Molecular profiling of cutaneous melanoma and functional characterization of *ASK/Dbf4*

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TO MY MOTHER

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CONTENTS

1 ABBREVIATIONS

A	adenine
aa	amino acid(s)
ASK	activator of S phase kinase
ATP	adenosine triphosphate
BCC	basal cell carcinoma
bp	base pair
BSA	bovine serum albumin
C	cytosine
ºC	degree Celsius
Cdc7	cell division cycle 7
CDK4	cyclin-dependent kinase 4
CDKN2A	cyclin-dependent kinase inhibitor 2a
C-terminal	carboxy terminal
cDNA	complementary DNA
CL	Clark level
CM	Cutaneous melanoma
CMM	Cutaneous malignant melanoma
cpm	counts per minute
DAPI	4´,6-diamidino-2-phenylindole, dilactate
Dbf4	Dumbbell former 4
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DNA	Deoxy ribonucleic acid
dNTP	Deoxy ribonucleotide triphosphate
DTT	1,4-dithiothreitol
DPBS	Dulbecco’s Phosphate buffered Saline

DMEM	Dulbecco's Modified Eagle Medium
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ERK1/2	extracellular signaling-regulated kinase 1/2
FBS	fetal bovine serum
G	guanine
GSTM1	glutathione S-transferase mu-1
GSTT1	glutathione S-transferase theta-1
H&E	hematoxylin and eosin
HEPES	N-(2-hydroxyethyl)piperazine-N´-(2-ethanesulfonic acid)
HRP	horse radish peroxidase
hrs	hours
IgG	immunoglobulin G
ICH	immunohistochemistry
IL	interleukin
kD	kilo Dalton
M	molar
m^2	square meter
MAPK	mitogen-activated kinase
MC1R	melanocortin 1 receptor
Mcm2	minichromosome maintenance 2
MCAM	melanoma adhesion molecule
min	minute
MM	malignant melanoma
mm	millimeter
MW	molecular weight
mRNA	messenger RNA

NaCl	sodium chloride
Na_3VO_4	sodium orthovanadate
NaF	sodium fluoride
NHM	normal human melanocytes
nm	nanometer
NS	normal skin
N-terminal	amino terminal
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
aRNA	anti-sense RNA
cRNA	complementary RNA
SiRNA	silencer RNA
RGP	radial growth phase
RNAse	ribonuclease
rpm	rounds per minute
RT	RT
RT-PCR	reverse transcription polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
SCC	squamous cell carcinoma
SDS	sodium dodecyl sulphate
SEER	surveillance epidemiology and end results
T	thymine
TBS	tris buffered saline

Tpr	Translocated to promoter region
UM	uveal melanoma
UV	ultraviolet light
VGP	vertical growth phase

2 INTRODUCTION

Cutaneous malignant melanoma (CMM) is the most aggressive amongst skin cancers. Melanoma arises from neoplastic transformation of neural crest-derived normal human melanocytes (NHM) residing in the basal layer of the epidermis to synthesize the pigment melanin. Melanoma has a strong propensity to metastasize, however important genetic changes responsible for melanoma development and progression remain poorly characterized, and the heterogeneity of the clinical course is still unexplained. Conventional diagnosis and prediction of prognosis of cutaneous melanoma using various clinical and histological features have proved to offer limited scope. As a consequence, melanoma continues to be an unpredictable cancer. Therefore a high-throughput gene expression-profiling platform would be an ideal investigative choice to understand various stages in its tumor progression model. As DNA microarrays are being used with the objectives of identifying regulated genes, patterns and pathways, leading to functional characterization and tumor sub-classification of several cancers, this study has focused on examining the gene expression signatures of known steps in the tumor progression model of CMM, from nevus to primary melanoma and to metastatic melanoma. Thus, the first introductory part of the thesis deals with the architecture of normal skin and introduces major neoplastic malignancies of the skin including melanoma. The latter part of the introduction deals with etiology and molecular basis of melanoma development as well as surveys DNA microarrays and their feasibility in molecular profiling cancer.

2.1 Normal skin

2.1.1 The function of normal skin

The skin is considered to be the largest organ of the human body, accounting for about 15% of the body weight with a surface area of 1.5 - 2.0 m^2. Besides its obvious property of enveloping the body, the skin has a wide range of diverse functions, including protection against injury, thermoregulation, excretion, absorption, and waterproofing and fluid conservation. The skin also serves as the first line of immunological defence producing sebum that inhibits the growth of certain bacteria and fungi. It is of considerable importance in the absorption of

ultraviolet radiation and in the production of vitamin D. It acts as a barrier to pathogenic organisms and functions in the detection of sensory stimuli.

2.1.2 Skin structure

The skin conventionally divides into two distinct layers, the **epidermis** and its appendages, derived from ectoderm, and the **dermis** with the underlying subcutaneous fat, derived from mesoderm (Figure 2-1, 2-2). The nerves and melanocytes are of neuroectodermal origin. The epidermis is a multilayered squamuos epithelium from which arise the pilosebaceous follicles, apocrine glands and eccrine sweat glands. The dermis consists of the ground substance and fibrous component collagen and elastin. There is considerable regional variation in skin structure and to some extent in function. The skin is divided into two types: glabrous and hairy. Hair production is maximal on the head, axillae, pubic region, and on the face of men. Sebaceous glands are especially numerous in the face and around the nose, whereas eccrine glands are most commonly found on the palms and soles. Skin differs from mucous membranes by the presence of both granular and horny layers.

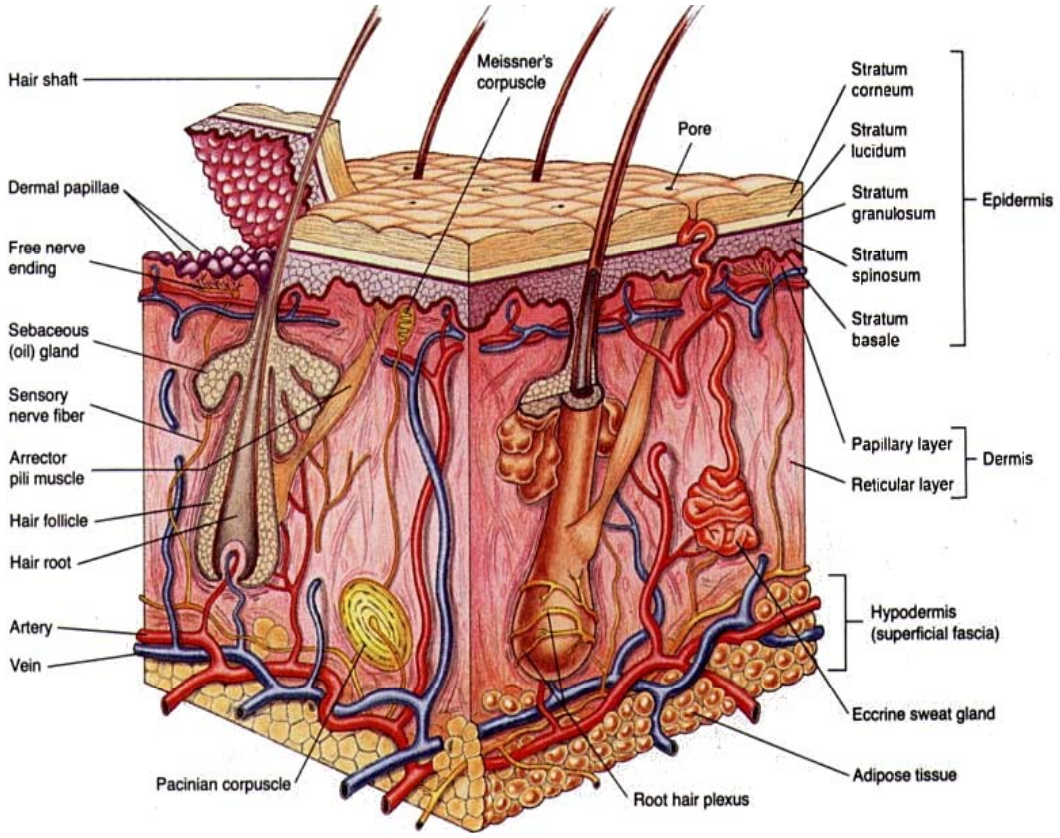


Figure 2-1: Schematic structure of normal skin (courtesy: www.cosmetique.ch)

2.1.2.1 Epidermis

Histologically, the epidermis consists of at least four cell types, such as keratinocytes, melanocytes, Merkel cells and Langerhans' cells, and has four clear defined layers: the basal, squamous, granular and horny layer. A fifth layer may be interposed between the granular and horny layers on the palms and soles. The thickness of the epidermis is about 0.1 mm, but it may vary with the anatomical site with the greatest thickness on the soles and palms (0.8 - 1.4 mm).

The average normal epidermal transit time is approximately 30 days [Halprin, 1972]. Maturation consists of the conversion of columnar basal cells into fully keratinized cells of the epidermal horn and involves the transformation of cellular polarity, basal cells being arranged at right angles to the basement membrane while the cellular residues of the horny layer are oriented parallelly.

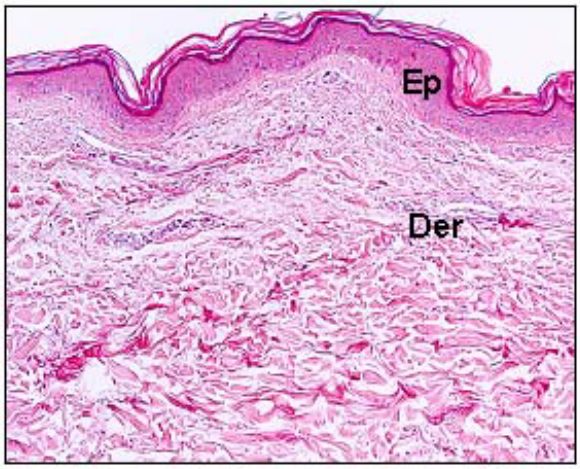


Figure 2-2: Histology of normal skin: epidermis (Ep) and dermis (Der). H&E, original magnification x100.

The exact mechanism of epidermis maturation is not fully understood. Maturation involves the increase in the number of tonofibers followed by their incorporation into the amorphous substance of the keratohyalin granules. Tonofilaments and keratohyaline granules are largely composed of protein, while membrane-coating granules contain large amounts of lipids and hydrolytic enzymes.

The ***basal cell layer***, *Stratum basale*, a germinative layer of the epidermis, consists mainly of a single layer of perpendicularly oriented columnar cells, which have a deeply basophilic cytoplasm and round to oval hyperchromatic nuclei (Figure 2-3). The cells in *Stratum basale* are attached to the *sub epidermal*

basement membrane via desmosomes and mainly consist of keratinocytes. However about 25% are melanocytes that produce the pigment melanin in vesicles and once mostly filled with melanin it is called melanosome. Melanosomes develop/mature distal to the cell body and move through dendritic process possibly via acto/myosin interactions and are “injected” into keratinocytes. Melanin taken up by keratinocytes and the melanosome localizes to the apical perinuclear region to protect the mitotic nuclei from UV light The basal undergo rapid cell division and with each division, approximately 50% of the daughter population contributes to the developing epidermis.

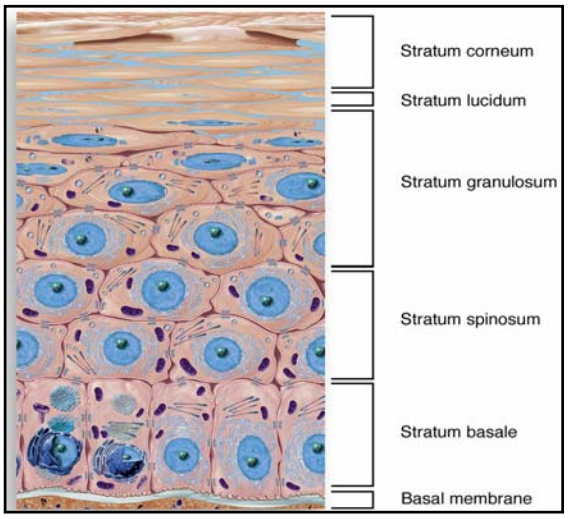


Figure 2-3: Schematical structure of the epidermis (www.eucerine.com).

The ***squamous cell layer,*** *Stratum spinosum,* consists of daughter basal cells, which have migrated upwards to form this multi-cell layer (4-10 layers). They become polygonal and arranged parallel with their long axis to the skin surface. Squamous cells contain granules with lipids and lipoproteins and actively synthesize intermediate filaments composed of keratin, which are anchored to the desmosomes. In this layer, the intercellular bridges desmosomes are best seen. The antigen-presenting Langerhans cells are also mostly found in the squamous cell layer.

In the ***granular layer***, *Stratum granulosum*, which consists of 2-3 cell layers, the cells become flattened or diamond-shaped and lose their nuclei. Their cytoplasm is filled with deeply basophilic and irregular in size keratohyaline granules. By protein composition, three types of those granules could be distinguished: F- (protein filaggrin), L- (protein loricrin), and S-granules. This layer represents the

keratogenous zone of the epidermis, where dissolution of the nucleus and other cell organelles is prepared.

The uppermost layer is the ***horny layer***, *Stratum corneum*, which is the final stage of the maturation of the keratinocytes. This layer consists of a sheet of overlapping polyhedral cornified cells without nuclei, and the cytoplasm is replaced by content of keratohyaline granules. Desmosomes between the adjacent cells are not present anymore. The cells of the horny layer are cemented together to form a tough and flexible membrane, the superficial aspect, which is continuously being shed as large clusters of squames. The horny layer contains lipid deposits between the horny cells that prevent the loss of body fluids and influx of water into the skin. The strength and integrity of the horny layer is believed to result from the presence of disulphide bonds between adjacent keratin molecules.

