing cells was compared with untransfected cells. One could say that only the MK5-WT variant exerts an anti –proliferative role in SK-MEL-2 cells, whereas the constitutively active MK5 mutant may contribute to cell proliferation. The fact that the inactive MK5 mutant reduces proliferation rate in a similar way that MK5WT does, may suggests that MK5 is not a single player in the proliferation process.

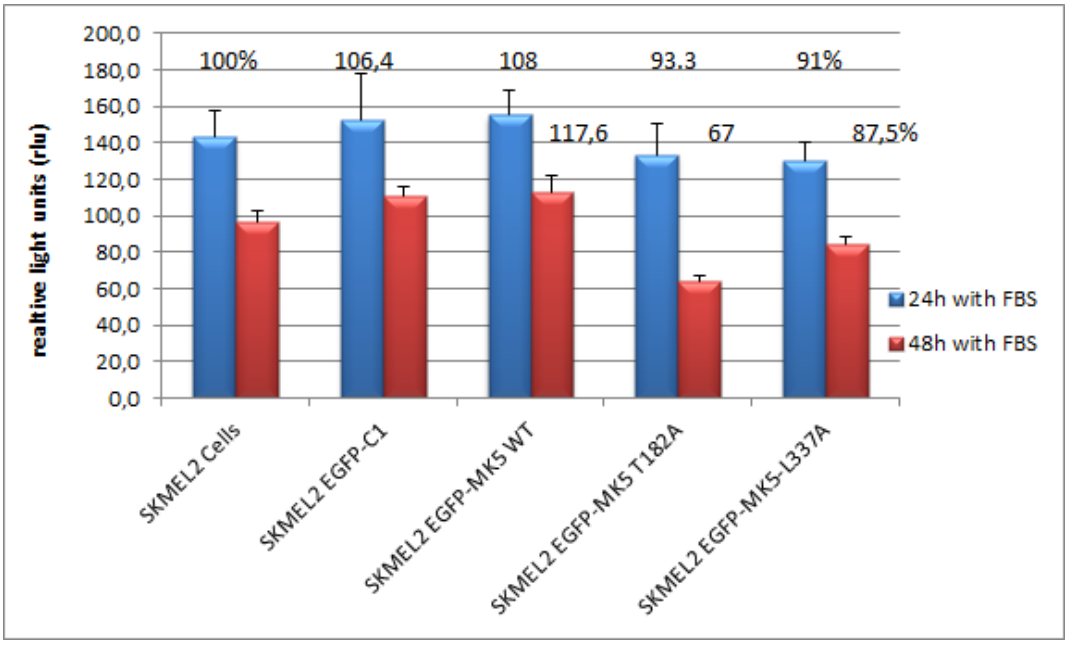


Figure 4.19: **ELISA** assay on SKMEL2 SCLs. The day after seeding, the cells were serum starved (1% FBS) for 18hrs, subsequently grown in medium containing 10% serum for 24hrs and 48hrs with the BrdU labeling in the last 24hrs of growth.

SK-MEL-2 stable cell lines used in ELISA assays were first serum starved (1%FBS) for 18hours then grown in complete medium with 10%FBS for24hrs and 48hrs with the BrdU labeling in the last 24hrs of growth. As general observation the proliferation rate was reduced in the

presence of the MK5 T182Avariant in both MTT and ELISA experiments, except MK5-L337A mutant and MK5-WT which stimulate proliferation in MTT and ELISA assay, respectively (**Figure 4.18,** panels A and B, red columns versus **Figure 4.19**). T-test indicates extremely significant difference in proliferation rate for MK5-T182 versus cells (P=0.0001, 48hrs) and very significant difference forMK5-L337A (P=0.0095, 48hrs) in the 95% confidence interval. Similar results were obtained in MTT assays performed with transiently transfected cells (results not shown).

Several ELISA experiments have been performed in SK-MEL-2 cells followed by the stimulation of cells with unspecific MK5 activator FSK. The reduction of cell proliferation was observed in all the experiments, but the obtained values varied, so the relative light units (rlu) values were presented as average percentage values **(Figure 4.20**). Transiently activation of MK5 by exposing the cells to FSK reduced proliferation rate with 11% to up to 25% (**Figure 4.20**) depending on the serum concentration and the time the cells were exposed to FSK. The MTT assays with SKMEL2 cells stimulated with FSK for 3hours also reduced the proliferation rate with 8% (results not shown).