The epidermis lies on a thin sheet-like structure ***basement membrane***, *Lamina basalis,* which links the epidermis tightly to the dermis. It is not visible in sections stained with hematoxylin-eosin but clearly visualized by periodic acid-Schiff (PAS) staining. The basement membrane is composed of neutral mucopolysaccharides and reticular fibers, collagens, laminins, nidogen and perlecan [Hallmann et al., 2005]. It restricts the passage of molecules between the epidermis and dermis on the basis of size and charge, but allows the passage of migrating cells under normal (i.e. melanocytes and Langerhans cells) or pathological (i.e. lymphocytes, neutrophiles and tumor cells) conditions. It also influences the behavior of keratinocytes by modeling cell polarity, proliferation, migration and differentiation [Burgeson & Christiano, 1997; Chan, 1997]. Both fibroblasts and keratinocytes synthesize components of basement membrane.

2.1.2.2 Dermis

The ***dermis*** is the supporting layer of the epidermis and consists of a fibrous component collagen and elastin together with the so-called ground substance. Lying within it are the epidermal appendages, the neurovasculature, fibroblasts and various inflammatory cells.

The dermis is divided into two layers: the ***papillary dermis***, including a periadnexal component, and the ***reticular dermis***. The papillary dermis is bounded superiorly by the epidermis, laterally by the epidermal ridges and

inferiorly by the superficial *vascular plexus* and *reticular dermis*. Collagen gives the dermis its structural stability. Elastic fibres are responsible for cutaneous elasticity and for prevention of overextension. Collagen and elastic fibres are produced by fibroblasts. The skin receives an extensive vascular supply from vessels within the subcutaneous fat. From these arise two vascular plexuses linked by intercommunicating vessels; one, deep vascular plexus, lies in the region of interface between dermis and subcutaneous fat and the other, the superficial vascular plexus, lies in the superficial aspects of the reticular dermis and supplies the papillary dermis with a candelabra-like capillary loop.

The subcutaneous tissue, ***hypodermis***, is another layer below the dermis, which consists of loose connective tissue and adipocytes, divided into lobules by fibrous septae and characterized by abundant amounts of lipid, which compress the nucleus against the cytoplasmic membrane. Subcutaneous fat is of great importance in thermoinsulation and also functions as a nutritional store.

2.2 Benign and malignant tumors of the skin

Skin cancers currently constitute the most common malignancies in Caucasians, and the incidence has reached epidemic proportions [Diepgen, 2002]. The development and progression of skin cancers is causally linked to cumulative UV-irradiation. While UVC (200-280nm) is absorbed by the atmospheric ozone layer, exposure to UVB (280-320nm) and UVA (320-400nm) is damaging the skin. Prolonged and repeated solar exposure in susceptible persons (skin phototypes I-III) leads to chronic solar damage and development of skin cancers. Recently, several genes and gene mutations, such as p53, p63, survin and hTERT have been linked to the occurrence of skin cancer [Park et al., 2004]. There are three main kinds of skin cancers.

- Basal cell carcinoma (BCC)
- Squamous cell carcinoma (SCC)
- Cutaneous malignant melanoma (CMM)

2.2.1 Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC)

Basal cell carcinoma arises in cells called basal keratinocytes in the deepest layer of the epidermis, hair follicles, and sweat ducts. The principal etiologic factors in human BCC tumorigenesis include UV and ionizing radiation, chemical carcinogens, and possibly infection with human papillomaviruses [Bastiaens et al., 1998]. Sauamous cell carcinoma involves the malignant transformation and proliferation of squamous (flat, scaly) cells, which are the most abundant type of cell in the epidermis. As in the case of BCC principal etiologic factors in SCC tumorigenesis include UV and ionizing radiation, chemical carcinogens, and possibly infection with human papillomaviruses. Non-melanoma skin cancer, such as BCC and SCC have an estimated incidence of more than 600,000 cases per year in the United States (with a ratio of BCC to SCC of 4:1), 20-times greater than that of melanoma [Diepgen, 2002].

2.2.2 Cutaneous malignant melanoma (CMM)

Cutaneous malignant melanoma arises from the pigment producing neural crest-derived normal human melanocytes (NHM) residing in the *Stratum basale* of the epidermis and has a strong propensity to metastasize [Albino et al., 1997]. Melanoma is the third most common skin cancer and represents from 3% to 5% of cutaneous malignancies. UV-light is the most important cause, and the incidence of CMM in those with a susceptible skin type increases with proximity to the equator. MM is rare in children and adolescence, but is frequently seen in those aged 20-30, and most commonly in those aged 30-50. The leg is the predilection site in women and the back in men. CMM may occur as a fast or moderately growing asymmetric, elevated lesion with irregular borders and exhibiting various amounts of melanin (Figure 2-4).

Clinically and histopathologically, CMMs are described as melanoma in situ, superficial spreading, acral lentiginous, nodular and lentigo malignan melanoma. These distinctions are made after examination of characteristics of the lesion like symmetry, border, diameter and pigmentation. Additionally, tumor thickness (Breslow method), level of invasion (Clark method), ulceration of the overlying epidermis and involvement of lymph nodes are also used for staging melanoma.

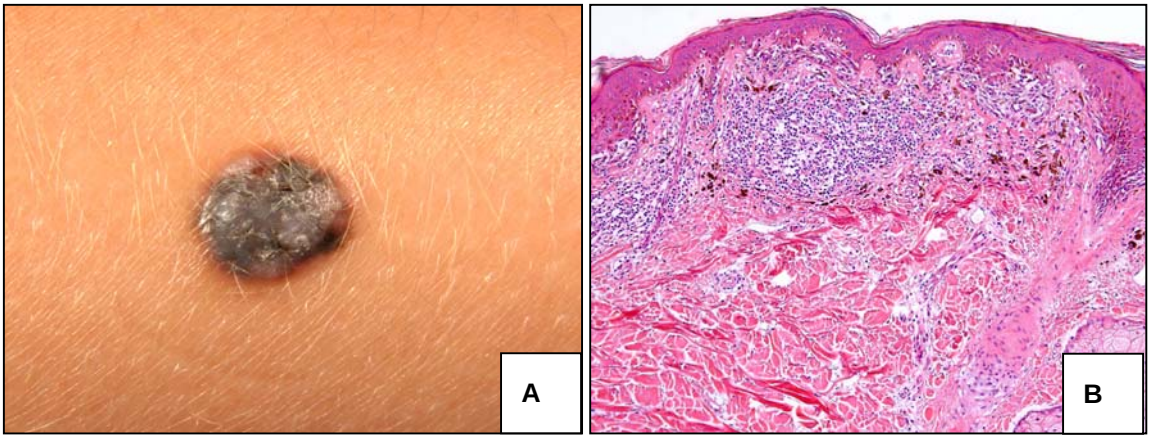


Figure 2-4: Superficial spreading melanoma: a black spot on the posterior tight of a female (A). Pleomorphic malignant melanocytes are scattered within the epidermis, infiltrate the superficial dermis and are surrounded by chronic inflammatory infiltrate (B). H&E, original magnification x100.

2.3 Tumor progression model of cutaneous melanoma

Clinical and histological studies have resulted in defining five major steps of melanoma development and progression (Figure 2-5): step 1, common acquired and congenital nevi with structurally normal melanocytes; step 2, dysplastic nevi with structural and architectural atypia; step 3, RGP, nontumorigenic primary melanomas without metastatic competence; step 4, VGP, tumorigenic primary melanomas with competence for metastasis; and step 5, metastatic melanoma [Clark et al., 1991]. About 66% of MMs develop de novo in previously normal skin; however, about 33% is associated with pre-existing congenital or dysplastic melanocytic nevi. As the presumed precursor for melanoma, both benign and dysplastic nevi are characterized by disruption of the epidermal melanin unit (Each epidermal melanocyte secretes melanosomes to approximately 36 adjacent keratinocytes, forming an epidermal melanin unit), leading to increased numbers of melanocytes in relation to keratinocytes. These precursor lesions may progress to *in situ* melanoma, which grow laterally and remain largely confined to the epidermis, so this stage is defined as the radial-growth phase (RGP). This is in contrast to the vertical growth-phase (VGP) melanoma, which both invades the upper layers of the epidermis and penetrates into the underlying dermis and subcutaneous tissue through the basement membrane, forming expansive nodules of malignant cells. It is believed that the transition from radial to vertical-growth phases is the crucial step in the evolution of melanoma that precedes the

acquisition of metastatic potential and poor clinical outcome. Consistent with that, the total thickness/height of a primary melanoma lesion is still one of the most predictive parameters for metastatic disease and adverse clinical outcome [Balch et al., 2001; Hengge et al., 2006]. Correlating with these histological features, RGP-melanoma cells remain dependent on exogenous growth factors supplied by surrounding keratinocytes, are incapable of anchorage independent growth, are not tumorigenic in immunodeficient mice and do not metastasize in patients. By contrast, VGP melanoma cells have completely escaped from keratinocyte control and established close communicative networks with fibroblasts, acquire growth factor- and anchorage-independent growth, are tumorigenic in animals and are highly metastatic both in patients and in experimental animal models [Hsu et al., 2002].

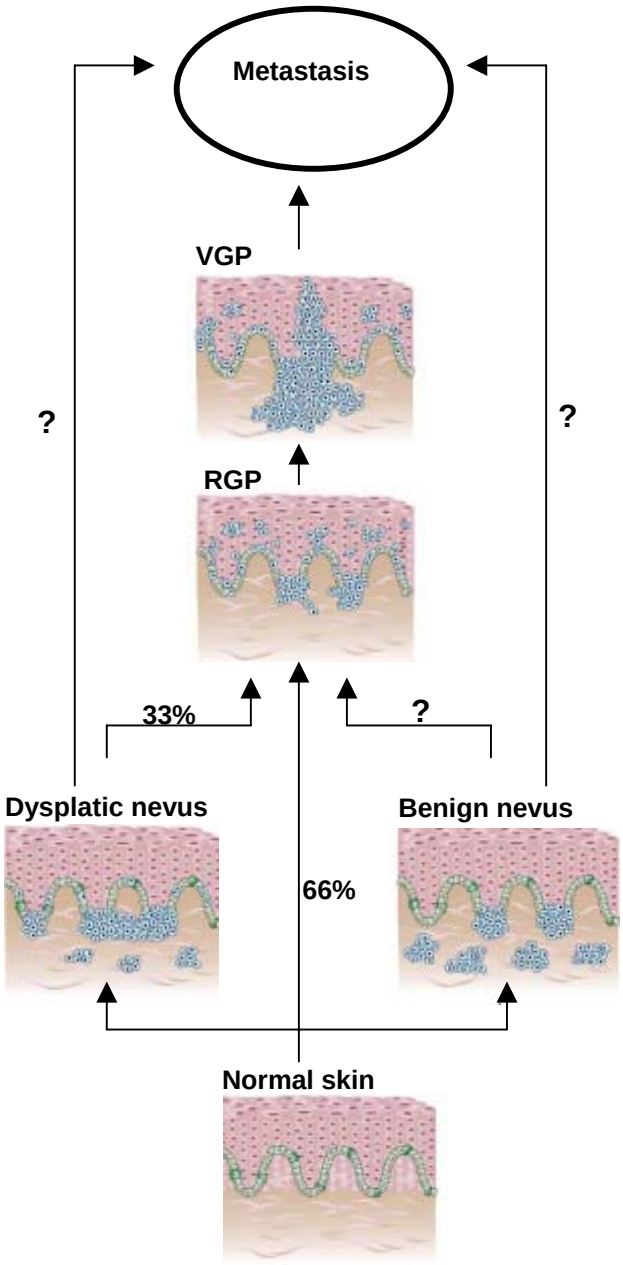


Figure 2-5: Five distinct stages have been proposed in the evolution of melanoma: common acquired and congenital nevi without dysplastic changes; dysplastic nevi with structural and architectural atypia; radial-growth phase (RGP) melanoma; vertical-growth phase (VGP) melanoma; and metastatic melanoma.

2.4 Etiology of melanoma development

2.4.1 Trends in cutaneous melanoma

Cutaneous melanoma has been one of the fastest-rising malignancies over the past several decades. Cutaneous melanoma represents approximately 4-5% of estimated cases for 2006 and ranks among the top 10 cancers (Figure 2-6), in

fact, incidence and mortality rates have sharply increased over the last 30 years (Figure 2-7,2-8).

Estimated New Cases*

Males			Females		
Prostate	234,460	33%	Breast	212,920	31%
Lung and Bronchus	92,700	13%	Lung and Bronchus	81,770	12%
Colon and Rectum	72,800	10%	Colon and Rectum	75,810	11%
Urinary Bladder	44,690	6%	Uterine Corpus	41,200	6%
Melanoma of the Skin	34,260	5%	Non-Hodgkin Lymphoma	28,190	4%
Non-Hodgkin Lymphoma	30,680	4%	Melanoma of the Skin	27,930	4%
Kidney and Renal Pelvis	24,650	3%	Thyroid	22,590	3%
Oral Cavity and Pharynx	20,180	3%	Ovary	20,180	3%
Leukemia	20,000	3%	Urinary Bladder	16,730	2%
Pancreas	17,150	2%	Pancreas	16,580	2%
All Sites	**720,280**	**100%**	**All Sites**	**679,510**	**100%**

Figure 2-6: Ten leading cancer types for the estimated new cancer cases, by sex, US, 2006 [Jemal et al., 2006]. Excludes basal and squamous cell skin cancers and in situ carcinoma except urinary bladder. Estimates are rounded to the nearest 10. Percentage may not total 100% due to rounding.

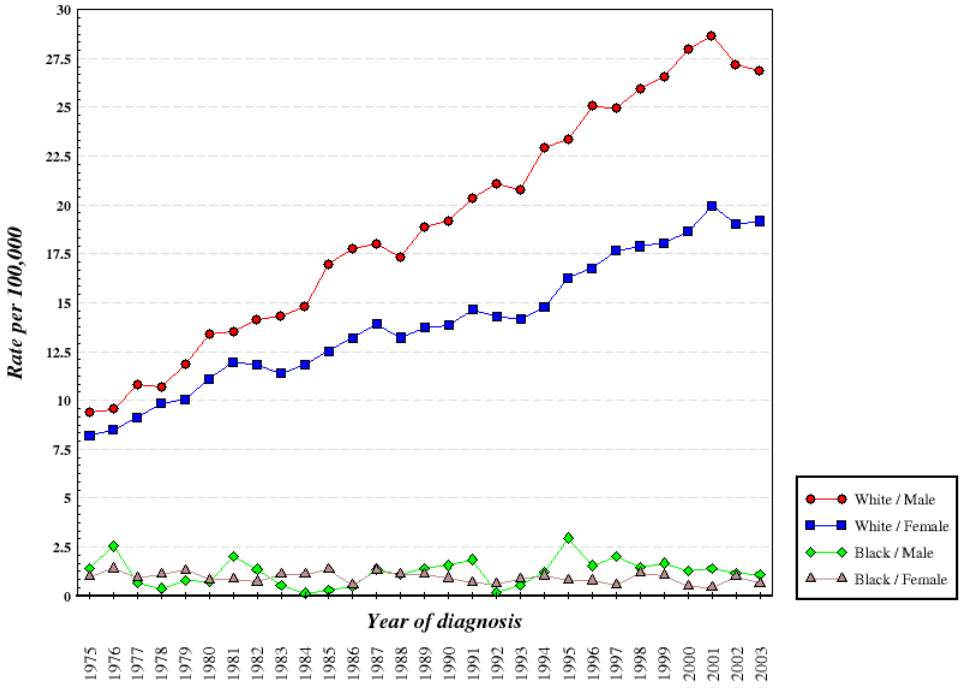


Figure 2-7: Epidemiology, and End Results (SEER) age adjusted incidence rates by race and sex for cutaneous melanoma from 1975-2003

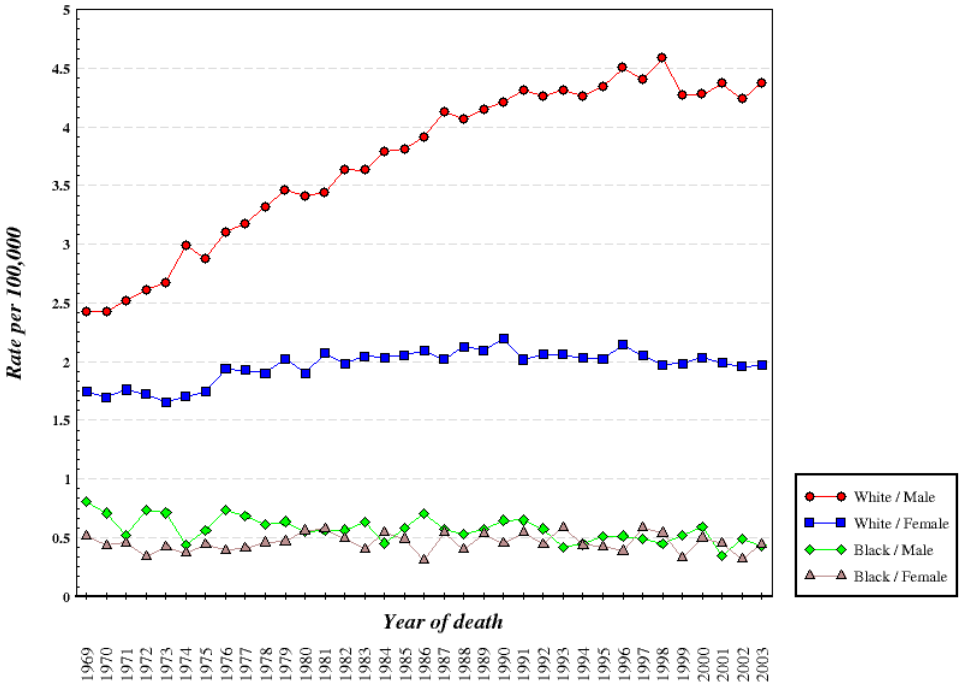


Figure 2-8: Epidemiology, and End Results (SEER) age adjusted total mortality rates by race and sex for cutaneous melanoma from 1969-2003

In the past 25 years, the incidence of melanoma has increased more rapidly than non-melanoma skin cancer, being now at 18 new cases per 100 000 population per year in United States [Schaffer, 2004]. In Europe the highest incidence rates have been reported from Scandinavia where up to 15 cases per 100,000 inhabitants and year have been registered [Armstrong et al., 1994; Osterlind et al., 1988; Thorn et al., 1990]. The figures from Germany [Garbe et al., 2001] are representative for middle Europe and similar incidence rates have been registered in England, Scotland and Italy [Carli et al., 1994; MacKie et al., 1992; MacKie et al., 1991]. Lower incidence rates where found in the Mediterranean countries. Therefore, in Europe we see a gradient in incidence rates with the highest rates in the northern and dropping incidence rates towards the southern countries. Much higher incidence rates have been reported from Australia. At the end of the 1980s an average annual incidence rate of 30 and 24 cases per 100,000 male and female inhabitants respectively was reported [Jelfs et al., 1994].

2.4.2 Role of UV radiation in development of melanoma

Development of melanoma is the pathologic consequence of environmentally initiated disruptions to cellular genetic control mechanisms. Epidemiological data support UV radiation as the major environmental carcinogen for melanoma. UV

exposure at the B (UV-B; 260 to 320 nm) and A (UV-A; 320 to 400 nm) range is the most biologically relevant factor for sporadic melanoma [Van schanke et al., 2005]. The exact mechanism, however, whereby UV irradiation induces melanoma has not been determined. Several theories of UV-induced carcinogenesis have been proposed; for example, UV has been suggested to induce permanent genotoxic damage, act as a melanocyte mitogen, induce the production of tumor promoting paracrine factors, or attenuate antitumor immune surveillance [Hengge et al., 2006]. It is now appreciated that transformation of melanocytes into malignant melanoma involves the interplay between genetic factors, UV exposure, and the tumor microenvironment.

The epidemiological data implicating sun exposure have recently been reviewed [Elwood and Jepson, 1997; Armstrong and Kricker, 2001]. Sunburns at any time in life conferred almost twofold increased risks [Elwood and Jopson, 1997]. Sunburns are a complex measure of both host susceptibility and exposure. Based largely on migration studies, it has been hypothesized that childhood may be a particularly susceptible time for sun exposure [Armstrong and Kricker, 2001; Whiteman et al., 2001]. In their review of childhood exposure, Whiteman *et al*. concluded that exposure to high levels of sunlight in childhood is an important risk factor for melanoma, but sun exposure in adulthood was also important. Part of the apparent variation in results of different studies may be due to behavioral patterns. Much of an individual's total life exposure to sun is obtained in the childhood and adolescent years, because of the greater time spent outdoors in youth [Fears et al., 2002]. All together, data are consistent with cumulative exposure being important, whether acquired as an adult or as a child. These findings imply that UV exposure is important in all stages of melanoma development from nevi through invasive melanoma, not just in the initiation of nevi. This association between UV radiation and melanoma has been clearly demonstrated in the epidemiological data emerging from Australia [Marks et al., 2002]. Australia has high levels of UV radiation and the highest per capita rate of melanoma in the world according to the Australian Institute of Health and Welfare (AIHW) & Australasian Association of Cancer Registries (AACR) report in 2001.

2.4.3 Molecular basis of melanoma development

Although causative molecular defects intrinsic to melanocytes have not been well defined in the majority of human melanomas, epidemiologic, genetic, and genomic investigations are uncovering the spectrum of intrinsic defects that are associated with melanoma.

2.4.3.1 *B-Raf* and *N-Ras*

Activating somatic missense mutations in the B-Raf protooncogene have been discovered in as many as 66% of human melanoma samples and cell lines, but at far lower frequency in a wide range of human cancers [Davies et al., 2002; Brose et al., 2002]. Although three types of mutations have thus far been identified [Tuveson et al., 2003], the most frequently occurring mutation (account accounting for 80% of total) is a single phosphomimetic substitution in the kinase-activation domain (V599E) that is known to confer constitutive activation of B-RAF. It was observed that V599E mutations are detectable in 68% of melanoma metastases, in 80% of primary melanomas, and, unexpectedly, in 82% of nevi [Pollock et al., 2003]. Interestingly even when nevi harbored *B-Raf* mutations, ERK1/2 (downstream components of the RAS/RAF/ MAPK pathway), were not phosphorylated, suggesting that in melanoma several mechanisms are responsible for activation of RAS/RAF/ MAPK pathway [Dong et al., 2003].

In melanoma, *N-Ras* mutations were identified in 5% to 15% of all sporadic melanomas [Ball et al., 1994]. Interestingly, an inverse correlation was found between mutations in the *N-Ras* and *B-Raf* genes because tumors carry only one and not both at the same time [Gorden et al., 2003].

2.4.3.2 *CDKN2A* and *CDK4*

Two genes conferring susceptibility to melanoma have been identified within high-risk families, *CDKN2A* and *CDK4* [Hussussian et al., 1994; Kamb et al., 1994; Zuo et al., 1996]. Both of these genes are important in controlling cell division.

CDKN2A codes for two proteins, $p16^{Ink4a}$ (important in the retinoblastoma pathway), and $p14^{ARF}$ (important in the p53 pathway). The $p16^{INK4a}$ is an important tumor suppressor and encodes for 16 kD protein. It functions as a D-type cyclin-dependent kinase inhibitor that blocks the ability of CDK4 to interact with cyclin D and hence prevents the progression of eukaryotic cells through the G1 phase of

the cell cycle [Serrano *et al.*, 1993]. *CDKN2A* mutations have been described in 10.3% of a population sample of high-risk families from Australia [Aitken et al., 1999]. They estimated that 0.2% of melanoma in Australia were due to mutations in *CDKN2A.* The likelihood of finding a mutation in *CDKN2A* is dependent on the number of affected family members overall, rising from about 5% in families with two affecteds to 20-40% in families with three or more affected individuals [Kefford et al., 1999]. Many of the recurrent mutations in p16 that have been described are founder mutations dating back up to 100 generations [Ciotti et al., 2000; Goldstein et al., 2001; Hashemi et al., 2001]. These founder mutations have been described in a number of different populations [Gruis et al., 1995; Borg et al., 1996; Platz et al., 1997; Pollock et al., 1998; Auroy et al., 2001; Ciotti et al., 2000; Goldstein et al., 2001]. In populations with a prevalent founder mutation, the likelihood of detecting a mutation in *CDKN2A* (frequently the founder mutation) may be higher in families with fewer affecteds than in populations without such founder mutations [Soufir et al., 1998; Ruiz et al., 1999; Mantelli et al., 2002]. Population prevalence of these founder mutations has not been well quantified yet. Families with mutations in *CDKN2A* that affect only $p14^{ARF}$ are much less common than mutations that affect $p16^{Ink4a}$ with or without affecting $p14^{ARF}$ [Randerson-Moor et al., 2000; Rizos et al., 2001; Bishop et al., 2002]. Recently, the Melanoma Genetics Consortium estimated the penetrance of melanoma among mutation carriers in 80 high-risk families with *CDKN2A* mutations from Europe, the US, and Australia [Bishop et al., 2002]. Overall, the cumulative risk of melanoma at age 50 was 0.30 and at age 80 was 0.67. The penetrance at age 80 was much higher in Australia (0.91) and the US (0.76) than in Europe (0.58). The penetrance was not altered by gender or whether the mutation altered $p14^{ARF}$.

Similarly a point mutation in CDK4 producing an amino acid substitution of arginine to cysteine at residue 24 (R24C) has been detected in SK-MEL-29 cells [Wolfel et al., 1995], as well as in three melanoma families. This mutation interferes with the binding of CDK4 to $p16^{Ink4a}$. Moreover, Cdk4R24C/R24C murine models appear susceptible to melanoma development [Sotillo et al., 2001].

Additionally silencing of, *CDKN2a* ($p16^{INK2a}$) mediated by aberrant promoter methylation has been extensively reported in melanoma [Merbs and Sidransky, 1999; Marini et al., 2006].

2.4.3.3 *PTEN*

PTEN is a tumor suppressor gene mutated in some human melanomas [Robertson et al., 1999]. It can up-regulate proapoptotic machinery involving caspases and BID and downregulate the antiapoptotic proteins such as Bcl2. It is involved in the inhibition of focal adhesion formation, cell spreading, and migration, as well as the inhibition of growth factor-stimulated MAPK signaling. A mutated PTEN can constitutively activate PKB/AKT to influence downstream genes. The combined effects of the loss of PTEN lipid and protein phosphatase activity may result in aberrant cell growth and escape from apoptosis, as well as abnormal cell spreading and migration. In melanoma, PTEN loss has been mostly observed as a late event, although a dose-dependent loss of PTEN protein and function has been implicated in early stages of tumorigenesis as well. Similar to $p16^{Ink4A}$, *PTEN* may be structurally normal in the vast majority of melanomas but functionally inactive, or expression is silenced due to hypermethylation [Stahl et al., 2003]. In addition, loss of *PTEN* and oncogenic activation of RAS seem to occur in a reciprocal fashion, both of which could cooperate with $p16^{Ink4A}$ loss in contribution to melanomagenesis. Recently epigenetic silencing of the PTEN promoter has been reported in melanoma [Mirmohammadsadegh et al., 2006].

2.4.3.4 *MITF*

Microphthalmia-associated transcription factor (*MITF*) acts as a master regulator of melanocyte development, function and survival by modulating various differentiation and cell-cycle progression genes [Levy et al., 2006; Carreira et al., 2005]. It has been demonstrated that *MITF* acts as an oncogene and is amplified in a fraction of human melanomas and that it also has an oncogenic role in human clear cell sarcoma [Garraway et al., 2005; Li et al., 2003]. However, *MITF* also modulates the state of melanocyte differentiation [Shibahara et al., 2000]. These data place *MITF* between instructing melanocytes towards terminal differentiation and/or pigmentation and, alternatively, promoting malignant behavior.

2.4.3.5 *MC1R* and *GSTM1*

MC1R and other pigmentation genes are under active investigation, both in family and in larger population epidemiologic studies. *MC1R* variants have been

associated with increased risk of melanoma in some melanoma-prone families with *CDKN2A* mutations [Box et al., 2001; van der et al., 2001]. Based on relatively small numbers, *MC1R* variants have also been associated with increased risk of melanoma (over phenotype only) among individuals with dark complexions [Palmer et al., 2000]. There is some suggestion that among individuals with red, reddish-brown, or blond hair, *GSTM1 null, GSTT1 null* and *GST null* may increase risk of melanoma [Kanetsky et al., 2001].