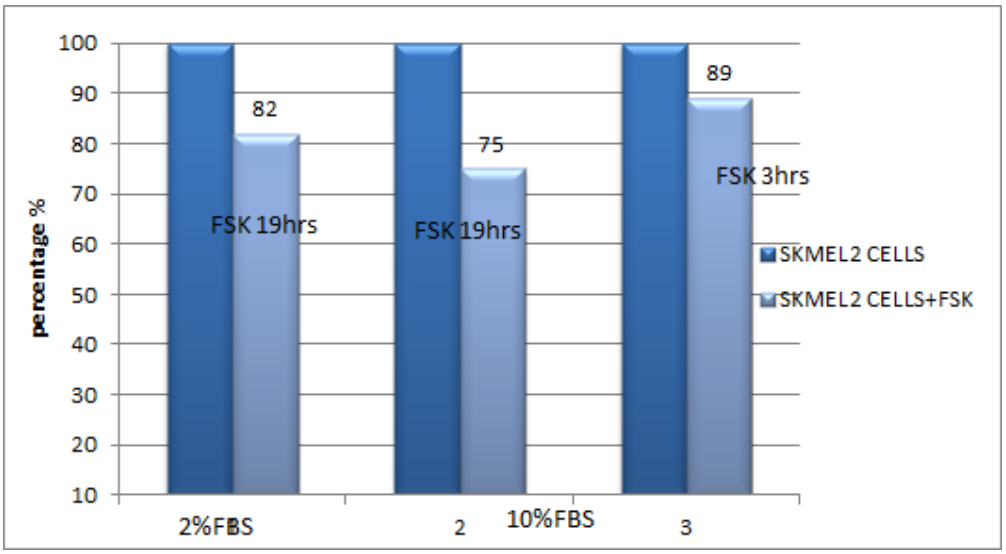


Figure 4.20: ELISA assay on SKMEL2 cells stimulated with 10µM FSK for different times as indicated. The results represent the mean of 3 experiments and relative light units (rlu) values are presented as average percentage values. The experiments follow these steps: seeding of cells, serum starvation next day for 18 hrs in medium containing only 1% FBS, growing of the cells in different concentrations of serum (2% and 10%FBS) with simultaneous stimulation of cells with FSK (19 hrs) and BrdU labeling. As an alternative, cells were stimulated for only 3 hrs with FSK.

SK-MEL-2 cell were also used in Luciferase reporter assay to investigate the role of MK5 in senescence. Stable cell lines expressing MK5 variants were transiently transfected with p21-Luc plasmid. Unexpectedly, cells expressing kinase dead MK5 show two fold higher luciferase activity, indicating a higher p21promoter activity than the control cells (**Figure 4.21).** This is in contradiction with the findings of Sun and colleagues who reported that MK5 kinase activity is required to activate the expression of p21 [Sun et. al., 2007]. The cells expressing wild type and active MK5 have a reduced p21 promoter activity compare to control cells, suggesting that senescence was not initiated. Furthermore, no p21 transcripts were found by RT-PCR assay for any of the SK-MEL-2 cells expressing MK5 (**Figure 4.10** and **4.11**). Overexpression of WT and constitutive active MK5 reduced p21 promoter activity, while the inactive MK5 variant (MK5 T182A) stimulated p21 promoter strength (**Figure 4.21**).