DNA repair genes are also of great interest both in family studies and in larger population studies. Several groups are also attempting to localize genes for nevi.

2.4.3.6 STAT3 and STAT5

STATs (signal transducers and activators of transcription) are cytoplasmic proteins, which dimerize after tyrosine phosphorylation and translocate into the nucleus, where they act as transcription factors. STAT3 and STAT5 were found to be involved in the signalling of RTKs such as the EGF and PDGF receptor [David et al., 1996; Vignais et al., 1996]. STAT3 activation regulates the expression of matrix metalloproteinase-2 and thus promotes melanoma invasion and metastasis [Xie et al., 2004] where as STAT5 phosphorylation in malignant melanoma is mediated through SRC and JAK1 kinases [Mirmohammadsadegh et al., 2006] and has been reported exert its pro-proliferative function in melanoma through expression of *pim*-1 and *bcl-x* [Morcinek et al., 2002].

2.4.3.7 Melanoma-associated antigens (*MAGE*s)

One of the first isolated tumor-specific antigens was the melanoma antigen 1 (MageA1) [Van der Bruggen et al., 1991]. It belongs to the MAGE gene family, characterized by a conserved domain (MAGE Homology Domain). Melanoma-associated antigens (MAGEs) were identified and classified into 2 subgroups, I and II. Subgroup I consists of antigens whose expression was generally restricted to tumor or germ cells, also named as cancer/testis (CT) antigen [Simpson et al., 2005]. Although the exact molecular role of MAGEs in melanomagenesis remains unknown, recently correlation between MAGE-A expression and resistance to apoptosis was validated in melanoma cell lines, where combined trichostatin A and etoposide treatment restores the p53 response and reverts the chemoresistance of melanoma cells expressing high levels of MAGE-A [Monte et al., 2006].

2.5 Molecular Profiling

2.5.1 Overview of microarray platforms

Microarrays constitute a group of technologies characterized by the common availability of measuring hundreds or thousands of items, including DNA sequences, RNA transcripts or proteins, within a single experiment using miniaturized devices. There are several variants of the DNA microarray technology for analysis of transcription profiles. However, there are two important variants in terms of the arrayed DNA sequences with known/unknown identity: Either cDNA (> 250 bases long) immobilized to a solid surface such as glass or nylon membrane using robotic spotting or oligonucleotides (20~80-mer oligos) synthesized either *in situ* (on-chip) or by conventional synthesis followed by on-chip immobilization [Fodor et al., 1991 and 1993; Schena et al., 1995; Lockhart et al., 1996]. Protein/peptide arrays are solid-phase ligand binding assay systems using immobilized proteins/peptides on surfaces, which include glass, membranes, microtiter wells, mass spectrometer plates, and beads or other particles. Protein arrays enable the parallel screening of thousands of interactions, encompassing protein-antibody, protein-protein, protein-ligand or protein-drug, enzyme-substrate screening and multianalyte diagnostic assays [Lueking et al., 2001]. Another popular array platform - the tissue microarray (TMA), is one where tissues are arrayed on modified glass slides. This technology allows rapid visualization of molecular targets in hundreds of tissue specimens at a time, either at the DNA, RNA or protein level. In particular, the signal is visualized on a per cell basis (*in situ*) [Schraml et al., 1999, Rao et al., 2002]. This technique facilitates rapid translation of molecular discoveries to clinical applications. By revealing the cellular localization, prevalence and clinical significance of candidate genes, TMAs are ideally suitable for genomics-based diagnostic and drug target discovery. In recent years, comparative genomic hybridization (array-CGH) has also emerged as a novel array-based platform for genome wide comprehensive analysis of chromosomal imbalances [Pinkel et al., 1998; Snijders et al., 2001]. Thus, array technologies represent a spectrum of high-throughput means to identify molecular targets associated with biological and clinical phenotypes by comparing samples representative of distinct pathophysiological states. However, irrespective of the type of the array platform, an appropriate experimental design and the use of well-

characterized *in vitro* or *in vivo* systems, as well as accurately annotated clinical specimens and appropriate controls are key to the success of any detailed analysis.

2.5.2 Molecular classification of cancer: Gene expression monitoring

The challenge of cancer treatment has been to target specific therapies to pathogenetically distinct tumor types, to maximize efficacy and minimize toxicity. Improvements in cancer classification have thus been central to advances in cancer treatment. Cancer classification has been based primarily on morphological appearance of the tumor, but this has serious limitations. Tumors with similar histopathological appearance can follow significantly different clinical courses and show different responses to therapy. In a few cases, such clinical heterogeneity has been explained by dividing morphologically similar tumors into subtypes with distinct pathogeneses. Key examples include the subdivision of acute leukemias, non-Hodgkin's lymphomas, and childhood "small round blue cell tumors" [tumors with variable response to chemotherapy [Trische., 2001] that are now molecularly sub classified into neuroblastomas, rhabdomyosarcoma, Ewing's sarcoma, and other types [Golub et al., 1999; Stephenson et al., 1992; Turc-Carel et al., 1986; Douglass et al., 1987; Dalla-Favera et al., 1982.]. For many more tumors, important subclasses are likely to exist but have yet to be defined by molecular markers. For example, prostate cancers of identical grade can have widely variable clinical courses, from indolence over decades to explosive growth causing rapid patient death. Cancer classification has been difficult in part because it has historically relied on specific biological insights, rather than systematic and unbiased approaches for recognizing tumor subtypes. Expression profiling and pattern identification using various available microarray platforms is becoming a novel tool for identifying new cancer classes (class discovery) like in the example of a novel class of lymphoblastic leukemia [Armstrong et al., 2002] or for assigning tumors to known classes (class prediction) as in human acute leukemias [Golub et al., 1999], cutaneous malignant melanoma [Bittner et al., 2000], lung carcinomas [Bhattacharjee et al., 2001], and embryonal tumors of the central nervous system (CNS) [Pomeroy et al., 2002]. Reports using different microarray platforms and analyzing specific cancer subgroups lead to consensus on subclasses and signatures of the disease with predictive utility.

2.6 Aim of the study: Molecular profiling cutaneous melanoma and functional characterization of *ASK/Dbf4* upregulation

Few reports have analyzed global gene expression patterns in cutaneous melanomas. By cDNA microarray analysis, Bittner *et al.* identified a gene expression cluster that correlated with invasive behavior *in vitro* [Bittner et al., 2000]. Tschentscher *et al.* described a correlation between gene expression profiles and monosomy of chromosome 3 in uveal melanomas, whereas Hoek *et al.* investigated expression profile differences between NHM and melanoma cells from advanced lesions [Tschentscher, 2003; Hoek, 2004]. Recently, Haqq et al. have defined melanoma progression profiles using a cDNA microarray approach [Haqq et al., 2005]. As the relationship between global gene expression and development and progression of cutaneous melanoma remains unachieved in patients, an oligonucleotide microarray based analysis was employed to investigate the molecular basis of malignant transformation and progression to evaluate the biological relationship between different stages and to identify molecular predictors. The goal of this work focused on the following:

- (1) comparative analysis of a series of benign congenital nevi, primary cutaneous melanomas, and cutaneous melanoma metastases using the human genome Affymetrix™ GeneChip system HG-U133A microarrays

- (2) generation of qRT-PCR confirmed multi-gene signature to differential benign nevi from malignant melanoma

- (3) functional characterization of *ASK/Dbf4* one of the components in the multi-gene signature.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, enzymes and antibodies

If not stated otherwise chemicals, enzymes and antibodies were of highest purity grade

3.1.1.1 Chemicals

Adenosine Triphosphate (ATP)	Sigma (St.Louis, USA)
Agar	Difco laboratories (Detroit, USA)
Advanced protein assay reagent (5X) #ADV01	Cytoskeleton Inc (Denver, USA)
Bovine Serum Albumin (BSA) solution (50 mg/ml) P/N 15561-020	Invitrogen Life Technologies (Carlsbad, USA)
Cis-PLATINUM(II)-Diammine dichloride # P 4394	Sigma-Aldrich (St.Louis, USA)
Dideoxynucleotide (dNTP)	Sigma (St.Louis, USA)
Glutathione-Sepharose	Pharmacia (Freiburg, Germany)
Herring Sperm DNA , P/N D1811	Promega Corporation (Madison, USA)
MTT #475989-1GM	Calbiochem (San Diego, USA)
NaCl	Difco laboratories (Detroit, USA)
Protease-inhibitor (Tablet)	Boehringer Mannheim (Mannheim, Germany)
R-Phycoerythrin Streptavidin, P/N S-866	Molecular Probes Invitrogen (Calrlsbad, USA)
RNAse A	Boehringer Mannheim (Mannheim, Germany)
RNAse-inhibitor	Boehringer Mannheim (Mannheim, Germany)
20x SSPE (3M NaCl, 0.2M NaH2PO4, 0.02M EDTA), P/N 51214	BioWhittaker MolecularApplications / Cambrex (New Jersey, USA)
TRIZOL® Reagent # 15596-018	Invitrogen Life Technologies (Calrlsbad, USA)
Ultralink immobilized protein A/G plus # 53135	Perbio science (Bonn, Germany)

3.1.1.2 Antibodies

I. Primary antibodies

Antibody	Description	Supplier
ASK/Dbf4	Customized rabbit polyclonal antiserum	Biogenes GmbH (Berlin, Germany)
Negative control	Rabbit pre-immune anti serum	Biogenes GmbH (Berlin, Germany)
Cdc7 kinase	Mouse monoclonal	Abcam (Cambridge, UK)
E2F1	Rabbit polyclonal	Genecraft (Muenster, Germany)
Actin (Ab-1)	Mouse monoclonal	Calbiochem (San Diego, USA)

Table 3-1: Description of primary antibodies

The anti-human ASK/Dbf4 polyclonal antibody was raised against the amino acids 336-349 (C-FDFVEYEKDTPKKK-amide) in rabbit (BioGenes, Germany) in conjugation with carrier protein LPH in animals 8809 and 8810, respectively. The pre-immune sera from the same animals were used as negative controls for Western Blot analysis.

II. Secondary antibodies

Secondary antibodies, anti-mouse IgG and anti-rabbit IgG coupled to horseradish peroxidase were purchased from cell signalling (Massachussets, USA). Goat IgG, Reagent Grade (P/N I 5256) was procured from Sigma-Aldrich. Biotinylated anti-streptavidin antibody (goat) (P/N BA-0500) was purchased from Vector Laboratories (CA, USA).

3.1.2 Kits for molecular biology

Kits	Supplier
RNeasy mini kit (250)	Qiagen
#74106	(Hilden, Germany)
MessageAmpTM aRNA Amplification kit	Ambion
#1750	(Austin, USA)
BioArrayTM High YieldTM RNA transcript labeling kit (#900182)	Enzo Life sciences (Farmingdale, USA)
GeneChip Human Genome U133A Array	Affymetrix
(# 900366)	(Santa Clara, USA)
Taqman® revere transcription reagents	Applied biosystems
# N808-0234	(Foster city, USA)
SilencerTM siRNA transfection kit	Ambion
(#1630)	(Austin, USA)
Cell proliferation ELISA BrdU (Colorimetric) #11 647 229 001	Roche applied science (Mannheim, Germany)
GeneChip Eukaryotic Hybridization Control Kit,	Affymetrix
P/N 900457 (150 reactions), contains Control cRNA and Control Oligo B2	(Santa Clara, USA)
Universal LSAB™2 Kit/AP, Rabbit/Mouse	Dako
#K0674	(Glostrup, Denmark)
Annexin V-PE Apoptosis Detection kit	PharMingen
	(San Diego, USA)

Table 3-2: Description of molecular biology kits

3.1.3 Patients and tissue samples

Primary cutaneous melanoma, cutaneous melanoma metastases, and congenital nevus samples were obtained from patients who underwent surgical tumor removal at the Department of Dermatology, University of Duesseldorf. Patient signed an informed consent. The disease history was obtained from their medical records. Histopathological classification of each sample was performed according to Breslow index and Clark level. Clinical stage was determined according to the TNM classification (Appendix 7.1). All tumor specimens and nevi were microscopically dissected from the surrounding tissue, and immediately frozen and stored at -80 degrees until used for experimental analysis.

3.1.4 Cell lines and primary cell culture

Cell line	Description	Source
Normal human melanocytes	Primary culture	PromoCell (Heidelberg, Germany)
A375	Primary melanoma	ATCC (CRL 1619)
BLM	Metastatic melanoma	
HeLa	Cervix carcinoma	ECACC (85060701)
M13	Metastatic melanoma	
MV3	Metastatic melanoma	
SK-MEL-28	Primary melanoma	ATCC (HTB 72)

Table 3-3: Description of cell lines and primary cultures

3.1.5 Media for cell culture

DMEM/F12 (1:1)-medium	Serva (Heidelberg, Germany)
Dulbecco's Phosphate buffered Saline (DPBS)	Sigma (Deisenhofen, Germany)
Dulbecco's Modified Egale Medium (DMEM)	Gibco BRL (Eggenstein, Germany)
Melanocyte growth medium M2	PromoCell (Heidelberg, Germany)
Fetal Bovine Serum (FBS)	Gibco BRL(Eggenstein, Germany)
Trypsine/EDTA solution	Seromed/Biochem (Berlin, Germany)

3.1.6 X-ray films

Hyperfilm ECL	Amersham Biosciences (Buckinghamshire, UK)

3.1.7 Buffers and solutions for olignucleotide microarray hybridization, washing and staining

12X MES Stock Buffer

(1.22M MES, 0.89M [Na+])

For 1,000 ml

MES hydrate	64.61 g
MES Sodium Salt	193.3 g
Molecular Biology Grade water	800 ml

Mixed and volume adjusted to 1,000 ml.

The pH adhusted to between 6.5 and 6.7.and Filtered through a 0.2 µm filter.