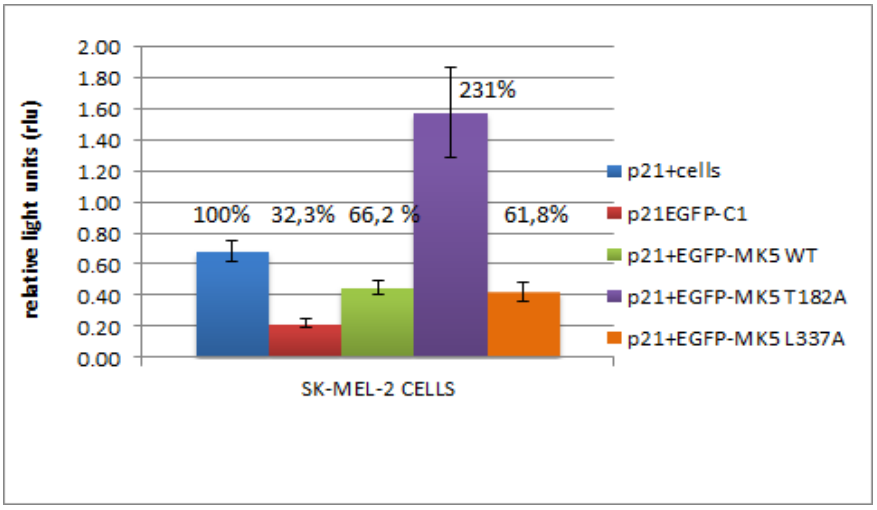


Figure 4.21: Luciferase assay on SKMEL2 SCL; cells were transiently transfected with 1µg p21-Luc DNA plasmid and next day luciferase activity was measured by Luminometer in a 96-well plate.

The cells expressing p21 and wild type/active MK5 showed extremely significant difference in p21 promoter activity (P=0.0001) in the 95% confidential interval, but not cells expressing p21 and kinase dead MK5 (P=0.4283).These results do not confirm the anti-proliferative role of MK5 in SK-MEL-2 cells.

The cellular senescence was also investigated using western blot against p16 ^{INK4a} tumor suppressor protein that inhibits cyclin-dependent kinases (CDKs) which regulates G1 check point through phosphorylation of retinoblastoma (Rb) protein [Rayess et al.,2012].

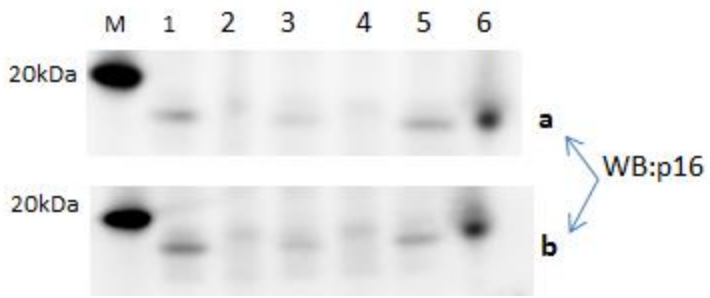


Figure 4.22: Western blot in SKMEL SCLs - expression of p16 protein. **Panel a:** cells harvested within 24hrs after seeding **Panel b:** cells were first serum starved (1% FBS) for 24hrs, then grown in complete medium with 10% serum for maximum 10hours before harvesting.
Lanes: M-molecular weight marker; 1-4 stable cell lines expressing (1) EGFP-C1, (2) EGFP-MK5 WT, (3) EGFP-MK5 T182A, (4) EGFP-MK5 L337A; (5) untransfected cells, (6) lysate from HeLa cells used as positive control for the expression of p16 protein.

HeLa cell lysate was used as positive control for the expression of p16 protein. As previously described, two experimental approaches were used to investigate expression of p16 in SKMEL-2 cells. In both cases, p16 protein was mainly found in untransfected cells or cells EGFP-C1 and HeLa cells (**Figure 4.22**, lanes 1, 5 and 6). Very week bands corresponding to p16 protein can be also observed in cell expressing EGFP-MK5 T182A mutant (**Figure 4.22,** lane 3). The loading control with ERK-2 antibody was presented in the WB with MK5 antibody (**Figure 4.4**).These results indicate that senescence is not initiated in cells expressing active MK5 mutants.

4.5 PCR-based sequencing of BRAF and NRAS genes in melanoma cells

Because MK5 was reported to interfere with proliferation induced by the RAS and RAF pathways [reviewed in Kostenko et al., 2012] , we wanted to verify the mutation state of the *BRAF* and *NRAS* genes in the melanoma cells used in this study.
DNA from A375 cells was sequenced with BRAF Fw primer, intron 14-2F. The nucleotide sequences were blasted against Homo sapiens v-raf murine sarcoma viral oncogene homolog B1 (BRAF), mRNA, accession number NM_004333.
Search for the mutation in exon 15(1803-1921; start codon 62 revealed a missense mutation at nucleotide 1860 corresponding to the codon 600, where thymine was replaced by alanine, resulting the **V600E** mutation. The amino acid valine (V = GTG) is replaced by Glutamic acid (E= GAG). The same mutation was found in WM266-4 cells. The results from PCR-sequencing

are enclosed in Appendix, **Supplementary Figure 5** (for A375 cells) and **Supplementary Figure 6** (for WM266-4 cells). The unambiguous A to T transversion suggests homozygous mutation (mutation in both alleles).

DNA from SK-MEL2 cells was sequenced with NRAS exon 2(fragment 1 and 2) Fw and exon 3 (fragment 1 and 2) Fw. Primers**.** The nucleotide sequences were blasted against Homo sapiens neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) mRNA, accession number NM_002524**.**

A missense mutation was found in exon3 (366-544; start codon 255) at nucleotide 436 corresponding to codon 182, where Alanine was replaced with Guanine, resulting in **Q61R** substitution. The amino acid glutamine (Q =CAA) is replaced by arginine(R= CGA)
The DNA was re-sequenced and R (single letter code: A or G) corresponds to G at codon 182. The presence of A or G can indicate a heterozygous mutation where one allele reads CAA and the other allele CGA. The results from RCR-sequencing are enclosed in Appendix, **Supplementary Figure 7.**

cDNAs from untransfected melanoma cells (A375, WM266-4, SK-MEL-2) were sequenced with MK5 primers (S115D Fw primer or MK5 433 Rev primer)**.** The nucleotide sequences (from amplification of cDNA) were blasted against Homo sapiens mitogen-activated protein kinase-activated protein kinase5 (MAPKAPK5), transcript variant 1, mRNA, accession number NM_003668. All nucleotide sequences were similar for A375, SK-MEL2 and WM266-4 cells, with 10-19 gaps and many nucleotide substitutions. It is possible that the aberrant growth of melanoma cells generates a protein with modified structure compare to normal cells. The results from PCR-sequencing are enclosed in Appendix, **Supplementary Figure 8.**

5. Conclusions and future perspectives

As it was shown in primary murine and human fibroblasts, MK5 mediates senescence by activating expression of p21 [Sun et al., 2007], or its overexpression can suppress proliferation in NHI3T3 cells [Li et al, 2008]. These findings underscore an anti-proliferative role of MK5. Moreover, in osteosarcoma U2OS cells the RNA-binding protein IGF2BP prevents translation of ERK4 mRNA, which impedes activation of MK5. This in turn induces tumor cell migration [Stöhr et al., 2012]. In osteosarcoma U2OS cells IGF2BP interferes with ERK4/MK5 pathway and induces tumor cell migration [Stöhr et al., 2012]. Hence, overexpression of MK5 may reduce cell proliferation and abrogate cell motility, making MK5 an attractive target in cancer therapy. However, a recent study showed that once the tumor is established, MK5 participates in tumor growth and progression by stimulating angiogenesis [Yoshizuka et al., 2012a]. Thus increased MK5 levels may actually stimulate tumor growth. MK5 acts in a yin-yang way because it can promote or inhibit tumor progression depending on the cellular context and the time point of its action.

The aim of this study was to investigate whether MK5 could play and anti-proliferative role in melanoma cells and as such be a target for cancer therapy. Our results indicate a minor role for MK5 in reduction of A375 proliferation rate sustained by the fact that both inactive and active MK5 have similar effects on reduction of cell proliferation in BrdU incorporation studies.

SA-β-galactosidase, p16 ^{INK4a} and p21 are hallmarks for senescence and their expression was therefore monitored in melanoma cells and melanoma cell liens stably expressing MK5 variants

Although no p16 ^{INK4a} protein expression was found in A375 cells, the p21 transcripts were expressed and p21 promoter activity was elevated in cells expressing constitutively active MK5, suggesting a possible role for MK5 in mediating senescence in A375 melanoma cells through modulating the cyclin-dependent protein kinase inhibitors

Experimental design plays an important role especially in MTT assays, since the use of low WM266-4 cell number could be sensitive enough to indicate a reduced proliferation rate in the presence of MK5(especially MK5 WT), whereas cell proliferation almost reaches the “plateau phase” when a higher number of cells is used. In that sense direct DNA synthesis measurement

by BrdU incorporation is more reliable than measurement of metabolic activity by MTT. These results neither clearly show an anti-proliferative role of MK5, nor totally exclude this hypothesis.