2X Hybridization Buffer

(Final 1X concentration is 100 mM MES, 1M [Na+], 20 mM EDTA, 0.01% Tween-20)

For 50 ml

12X MES Stock Buffer	8.3 ml
5M NaCl	17.7 ml
0.5M EDTA	4.0 ml
10% Tween-20	0.1 ml
H_2O	19.9 ml

Stored at 2°C to 8°C, and shielded from light

Streptavidin-phycoerythrin (SAPE) staining solution

2X stain buffer	600 µl
50mg/ml BSA	48 µl
1mg/ml SAPE	12 µl

RNase free water made upto 1200 µl

Antibody stain solution

2X stain buffer	300 µl
50mg/ml BSA	24 µl
10mg/ml Goat IgG stock	6 µl
0.5mg/ml biotynylated Ab	3.6 µl

RNase free water made upto 600 µl

Wash buffer A: Non-stringent wash buffer

(6X SSPE, 0.01% Tween-20)

For 1,000 ml

20X SSPE	300 ml

10% Tween-20	1.0 ml
H_2O	699 ml

Filter through a 0.2 µm filter

Wash buffer B: Stringent wash buffer

(100 mM MES, 0.1M [Na+], 0.01% Tween-20)

For 1,000 ml

12X MES Stock Buffer	83.3 ml
5M NaCl	5.2 ml
10% Tween-20	1.0 ml
H_2O	910.5 ml

Filter through a 0.2 µm filter

Store at 2°C to 8°C and shield from light

2X Staining buffer

(Final 1X concentration: 100 mM MES, 1M [Na+], 0.05% Tween-20)

For 250 ml

12X MES Stock Buffer	41.7 ml
5M NaCl	92.5 ml
10% Tween-20	2.5 ml
H_2O	113.3 ml

Filter through a 0.2 µm filter

Store at 2°C to 8°C and shield from light

10 mg/ml Goat IgG Stock

Resuspend 50 mg in 5 ml of 150 mM NaCl

Store at 4°C

3.1.8 Buffers and solutions for the preparation of nuclear extracts, whole cell extracts and cell extrats for immunoprecipitation

RIPA buffer

50 mM	Tris (pH 8.0)
150 mM	NaCl
1.0%	NP-40
0.5%	DOC
0.1%	SDS

Modified RIPA buffer

50 mM	Tris (pH 7.4)
150 mM	NaCl
1 mM	EDTA
1 mM	Na_3VO_4
1 mM	NaF
1.0%	NP-40
0.25%	DOC

NP-40 Lysis buffer

50 mM	Tris (pH 8.0)
150 mM	NaCl
1.0%	NP-40

High salt lysis buffer

50 mM	Tris (pH 8.0)
500 mM	NaCl
1.0%	NP-40

Low salt lysis buffer

50 mM	Tris (pH 8.0)
1.0%	NP-40

Buffer A

20 mM	HEPES (pH 7.9)
10 mM	NaCl
0.2 mM	EDTA
2 mM	DTT

Store at 4°C until use. Before use add the protease inhibitor to buffer A (1 volume protease stock solution: 24 volumes buffer A).

Buffer C

20 mM	HEPES; pH 7.9
0.75 mM	spermidin
0.15 mM	spermin
420 mM	NaCl
0.2 mM	EDTA
2 mM	DTT
25%	Glycerol

Store at 4°C until use. Before use add the protease inhibitor to buffer C (1 volume protease stock solution: 24 volumes buffer C).

KALB lysis buffer

50mM	Tris-HCl, pH 7.5
1% vol/vol	Triton-X-100
1mM	Na_3VO_4
1 mM	NaF
150 mM	NaCl
1X	Protease inhibitor

Protease inhibitors

Prepare as 25x stock solution; store at -20°C; and add fresh to the lysis buffer

3.1.9 Buffers and solutions for protein measurement, electrophoresis and Western blot

1X protein measurement reagent

Advanced protein assay reagent (5X) 200 ml

Adjust volume to 1l with deionized H_2O. Store at RT until use.

20x running buffer

MOPS	104.6 g
Tris-base	60.0 g
SDS	10 g
EDTA	3 g

Adjust volume to 500 ml with deionized H_2O

1x buffer is prepared by diluting 50 ml of the 20x stock in 1l of deionized H_2O

Staining solution

25%	Isopropanol
10%	Glacial acetic acid
0.25%	Coomassie brilliant (R250)

Adjust the volume to 800 ml with H_2O

Destaining solution

7% glacial acetic acid

Transfer buffer

Glycine	2.93 g
Tris-base	5.82 g
10% SDS	3.7 ml
Methanol	200 ml

Adjust the volume to 1l with deionized H_2O.

10x TBS buffer

100 mM	Tris-base
1.5 M	NaCl

Adjust the volume to 1l with H_2O and the pH to 8.3.

Blot solution A

5%	Milk powder blotting grade
0.10%	Triton X-100
0.05%	Tween 20

Adjust the volume to 100 ml with 1x TBS. The solution is prepared fresh before use.

Blot solution B

0.5%	Milk powder blotting grade
0.10%	Triton X-100
0.05%	Tween 20

Adjust the volume to 100 ml with 1x TBS. The solution is prepared fresh before use.

Washing solution

0.10%	Triton X-100
0.05%	Tween 20

Adjust the volume to 1l with 1x TBS.

Stripping solution

2%	SDS
100mM	ß- mercaptoethanol
50mM	Tris.HCl pH 6.8

Store at RT until use.

3.1.10 Buffers and solutions for cell survival and proliferation assays

Stop solution for MTT assay

10%	SDS
0.6%	Glacial acetic acid

Adjust the volume to 1l with DMSO. Store at RT until use.

3.1.11 Buffers and solutions for electrophoretic mobility shift assay

5x gel shift binding buffer

25%	Glycerol
10mM	$MgCl_2$
100mM	Hepes pH 7.9
0.5mM	EDTA
300mM	KCl
2.5mM	DTT
0.25 mg/ml	Poly (di-dc)

10x TBE buffer

Tris-base	107.80 g
Boric acid	55.0 g
EDTA	7.44 g

Adjust the volume to 1l with deionized H_2O.and adjust pH to 8.3

4% non-denaturing polyacrylamide for analysis of DNA-protein complexes

10x TBE buffer	2.5 ml
30% acrylamide	10 ml
Deionized water	37.0 ml
TEMED	30µl
10% APS	500µl

10x gel loading buffer

250mM Tris.HCl, pH 7.5

0.2% Bromophenol blue

40% Glycerol

3.1.12 Equipment and applications

- Centrifuges:
    - Centrifuge 5415 D/R (Eppendorf)
    - EBA 20 (Hettig)
- Biofuge 28 RS (Heraeus, Sepatech)
- GeneChip Fluidics Station 400: Washing and Staining (Affymetrix)
- GeneChip Hybridization Oven 640: Hybridization (Affymetrix)
- GeneChip Scanner G2500A (Agilent)
- ABI PRISM 7300 Real Time PCR System (Applied Biosystems)
- PCR T3-Thermocyclers (Biometra)
- Thermomixer 5437 (Eppendorf)
- Photometer: Spectrophotometer ultraspec 3000 (Pharmacia Biotech)
- Heidolph Polymax 1040 shaker (?)
- MS1 Minishaker (Ika)
- Western Blot gel running apparatus: E19001-Xcell^{TM} Mini cell (Novex)
- Trans-Blot SD Semi-Dry Tranfer cell (Bio-rad)
- Air incubator (Bachofer)
- ELISA Reader MR 5000 (Dynatech)

3.2 Methods

3.2.1 Cell culture

The melanoma cell lines were maintained in Dulbecco's Modified Eagle medium (DMEM-F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2.

Normal human melanocytes obtained from PromoCell (Heidelberg,Germany) were seeded to the recommended seeding density for NHEM, M2 (5.000-10.000 cells per cm2) in melanocyte growth medium M2 and incubated at 37°C in a humidified atmosphere of 5% CO_2.

3.2.2 Total RNA isolation

Total RNA from fresh tumors, NHM and melanoma cell lines was obtained using TRIzol© (Invitrogen, Carlsbad, CA). The tissue was flash frozen by immersion in liquid nitrogen followed by pulverization in a dismembrator at 2,000 for 2 min. This was dissolved in TRIzol© (1.0 ml per 50 mg of tissue) and homogenized by passing several times through a pipet. In case of cell lines/primary cells, after two washings with ice-cold PBS, A375, BLM, NHM, M13, MV3, SK-MEL-28 cells were scraped off from 25 cm^2 cell culture flasks with a plastic scraper with the addition of 1400 µl of TRIzol©. Subsequently, 200 µl of chloroform were added for each 1 ml of TRIzol© used for homogenization, vortexed vigorously for 30 seconds and incubated at RT for 10 min. Phase separation was performed by centrifuging at 12,000g for 20 min at 4°C followed by careful removal of the upper aqueous phase into a new microcentrifuge tube. 600 µl of TRIzol© and 150 µl of chloroform was added to this mixture and vortexed vigorously for 30 seconds and incubated at RT for 10 min. Phase separation was repeated by centrifuging at 12,000g for 20 min at 4°C followed by careful removal of the upper aqueous phase into a new microcentrifuge tube. To this, equal volumes of isopropanol were added and gently mixed by inverting three times and incubated at RT for 30 min to precipitate the RNA followed by centrifugation at 22,000 rpm for 30 min. The supernatant was then removed and the pellet was washed using 1 ml of 75% ethanol. The samples were centrifuged at 7500g for 3 min at 4°C to ensure that the pellet was sedimented before removal of ethanol. The RNA was allowed to air dry for 10 min

at RT. After drying the RNA, samples were resuspended in 50 µl RNase-free water, heated at 65°C for 10 min and placed on ice. Removal of melanin was performed using RNAeasy kits (Qiagen, Valencia, CA) by using the RNAeasy mini protocol for RNA clean up as described by the manufacturer. RNA specimens were analyzed by microcapillary electrophoresis on RNA600 LabChips using the Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Boeblingen, Germany) following the manufacturer's instructions. The bioanalyzer fractionates RNA molecules accrding to size and amounts. The integrity of the RNA was confirmed by the presence of 18S and 28S RNA peaks and an absence of lower peaks corresponding to degradation products in the electropherogram. The ratio of 28S to 18S rRNA approached 2:1 in RNA samples comprised of primary full length RNA. The integrity-confirmed RNA samples were subsequently quantified in a photometer (Ultraspec 3000, Pharmacia Biotech) at A_{260} and stored at -80°C until further analysis.

3.2.3 Affymetrix HGU133A analysis

The human genome Affymetrix GeneChip system HG-U133A microarray (Affymetrix Inc., Santa Clara, CA) containing a total of 22,283 probe-sets representing 18,400 transcripts was used. 2 µg of total RNA was used for synthesizing cRNA using MessageAmp™ kit (#1750) from Ambion, Inc.

3.2.3.1 First strand cDNA synthesis

Total RNA	2 µg
T7 oligo dT Primer	1 µl

made up to 12 µl with nuclease free water

The mixture was incubated for 10 min at 70°C in a thermoblock, pulse spun for 5 seconds and placed on ice. The reverese transcription master mix was prepared as explained below.

10x First strand Buffer	2 µl
Ribonuclease Inhibitor	1 µl
dNTP mix	4 µl

The microcentrifuge tube was flicked, pulse spun and placed on ice. 7 µl of RT master mix were added to each sample (mix) and placed at 42°C in a humidified

incubator. 1 µl of reverse transcriptase was then added to each reaction and incubated for 2 hrs at 42°C in a humidified incubator. After the completion of the reaction the microcentrifuge tube was pulse spun and placed on ice immediately before proceeding for second strand synthesis.

3.2.3.2 Second strand cDNA synthesis

After completion of first strand cDNA synthesis the following were added on ice in the order listed

cDNA sample (post step2)	20 µl
Nuclease free water	63 µl
10x Second strand buffer	10 µl
dNTP Mix	4 µl
DNA Polymerase	2 µl
RNase H	1 µl

The reaction mixture was mixed using a pipet and pulse spun. This was then incubated for 2 hrs at 16°C in a refrigerated water bath (Julabo Labortechnik GmbH, Germany)

3.2.4 cDNA purification

The cDNA filter cartridge was placed in a 2 ml tube, equilibrated by adding 50 µl of cDNA binding buffer and was incubated at RT for 5 min. 250 µl of cDNA binding buffer were added to each cDNA sample and mixed gently. This mixture was pipetted to the centre of an equilibrated cDNA filter cartridge and spun at 10,000 g for 1 minute. The flowthrough was discarded and and placed in a fresh collection tube. Subsequently, 650 µl cDNA wash buffer were added to the filter centrally and spun at 10,000 g for 1 minute. The flowthrough was discarded and an additional spin was performed to remove traces of ethanol. The filter was then placed in a cDNA elution tube and ds cDNA was eluted after adding 10 µl of RNase free water preheated to 50°C, for 2 min and spinning at 10,000 g until the water had passed through the column. This was repeated twice and the eluate was placed on ice.

3.2.4.1 *In vitro* transcription

dscDNA after step 5	16 µl
rATP(75nM)	4 µl
rUTP(75mM)	4 µl
rGTP(75mM)	4 µl
rCTP(75mM)	4 µl
T710x Reaction Buffer	4 µl
T7 enzyme mix	4 µl

The mixture was gently mixed and pulse spun and incubated for 12 hours at 37°C in a humidified incubator.

3.2.4.2 aRNA purification

Sixty microliter of nuclease free water were added to each aRNA sample to make up the volume to 100 µl and mixed gently. The aRNA filter cartridge was placed in a 2 ml collection tube and equilibrated with 100 µl aRNA binding buffer at RT for 5 min. Three hundred and fifty microliter aRNA binding buffer and 250 µl of ACS grade 100% ethanol were then added to each aRNA sample and mixed thoroughly. The mixture was then applied to the equilibrated aRNA filter catridge and spun at 10,000 g for 1 min. The flowthrough was discarded and the filter replaced in a separate tube. Six hundred and fifty microliter aRNA wash buffer were added to each aRNA filter cartridge and spun at 10,000 g for 1 minute. The flowthrough was discarded and an additional spin to remove traces of ethanol was performed. The filter cartridge was transferred to a fresh aRNA collection tube and 50 µl of RNase free water preheated to 50°C, was applied and incubated for 2 min followed spinning at 10,000 g until the water passed through the column. The samples were subsequently quantified in a photometer (Ultraspec 3000, Pharmacia Biotech) at A_{260}.