When WM266-4 cells were exposed to FSK and MK5 was in this way transiently activated via the cAMP/PKA pathway, the proliferation rate slightly had reduced to 5-7% in ELISA but not MTT assays. It does not excluded the idea that, besides MK5, other proteins might be involved in cell proliferation since FSK stimulation is an indirect way to determine the role of MK5 in these cells.

A possible implication of MK5 in cellular senescence was suggested by the expression of p21 transcripts and a weakly expression of p16 protein in WM266-4stable cell lines and untransfected cells. Furthermore, higher levels of SA-β-Gal activity were detected in cells expressing MK5-L337A mutant than in control cells or cells expressing WT or kinase dead MK5.

The results from SK-MEL-2 experiments suggest that no senescence occurred in these cells as p21 or p16 expression was undetectable, except in control cells. It would have been a better choice to actually quantify the copy number of transcript of interest involved in senescence process. Therefore a further investigation using quantitative PCR instead of RT-PCR could overcome undetectable levels of p21 or p16.

The cell proliferation results in SK-MEL-2 are controversial and difficult to interpret. First, an important reduction of proliferation rate was observed with all MK5 variants, 50% with MK5 WT and T182A and 15% with MK5 L337A, independently of low or high serum concentration growth condition. Transient activation of MK5 through the forskolin/cAMP/PKA pathway also reduces cell proliferation .Secondly, the inactive MK5 mutant reduces proliferation rate in a similar way to the other variants and also enhances p21 promoter activity, suggesting that MK5 stimulates cell proliferation process, but that the enzymatic activity of MK5 is not required. This is in contradiction with the findings of another group who showed that kinase dead MK5 did not inhibit oncogenic Ras-induced cell proliferation [Sun et al., 2007; Li et al., 2008]. In fact, our ELISA results actually indicate that MK5 WT may participate in cell proliferation. One could say that inactivation of MK5 reduces proliferation, whereas MK5WT overexpression has opposite effect.

This study does not clearly confirm the suppressive role of MK5 in melanoma cells, but this possibility is not excluded either. New approaches could be further tried such as infecting melanoma cells with adenovirus expressing only MK5 WT, thus avoiding differences in expression of various MK5 variants or the control. To have a better understanding of the role that MK5 actually plays in cell proliferation the approach mentioned above could be combined with siRNA –mediated depletion of MK5 in melanoma cells. Human primary melanocytes could be also used in experiments. A previous study in mouse embryonic fibroblasts (MEF) showed that knockout of the *mk5* gene resulted in cell cycle arrest and MK5 deficient MEF cells proliferate slower than wild type MEFs [Gong et al., 2009]. Alternatively, specific MK5 inhibitors can be used to unravel the role of MK5 in cell proliferation. A number of compounds have been described that can inhibit MK5's kinase activity, but their specificity is not known or they are not commercially available [Anwar et al., 2011; Andrews et al., 2011]. Our group has previously identified a specific MK5 inhibitor [Kostenko et al., 2011b], but unfortunately this drug was not available at the time of the studies presented in this work.

A recent study revealed a missense mutation found in *PRAK* gene during genome screening of melanoma samples from patients [Berger et al., 2012]. The G297E mutation, in which glycine is replaced by glutamic acid, is localized the kinase domain of MK5 protein. It would be interesting to further investigate whether this mutation affects the biological functions of MK5 and whether it may be implicated in tumorigenesis.

In conclusion, the anti-proliferative function of MK5 in melanoma remains elusive. Interestingly, MK5 expression is higher in normal colon tissue than in colorectal tumors and MK5 was shown to down-regulate of c-Myc through a pathway involving the transcription factor Foxo3a and the microRNA miR34b/c which targets *c-myc* mRNA[Kreb et al., 2011]. Thus MK5 may prevent cell cycle progression by preventing translation of c-*myc* transcripts. Different roles of MK5 in cancer are emerging so that elucidation of the precise function of MK5 role in malignancy would have great impact on designing therapeutic strategies.