3.2.4.3 Second round first strand cDNA synthesis

Total RNA	2 µg
Second round Primer	2 µl

made up to 12 µl with nuclease free water

The mixture was incubated for 10 min at 70°C in a thermoblock, pulse spun for 5 sec and placed on ice.

The reverse transcription master mix was prepared as follows:

10x First strand Buffer	2 µl
Ribonuclease Inhibitor	1 µl
dNTP mix	4 µl

The tube was flicked, pulse spun and placed on ice. Seven microliters of the RT master mix were added to each sample, mixed and placed at 42°C. One micorliter of reverse transcriptase was added to each reaction and incubated for 2 hrs at 42°C in an humidified incubator. After completion of the reaction, the microcentrifuge tube was pulse spun and 1 µl of RNaseH was added and incubated for 30 min at 37°C.

3.2.4.4 Second round second strand cDNA synthesis

Five microliters of T7 Oligo dT primer were added to this reaction mixture after the second round first strand cDNA synthesis and incubated at 70°C for 10 min. The following were added on ice in the order listed (>1 sample make master mix):

cDNA sample(Post step2)	26 µl
Nuclease free water	58 µl
10x second strand buffer	10 µl
dNTP Mix	4 µl
DNA Polymerase	2 µl

The reaction mixture was mixed, pulse spun and incubated for 2 hrs at 16°C in a refrigerated water bath. After completion of the reaction the cDNA purification was performed as described in 2.2.3.3.

3.2.4.5 Second round *In vitro* transcription

ds cDNA after step 5	16 µl
10x biotinylated rNTPs (Enzo Life sciences, USA)	4 µl
T7 10x reaction buffer	4 µl

T7 enzyme mix	4 µl
Nuclease free water	12 µl

The mixture was gently mixed, pulse spun and incubated for 12 hrs at 37°C in a humidified incubator. ARNA purification was performed as described in 2.2.3.5. The samples were subsequently quantified in a photometer (Ultraspec 3000, Pharmacia Biotech) at A_{260}.

3.2.4.6 aRNA fragmentation

aRNA	15 µg
5x fragmentation buffer	6 µl

made up to 30 µl with RNase free water

The mixture was gently mixed, pulse spun and incubated for 30 min at 95°C in a thermal block. The fragmented aRNA samples were stored at -80°C until further analysis.

3.2.5 Hybridization, priming, washing, staining and scanning

3.2.5.1 Hybridization

Fragmented cRNA	15 µg
Control oligonucleotide B2 (3 nM)	5 µl
20x hybridization controls (bioB, bioC, bioD, cre)	15 µl
Herring sperm DNA (10 mg/ml)	3 µl
BSA (50 mg/ml)	3 µl
2x hybridization buffer	150 µl
DMSO	30 µl

made upto 300 µl with RNase free water

The hybridization cocktail was heated to 99°C for 5 min on a heating block. The Affymetrix HG-U133A array was equilibrated with 1x hybridization buffer at 45°C for 10 min with rotation. The 1x hybridization buffer was removed and replaced

with clarified hybridization cocktail and the hybridization was performed at 45°C for 16 hours at a rotation of 60 rpm GeneChip Hybridization Oven 640 (Affymetrix)

3.2.5.2 Priming, washing, staining and scanning

For priming the fluidics station, GeneChip Fluidics Station 400 (Affymetrix): “Prime module” or “Prime_450 module” was used after changing the intake buffer reservoir A to non-stringent wash buffer, and intake buffer reservoir B to stringent wash buffer. The washing and staining was performed in the GeneChip Fluidics Station 400: (Affymetrix) with the manufacturer´s recommended protocol EukGE-WS2v4.

EukGE-WS2v4 protocol:

Post hyb wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post hyb wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 min in SAPE solution at 25°C
Post stain wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C
2nd stain	Stain the probe array for 10 min in antibody solution at 25°C
3rd stain	Stain the probe array for 10 min in SAPE solution at 25°C
Final wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C

Wash Buffer A = non-stringent wash buffer

Wash Buffer B = stringent wash buffer

The Affymetrix UG-133A cartridges were then scanned using GeneChip Scanner G2500A (Agilent)

3.2.6 Data analysis

Image acquisition, comparative analysis to the baseline array and scaling/normalization were performed using Affymetrix GeneChip® Operating Software (GCOS). To normalize for sample loading and staining variation, the average of the fluorescent intensities of all probe sets on an individual array were

scaled to a constant target signal intensity (target signal factor 100) for all arrays used. Additionally, all experiments were corrected for 3´/5´ bias differences that may have arisen during labeling or hybridization experiments. After normalization, the log (base 2) transformation data table was imported into the significance analysis of microarrays (SAM) software package [Tusher et al., 2001]. For multiclass SAM, groups were defined based on the comparison performed; for example, group 1 = benign congenital nevi, group 2 = primary cutaneous melanoma, group 3 = cutaneous melanoma metastases, group 4 = melanoma cell lines. Delta was chosen to limit the output gene list so that less than 0.11% predicted false-positives would be included. Statistical testing, clustering and projection techniques were performed using TIGR MeV 3.1 software [Saeed et al., 2003]. Supervised classification was based on biological annotations from gene ontology (GO). Data from the above mentioned techniques were collated and curated to obtain the final list of genes. Input parameters for multiclass SAM analysis: imputation engine is row average imputer; data are in log scale; number of permutations is 100; RNG seed is 1234567; delta is 0.22797. Input parameters for two class-unpaired SAM analysis: imputation engine is row average imputer; data are in log scale; number of permutations is 100; RNG seed is 63158815; delta is 0.36811.

3.3 Quantitative real time-polymerase chain reaction (qRT-PCR)

qRT-PCR reaction was carried using a two-step RT-PCR gene expression quantification experiment.

3.3.1 cDNA synthesis

cDNA synthesis was performed with TaqMan® Reverse Transcription Reagents # N8080234 (Applied Biosystems Inc., Foster City, CA, USA) using random hexamers and manufacturer recommended protocols.

10x RT Buffer	2 µl
25 mM $MgCl_2$	4.4 µl
DeoxyNTP mixture	4 µl
Randomn hexamers	1 µl

RNase inhibitor	0.4 µl
RNA	100 ng

made upto 20 µl with RNase free water

Reverse transcription was carried in a PCR machine (Biometra) using the following conditions:

20°C	10 min
42°C	15 min
99°C	5 min
5°C	5 min

3.3.2 qReal Time PCR

After completion of the reverse transcription reaction the mixture was pulse spun and the following reaction was set in a 96 well format in a ABI Prism 7300 Real Time PCR systems (Applied Biosystems).

Taqman assay on demand (20x)	1.25 µl
cDNA	1 µl

made upto 25 µl with RNase free water

Thermal cycler protocol was as follows :

Step 1	50°C	2 min
Step 2	95°C	10 min
Step 3	95°C	15 sec
Step 4	60°C	1 min

Step 3 and 4 were repeated for 40 cycles.

All samples were amplified simultaneously in triplicates with 18S was used as the endogenous control for all samples.

3.3.3 Primers and probes

All gene expression detection was performed using the Applied Biosystems Assays-on-Demand™ Gene Expression system (Table 3-4 and 3-5). Each pre-formulated assay comes in a 20x mix and contains two unlabeled PCR primers and a FAM™ dye-labeled gene-specific TaqMan® MGB probe.

Gene	Assay ID
ASK/Dbf4	Hs00272696_m1
Tpr	Hs00162918_m1
MCAM/MUC18	Hs00174838_m1
c-MET	Hs00179845_m1
18S	Hs99999901_s1

Table 3-4: Description of Assays-on-Demand™ Gene Expression system from Applied Biosystems (Foster city, U.S.A)

Primers and FAM-MGB (a 6-carboxyfluorescein fluorescent dye and a minor groove binding [MGB] molecule attached to the 3' end) probe used for detection of *Tpr-MET* junction were custom synthesized and sequences were as follows:

Primer/probe	Sequence
Forward primer	5´-CAATGAGAGACTATCTCAAGAACTTGAA-3´
Reverse primer	5´-AGTCTTGCCAAGTACGGCT-3´
FAM MGB probe	5´-CTTAACAGATCAGTTTCCTAATTCA-3´

Table 3-5: Description of TaqMan® MGB probe and primers from Applied Biosystems (Foster city, U.S.A)

All data were analyzed by using the relative standard curve method with 18S as the endogenous control.

3.4 Preparation of cell extracts and protein quantification

3.4.1 Preparation of total cell lysates

After washing twice with ice-cold PBS, A375, BLM, HeLa, NHM, M13, MV3, SK-MEL-28 cells were scraped off from 25 cm^2 cell culture flasks with a plastic scraper. In case of patient tissue samples, the tissue is flash frozen by immersion in liquid nitrogen followed by pulverization in a dismembrator at 2,000 rpm for 2 min. Cells/pulverized tissue were resuspended in 200 µl/25 cm^2 ice-cold cell lysis buffer and lysed by ultrasonication for 15 sec. Lysates were stored frozen at -20°C until analysis by SDS-PAGE /Western blotting.

3.4.2 Preparation of nuclear extracts

Unless indicated otherwise, all procedures were performed at 4°C. Briefly, cells were washed with ice-cold PBS buffer and harvested by adding 500 µl of buffer A (20 mM HEPES, pH 7.9; 10 mM NaCl, 0.2 mM EDTA; and 2 mM DTT) containing protease inhibitor and incubated on ice for 10 min. The supernatant was discarded after centrifugation at 14.000 rpm for 3 min. The pellet was resuspended in 50 µl of buffer C (20 mM HEPES, pH 7.9; 420 mM NaCl, 0.2 mM EDTA; 2 mM DTT; 1 mM Na_3VO_4, 25% glycerol) containing protease inhibitor, incubated for 20 min at 4°C and then centrifuged at 14.000 rpm for 3 min. The supernatant was collected without disturbing the pellet and stored at -80°C until use.

3.4.3 Preparation of cell extracts for immunoprecipitation

Unless indicated otherwise, all procedures were performed at 4°C. Briefly, cells were washed with ice-cold PBS buffer and harvested by adding 500 µl of KALB lysis buffer (50 mM Tris-HCl, pH 7.5, 1% vol/vol Triton-X-100, 1 mM Na_3VO_4, 1 mM NaF, 150 mM NaCl) containing protease inhibitor and incubated on ice for 10 min. Thereafter, cells were scraped off with a plastic scraper, transferred to a micro centrifuge tube and centrifuged at 12,000 rpm for 20 min. The supernatant was collected after centrifugation without disturbing the pellet and stored at -80°C until further analysis.

3.4.4 Protein quantification

The protein samples were quantified in a photometer (Ultraspec 3000, Pharmacia Biotech) at 590 nm after 1:100 dilution in 1x advanced protein assay reagent.

3.5 SDS-Polyacrylamide Electrophoresis (SDS-PAGE)

3.5.1 Electrophoresis and blotting

SDS-PAGE was performed using NuPAGE 4-12% Bis-Tris gels (Invitrogen, USA). SeeBlue plus2 pre-stained standard molecular weight marker (Invitrogen, USA) and Magic Mark XP Western standard (Invitrogen, USA) were used as protein standards. Samples were mixed with 5x SDS sample buffer (Carl Roth GmbH, Germany) and boiled for 10 min at 95°C. After brief centrifugation, samples were loaded onto SDS-PAGE gels. Minigels were run at 200 V in a E19001-XCELL II mini cell (Novex, USA).

3.5.2 Protein transfer and detection of the specific signal (Western blot)

Transfer of protein from NuPAGE 4-12% Bis-Tris gel (Invitrogen, USA) was accomplished in a trans-blot SD semi dry transfer unit for 25 min at 15 V to Protan (PVDF, 0.45 µm) (Amersham, Braunschweig, Germany) transfer membrane pre-incubated with methanol. The Western blots were blocked in Blot solution A overnight at 4°C with constant agitation. The blots were washed three times for 15 min at RT in Western blot washing buffer. The washed blots were allowed to incubate with the primary antibody diluted in Blot solution B (monoclonal antibody 1:2,000 or polyclonal antibody/antiserum 1:500) with constant agitation overnight at 4°C. The blots were washed three times for 15 min at RT in Western blot washing buffer. The Western blots were allowed to incubate for 60 min at RT with the secondary antibody diluted in blot solution B (1:2,000). After the washing of Western blot three times for 15 min at RT in Western blot washing buffer, the specific signal was detected by using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Braunschweig, Germany).

3.5.3 Stripping of Western blots

Bound antibody was removed from Western blots by incubation for 30 min at 60°C in Western blot stripping buffer. After washing three times for 15 min at RT in Western blot washing buffer, the blots could be used for another immunodetection reaction.

3.6 Immunohistochemistry

The slides containing the cryosection (5 µm thick) of normal skin, benign nevi, primary melanoma and melanoma metastasis specimens were fixed in ice cold acetone for 5 min. After rinsing the slides in two changes of phosphate buffer saline (PBS), sections were incubated with the pre-immune serum at RT for 30 min followed by incubation with primary antibody (ASK antiserum 1:500) for 90 min at RT. Sections were rinsed in two changes of PBS, 5 min each, and then incubated with the secondary antibody (biotinylated link, antirabbit IgG, Dako LSAB 2 System kit, prediluted) for 10 min at RT. Following rinses in two changes of PBS, 5 min each, the sections were incubated for 10 min at RT with soluble complexes of alkaline phosphatase streptavidin, washed again in two changes of PBS, 5 min each, and incubated in buffered substrate-fast red chromogen solution (3 mg of fast red chromogen in 3 ml of naphthol phosphate in Tris-HCl buffer). Sections were then rinsed gently in distilled water and counterstained with Mayer's hematoxylin (Merk, Darmstadt, Germany) for 1 min each, washed in tap water for 5 min and mounted in an aqueous based-mounting medium (DAKO, Glostrup, Denmark). In each staining, negative controls were tissue sections immunostained with rabbit nonspecific IgG (Pre-immune serum). The slides were examined under a bright-field microscope.

3.7 Immunofluorescence

A375 human melanoma cells were grown to medium density on glass coverslips and rinsed twice with phosphate-buffered saline (PBS) before fixation in cold acetone for 5 min at 4°C. Fixed cells were washed twice with PBS and incubated for 1 h in blocking solution (5% normal goat serum, PBS, and 0.05% Tween 20). Primary antibodies (1:500 anti-ASK, 1:500 anti-Cdc7) were then added to fixed cells in PBS-0.05% Tween 20 and left for 2 h. After three washes with PBS, the samples were incubated for 30min in PBS-0.5% Tween 20 containing Alexa-488 anti-rabbit antibody or Rhodamine anti-mouse antibody or both (Molecular probes; 1:200 dilution). After three washes in PBS, samples were incubated for 10min PBS-0.5% Tween 20 containing DAPI (4´,6-diamidino-2-phenylindole, dilactate; Invitrogen, USA 1:200 dilution) and signals were detected in a fluorescence microscope (Axioskop; Zeiss). All the antibody reactions were conducted at RT.

3.8 Immunoprecipitation of human Cdc7

In order to precipitate Cdc7 from a cell lysate with a specific Anti-Cdc7, mouse monoclonal IgG (Abcam) cell cultures in 10 cm tissue culture petri dishes were harvested in KALB lysis buffer as described in 2.5.3. Four hundred micrograms of protein were mixed with 1 to 2 µg of anti-human Cdc7 antibody (abcam) for 12 hrs at 4°C. 50% slurry of Protein A Protein G (1:1) Sepharose beads (Pierce, Germany) were pre-incubated with 2% BSA in KALB Lysis buffer for 1 hr followed by 4 washes in KALB lysis buffer. In order to capture the antibody protein complexes, 10 µl of pre incubated protein A/G sepharose were added and incubated with rotation for another 2-3 hrs at 4°C. Thirty microlitre slurry per ml lysate were added. The IP beads were pelleted by centrifugation with 12.000x g at 4°C for 30 sec. The supernatant was discarded and the beads were washed 8 times with 1 ml ice-cold KALB lysis buffer. Thereafter, IP beads were ready for SDS-PAGE analysis. For short term storage, beads were frozen in 30% glycerol at -20°C. Cell extracts incubated with Protein A/G sepharose in the absence of ant-Cdc7 antibody were used as negative control

3.9 Small interference RNA (siRNA) mediated knock down

SiRNA mediated knock down was performed using the silencer siRNA transfection kit (# 1630) (Ambion, Inc) . Fifty thosand MV3 cells (for 6-well plate) and 200 cells (for 96-well plate) were synchronized by 24 hrs serum deprivation. Transfection of siRNA was conducted by using SiPort amine reagent (Ambion, Inc) as per the manufacturer´s recommendation (Table 3-6).

	96 well	**6 well**
Plate cells	0.2 X 10^3	0.5 X 10^5
SiPORT amine	2 µl	10 µl
DMEM medium (-FBS; -antibiotics)	19 µl	197 µl
siRNA (20 µM) stock	0.5 µl	5 µl
Cell culture volume	to 80 µl	to 800 µl
Final transfection volume	100 µl	1,000 µl
Fresh DMEM medium (+FBS; + antibiotics)	100 µl	3 ml

Table 3-6: Protocol of SiPort amine based siRNA transfection from Ambion, Inc (Austin, USA)

SiPORT amine was added to DMEM medium dropwise, followed by thorough vortexing and incubation at RT for 30 min whereas the addition of siRNA ID# 12688 [silencer® validated siRNA for *ASK/Dbf4* (Ambion)] was followed by gentle flicking of the microcentrifuge tube prior to incubation at RT for 20 min. The transfection agent-siRNA complex was always overlayed dropwise with gentle rocking of the 96/6-well plates to achieve the respective final transfection volume. All siRNA treatments were repeated every 24 hrs and total proteins were extracted at 24, 48 and 72 hrs, respectively, for Western blot analysis.

3.10 Measurement of cell viability, proliferation and apoptosis

Cell viability and proliferation are the two parameters that are mostly used to measure growth kinetics of cells. Cell viability can be defined as the number of living cells in a sample. The most straightforward method for determining viable cell or cell proliferation is direct cell counting, staining the cells with trypan blue, counting the cpm of [^{3}H] thymidine incorporation or 5-bromo2'-deoxy-uridine (BrdU) incorporation. Alternatively, as an indirect measure of viable cell number, the overall metabolic activity in a cell population may be determined. Tetrazolium salts like 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) or (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium.Na-salt (WST-1) are metabolized by NAD-dependent dehydrogenase activity to form a colored formazan product. In these assays the amount of dye formed directly correlates to the number of viable cells.

Apoptosis or programmed cell death is characterized by morphologic features, such as loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. However, loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS Annexin V can be conjugated to fluorochromes such as Phycoerythrin (PE). This

format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis

3.10.1 5-bromo2'-deoxy-uridine (BrdU) incorporation assay

To measure DNA synthesis or cell proliferation, 5-bromo2'-deoxy-uridine (BrdU) can be incorporated into DNA in place of thymidine and cells that have incorporated BrdU into DNA can be detected by immunoassay. After completion of siRNA mediated knockdown in a 96 well plate for 24, 48 and 72 hours, the cells were incubated with 10 µl of 100 µM BrdU labeling solution per well for 2 hrs at 37°C. The labeling medium was then removed by tapping off and the cells were fixed by adding 200 µl of FixDenat reagent per well and incubated for 30 min at RT. Subsequently, the FixDenat reagent was tapped off and 100 µl of (1:100 diluted) anti-BrdU-POD were added and incubated at RT for 90 min. The antibody conjugate was then removed by flicking off and rinsing wells three times with 200 µl of 1x PBS. After completion of the washining, 100 µl per well of substrate solution were added and incubated at RT for 5-30 min until colour development was sufficient for photometric detection. Twenty-five microliters of 1M H_2SO_4 were added to each well to stop the reaction.The absorbance was then measured at 450nm (refernce wavelength 690 nm) in ELISA Reader MR 5000 (Dynatech, Denkendorf, Germany).

3.10.2 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

After completion of siRNA mediated knockdown in a 96 well plate for 24, 48 and 72 hours, the cells were incubated with 10 µl of 5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] solution per well for 2 hours at 37°C. Mitochondrial dehydrogenase from viable cells cleaves the tetrazolium rings of the pale yellow MTT to form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells was achieved by the addition of 100 µl of stop solution (10% SDS; 0.6% glacial acetic acid in DMSO) per well that resulted in the liberation and solubilization of the formazan crystals. The number of surviving cells is directly proportional to the level of the formazan product created. The color was

then quantified using a simple colorimetric assay and was measured at 540nm in ELISA Reader MR 5000 (Dynatech, Denkendorf, Germany).

3.10.3 Assessment of apoptosis by Annexin V-staining and Fluorescence activated cell sorting (FACS)

Cells used for flow cytometry were harvested in 2 ml of nonenzymatic cell dissociation solution (10 mM EDTA in PBS without Ca^{2+} and Mg^{2+}) followed by spinning at 1200 rpm for 4 min at RT. Annexin V-PE Apoptosis Detection kit (PharMingen, San Diego, CA) was used. The cells were then resupended in 50 µl of 1x Annexin binding buffer and incubated with 2.5 µl of Annexin V-PE at RT for 15 min in the dark. The staining was stopped by adding 400 µl of 1x annexin binding buffer and cells were analyzed by flow cytometry FACS Calibar using the Cell Quest software system (Becton Dickinson, San Diego, USA). The percentages of apoptosis of the cells were calculated by data from FACS analysis and the result (% Apoptosis) was presented in the bar graph.

3.11 Electrophoretic mobility shift assay (EMSA)

The gel shift or electrophoretic mobility shift assay (EMSA) provides a simple and a rapid method for detecting DNA-binding proteins [Ausubel et al., 1989]. This method has been used widely in the study of sequence-specific DNA binding proteins, such as transcription factors. The assay is based on the observation that complexes of proteins and DNA migrate through a native polyacrylamide gel more slowly than free DNA fragments or ds oligonucleotides. The gel shift assay was performed by incubating the purified protein, a complex mixture of proteins (nuclear or whole cell extract preparations) with alpha-^{32}P end-labelled DNA specific sequences. The reaction products are then analysed on a native polyacrylamide gel. The specificity of the DNA binding protein for the putative binding site is established by competition experiments using DNA fragments or oligonucleotides containing a binding site for the protein of interest or other unrelated DNA sequences.

3.11.1 Preparation of nuclear extracts

Nuclear extracts from A375, BLM, MV3, M13 and SK-MEL-28 melanoma cells were performed as described in 2.4.2.

3.11.2 End labelling of nucleotides

In this technique, a dephosphorylated oligonucleotide receives phosphate onto ist 5´ hydrodxy termius, in the form of gamma-^{32}P-ATP labeled in the gamma donor position. This reaction is catalyzed by T4 polynucleotide kinase. The labeling of the oligonucleotides was performed as follows in a sterile microcentrifuge tube:

Oligonucleotide (1.75 pmol/µl)	1 µl
10x polynucleotide kinase buffer	1 µl
Gamma-^{32}P-ATP (3000 Ci/mmol)	1 µl
Polynucleotide kinase (5-10 U/µl)	1 µl
Nuclease free water	5 µl

The reaction mixture was incubated for 10 min at 37°C. After the incubation the reaction was stopped by the addition of 1 µl of 0.5 M EDTA and the reaction volume was adjusted to 100 µl with TE buffer.

Oligonucleotide	Description	Sequence
ASK MP (minimal promoter)	63 bp wild type minimal promoter capable of E2F1 binding	5´gggcggggcgcgcgtatcggcgc cgcggccgcgtgacgcgttttcaaatc ttcaaccgccgc 3´
Mutant MP	Modified sequence incapable of E2F1 binding	5´ggggcggggcgcgcgtatcggcg ccgcggccggaattcgcgttttcaaat cttcaaccgccgc 3´

Table 2-7: Description and sequence of oligonucleotides used for EMSA

The double stranded synthetic oligonucleotides carrying the non-canonical E2F1 binding site of ASK minimal promoter (MP) and mutant of ASK MP (mutant MP) were synthesized from (MWG, Ebersberg, Germany)

3.11.3 Removal of unincorporated label

To improve the qualtity of gel shifts, two methods were used to remove the unincorporated nucleotides from the DNA probes. The first method was used to

purify the very small oligonucleotides by using oligo purification kit (Qiagen, Hilden, Germany). The second method was used to purify the large oligonucleotides by ethanol precipitation. The labelled samples were mixed with ¼ volume of 5 M ammonium acetate and 2 volumes of absolute ethanol. Following the incubation at 20°C for 30 min, the samples were centrifuged at 15,000 rpm and the supernatant was removed carefully by aspiration. The pellet was resuspended in 100 µl of 1 M ammonium acetate and 200 µl absolute ethanol and placed at - 20°C for 30 min. After the centrifugation for 15 min at 15,000 rpm, the supernatant was carefully removed and the pellet was allowed to dry under vaccum. The dried pellet was resuspended in 100 µl TE buffer and stored at - 20°C until further use.

3.11.4 DNA binding reaction using nuclear extract

The DNA binding reaction was made by mixing 4 µg of the nucelar extracts from (BLM, A375, MV3, M13,SK-MEL-28 and cisplatin treated BLM) with 2 µl of 5x gel shift binding buffer in a sterile microfuge tube. The total volume was adjusted to 9 µl with nuclease free water. After the incubation of the reaction at RT for 10 min and the addition of 1 µl of gamma-^{32}P-ATP (50,000-200,000 cpm) labeled oligonucleotide. After the incubation of the reaction at RT for further 20 min, the sample was mixed with 1 µl of 10x gel loading buffer and analyzed on 4% polyacrylamide native gel.

3.11.5 Electrophoresis of DNA-protein complexes

The DNA-protein complexes were analyzed on a 14 x 22 cm, native, 4% acrylamide gel, 0.75 mm thick. Before loading the sample, the gel was allowed to pre run for 30 min in 0.5 x TBE buffer at 100 V. After loading the samples, the gel was allowed to run at 100 V at RT in 0.5 x TBE buffer for approximately 3 hrs. At the end of the run the gel was wrapped in plastic wrap and exposed to X-ray film overnight at - 70°C with an intensifying screen. The specific signal was quantified by the Reytest software (Reytest, Straubenhardt, Germany)

3.11.6 Competition assay and Supershift assay

The competition assay was performed in the same manner, except that unlabeled probes containing either ASK MP or mutant MP sequences were incubated with

nuclear extracts for 20 min at room temperature prior to the addition of the labeled probes in 3.11.4

For supershift experiments, 0.5 µg anti-E2F1 antibody (Genecraft, Muenster, Germany) were added to the reaction mixture and incubated for 30 min at 37 C prior to the addition of the labeled probes in 3.11.4.

3.12 Cisplatin treatment of melanoma cell lines

BLM cells were maintained in Dulbecco's Modified Eagle medium (DMEM-F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine and 1% antibiotic solution at 37°C in a humidified atmosphere of 5% CO_2 until 50% confluency was acheived. The existing medium was then replaced with medium containing 100 µM final concentration of cisplatin and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 and 48 hrs respectively.

For cisplatin treatment post siRNA treatment of cells, the siRNA treatment was performed as described in 2.9. At 24 and 48 hrs post siRNA treatment, the medium was replaced with medium containing 100 µM final concentration of cisplatin and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 hours.