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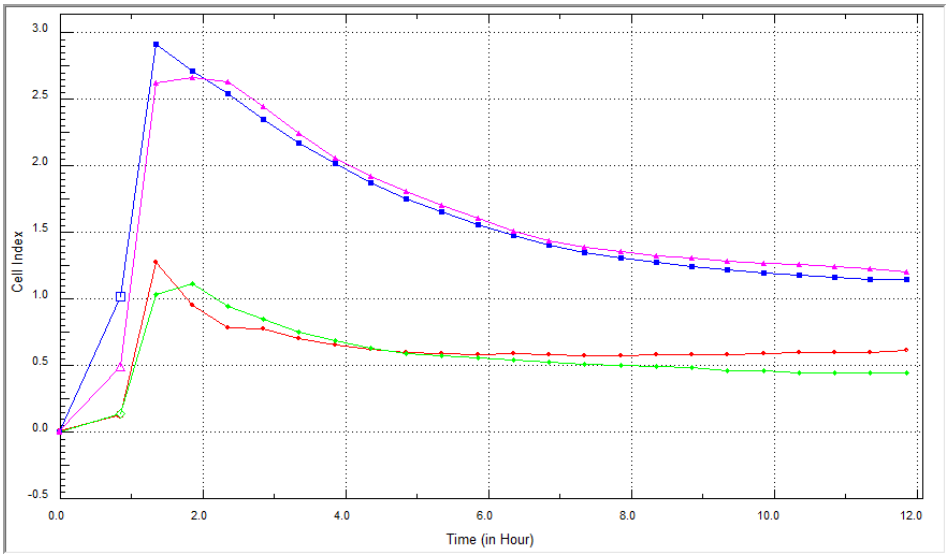
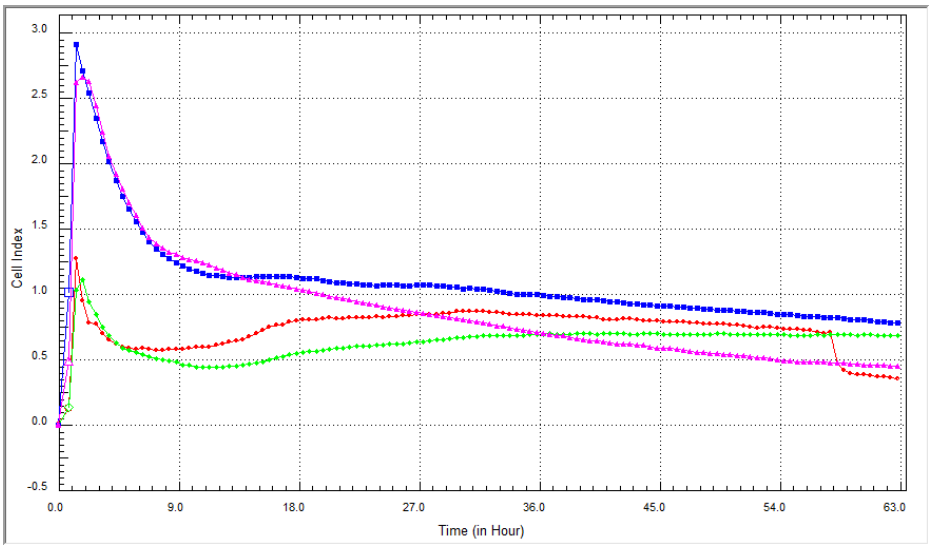
Appendix

Supplementary Figure 1: Impedance measurement with xCELLigence system in WM266-4 cells; cell index in the first 12 hrs (A) and after 63 hrs of growth (B)

10.000WM266-4 cells were seeded in E-plate in several variants: uncoated and fibronectin coated cells and cells expressing EGFPC-1/ EGFP-MK5 T182A.

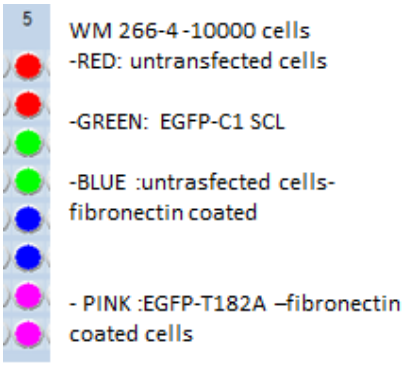


A



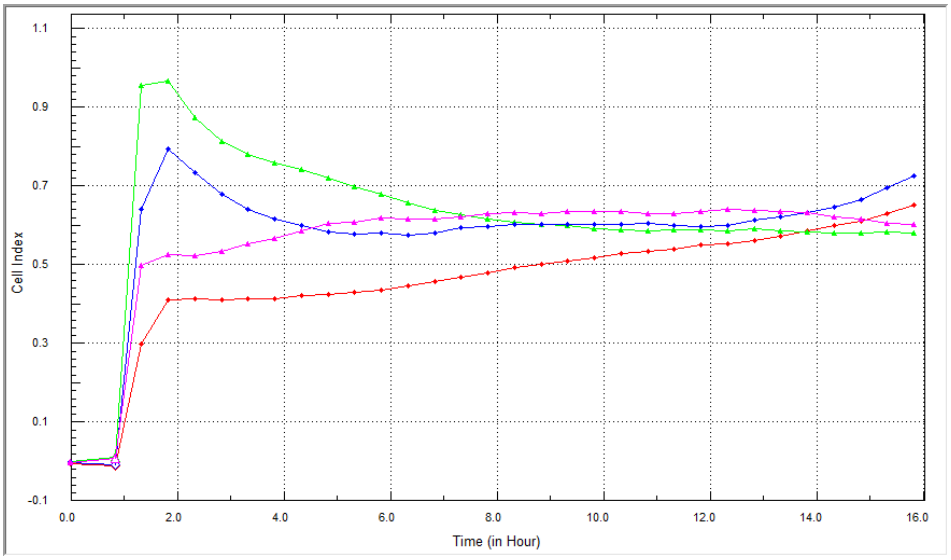
B

Supplementary Figure 2: Well map selection for **Supplementary Figure 1**



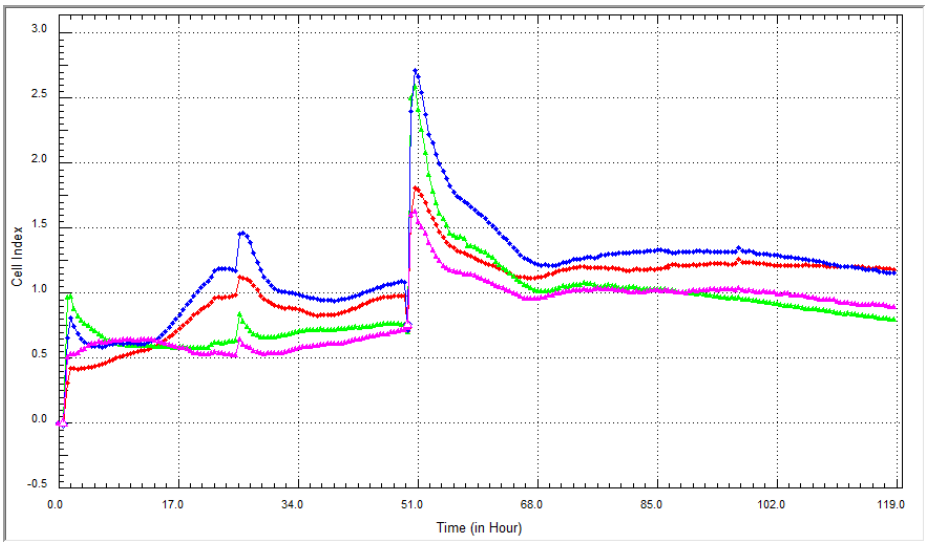
Supplementary Figure 3: Impedance measurement with xCELLigence system in WM266-4 cells; cell index in the first 16 hrs (A) and after 119 hrs of growth (B). The peeks correspond to the time when medium was changed from the wells.

Uncoated WM266-4 cells were seeded in E-plate in several variants: 5000 and 10.000cells/well; untransfected cells and cells expressing EGFPC-1.

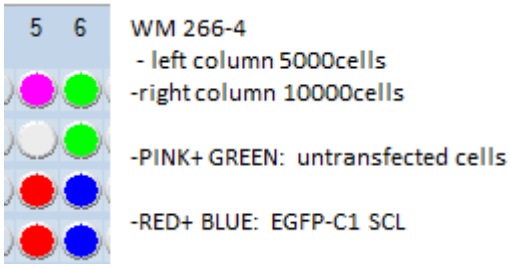


A

B



Supplementary Figure 4: Well map selection for **Supplementary Figure 3**

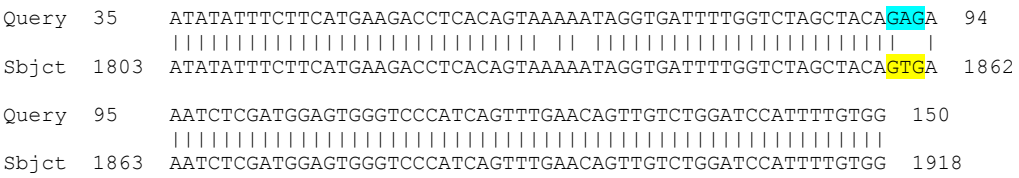


Supplementary Figure 5: PCR-sequencing of A375 cells with BRAF Fw primer, intron 14-2F, (**V600E** mutation).

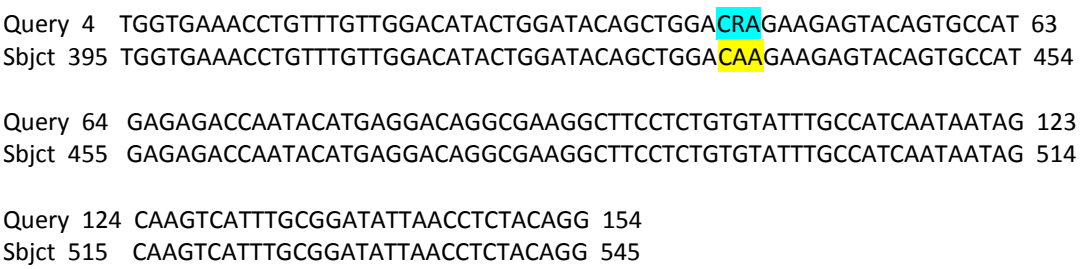
```
Query  35    ATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGAGA  94
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  1803  ATATATTTCTTCATGAAGACCTCACAGTAAAAATAGGTGATTTTGGTCTAGCTACAGTGA  1862

Query  95    AATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGG  150
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1863  AATCTCGATGGAGTGGGTCCCATCAGTTTGAACAGTTGTCTGGATCCATTTTGTGG  1918
```

Supplementary Figure 6: PCR-sequencing of WM266-4 cells with BRAF Fw primer, intron 14-2F; (V600E mutation)



Supplementary Figure 7: PCR-sequencing of SK-MEL2 cells with NRAS exon 3 fragment 2, Fw. Primer (Q61R mutation)



Supplementary Figure 8: PCR-sequencing of melanoma cells (A375, WM266-4, SK-MEL2) with MK5 primers (S115D Fw primer or MK5 433 Rev primer)

```
CTGGCATCATACCTACCTCACCGACGCCCTACACTTACAACAAGAGCTGTGACTTGTGGT  935
Sbjct  876
Query  320
CCCTAGGGGTGATAATTTATGTGATGCTGTGCGGATATCCTCCTTTTTACTCCAAACACC  379
||||||||||||| || ||||||||||||||||||| ||||||||||||||||||||||
CCCTAGGGGTGATTATCTATGTGATGCTGTGCGGATACCCTCCTTTTTACTCCAAACACC  995
Sbjct  936
Query  380
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| || |||||||||||||||||||||| | ||||||||||||||| ||||| ||||||||
ACAGCCGGACTATCCCAAAGGATATGCGAAGAAAGATCATGACAGGCAGTTTTGAGTTCC  1055
Sbjct  996
Query  440
CAGAAGAAGAGTGGAGCCAGATCTCAGAGATGGCTAAAGATGTTGTGAGGAAGCTTCTGA  499
|||| |||||||||||| |||||||||||||||| |||||||||||||||||||| ||||
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Sbjct  1526  TATAACCG  1533
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