3.13 UVB-treatment of normal human melanocytes (NHM)

Normal human melanocyte (NHM) cultures were maintained in 10 cm^{-2}petri plates as described previously in 2.2.1 and grown to 40% confluency. The NHM were then washed twice in PBS and was briefly, irradiated with 200 Jm^{-2} UVB. Fresh melanocyte growth medium was then added and the plates were incubated at 37°C in a humidified incubator For UVB irradiation, a single FS20T12 UVB lamp (Light Sources), with maximal output at 313 nm was used. Controls consisted of samples incubated in fresh melanocyte growth medium alone

4 RESULTS

4.1 Gene expression profiling data analysis and identification of differentially expressed candidates in cutaneous melanoma development

Well characterized specimens of 4 different classes (11 congenital nevi, 10 primary cutaneous melanoma, 11 cutaneous melanoma metastases and 5 melanoma cell lines) were analyzed. Gene selection from Affymetrix HGU133A (containing a total of 22,283 probe-sets representing 18,400 transcripts) oligonucleotide array analysis was based on comparison with normal human melanocytes (NHM) as calibrator. Multiclass significance analysis of microarrays (SAM) was performed to identify genes whose expression significantly differs among the 4 classes [Tusher et al., 2001] and projection methods [Brown et al., 2000; Lefkovits et al., 1988] were applied. Additionally a two-class SAM was performed to identify gene whose expression differentiate between primary cutaneous melanoma and cutaneous melanoma metastasis albeit with a high median false-significant rate. The post SAM data was independently subjected to unsupervised and supervised data analysis as well as principal component analysis (PCA). Unsupervised hierarchical clustering [Eisen et al., 1998] yielded a general separation of three patterns (Pattern A, B, C) based on their expression profiles (Figure 4-1). **Patterns A** and **B** represented genes whose expression distinguished benign nevi from malignant melanomas whereas **Pattern C** not only distinguished primary tumors from nevi but also primary tumors from cutaneous metastases (Figure 4-1).

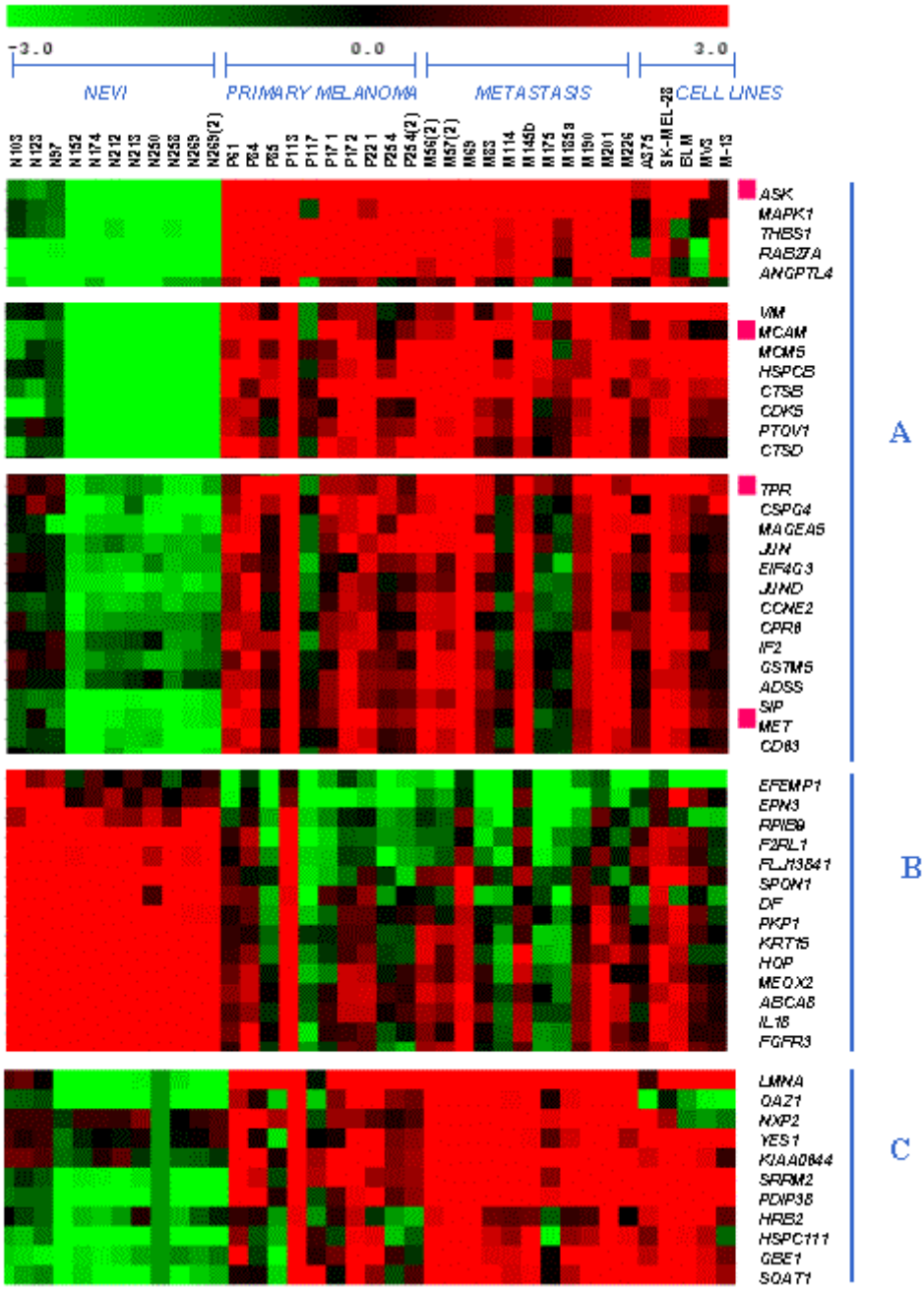
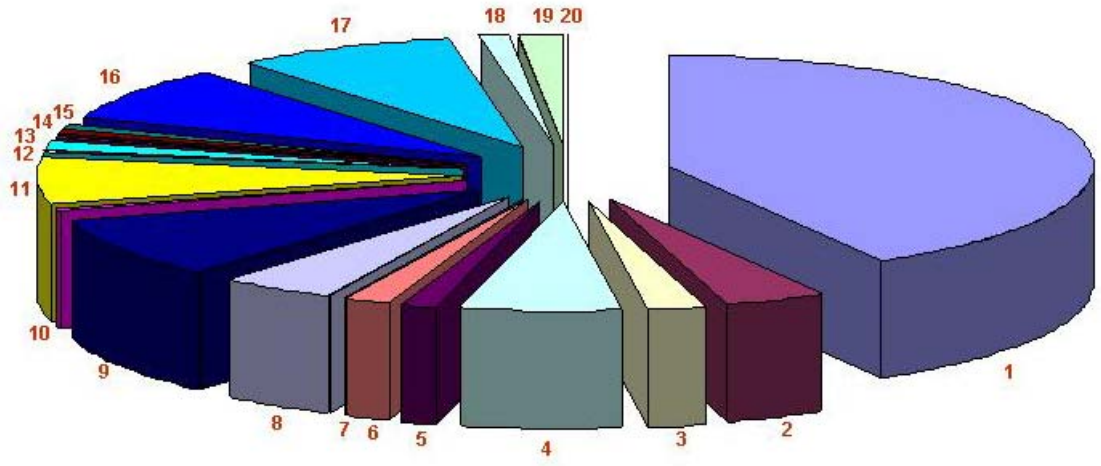


Figure 4-1: Unsupervised hierarchical cluster analysis of gene expression profiles of different stages in the tumor progression model of cutaneous melanoma. **Pattern A:** Genes with differential expression during melanoma development (Upregulated during benign to malignant transition).**Pattern B:** Genes with differential expression during melanoma development (downregulated during benign to malignant transition. **Pattern C:** Genes with differential expression during melanoma development and progression (Benign to malignant to inasive transition.

The supervised classification involved functional categorization based on the annotation of biological processes to 20 predefined categories (Figure 4-2).



	Functional categories	%
1	Unknown	41.71
2	Miscellaneous	3.91
3	Cellular respiration / proton transport	2.04
4	Cellular protein/small molecule trafficking	6.17
5	Apoptosis / apoptosis regulation	1.37
6	Cell differentiation / cell fate determination / development and differentiation	1.69
7	Drug resistance	0.12
8	Cell proliferation and cell cycle regulation	4.38
9	Metabolism	8.80
10	Cell -cell signalling	0.54
11	Signal transduction	6.64
12	Cell adhesion and ECM regulation / cell motility	1.83
13	Oncogenesis	0.19
14	Cytoskeltal organization, biogenesis, regulation	0.57
15	Chromatin organization and remodelling	0.80
16	Translation/protein synthesis and post translational modification/protein assembly	8.16
17	Transcription regulation and post transcriptional processing	8.34
18	DNA repair	1.11
19	Immune response	1.62
20	Angiogenesis	0.01

Figure 4-2: Supervised analysis of gene expression profiles of of cutaneous melanoma development and progression - functional categorization based on gene ontology descriptors.

The PCA produced a low-dimensional projection of an originally high-dimensional data set and allocated these points into a two- or three-dimensional subspace to display associations between genes and experiments as closely as possible. The molecular changes associated with transition of benign nevi to primary cutaneous melanoma (**Patterns A** and **B**) were distinct and discernible. Nevertheless, our data showed no absolute distinction between primary cutaneous melanoma and cutaneous melanoma metastasis but rather reflects the close biological relationship between them, suggesting the progression to cutaneous metastasis to be gradual and more diffuse (Figure 4-3).

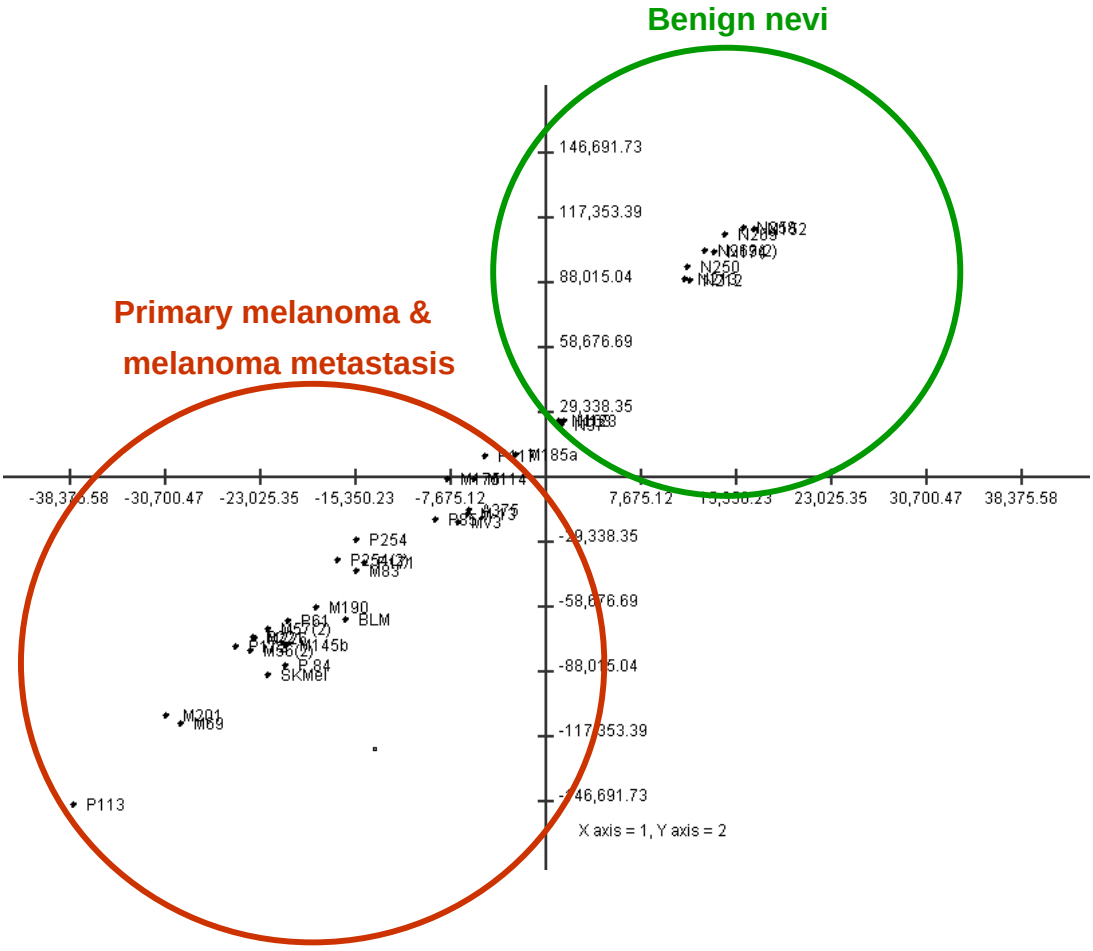


Figure 4-3: Principal component analysis of tumor progression model cutaneous melanoma. A two dimensional projection of benign nevi,primary melanomaand melanoma metastasis samples based on their expression profile.X axis represents dimension 1 and Y axis represents dimension 2

Finally, the information from the above-mentioned procedures was collated and curated to generate gene lists that were useful as molecular discriminators (Table

4-1A,.4-1B,4-1C). The genes upregulated in cutaneous melanoma development (**Pattern A**) represented a variety of functions including genes associated with signal transduction pathways e.g. mitogen-activated protein kinase 1 *(MAPK1)*, signal transducer and activator of transcription 3 (*STAT3)*, *ELK1* (member of EIS oncogene family), cell cycle regulation e.g. activator of S phase kinase (*ASK/Dbf4)*, cyclin A1 (*CCNA1*), cyclin B1 (*CCNB1*), cyclin E2 (*CCNE2*), cell motility and extracellular matrix e.g. vimentin (*VIM)*, chondroitin sulfate proteoglycan 4 (*CSPG4*) and other functions such as translocated promoter region/tumor potentiating region (*Tpr)*, dihydrofolate reductase (*DHFR)* etc.

Genes downregulated during cutaneous melanoma development (**Pattern B**) included spondin 1 (*SPON1*), interleukin-18 (*IL-18*) etc., whereas **Pattern C** represented genes whose expression distinguished nevi from primary cutaneous melanoma as well as primary cutaneous melanoma from cutaneous melanoma metastasis, including nuclear matrix protein (*NXP2)* and lamin A/C (*LMNA)*, for example.

Gene	Description	Unigene ID	Score(d)
ASK	**activator of S phase kinase**	**Hs.152759**	**1.406716**
ANGPTL4	angiopoietin-like 4	Hs.9613	1.048992
ACLY	ATP citrate lyase	Hs.387567	0.990791
RAB27A	RAB27A, member RAS oncogene family	Hs.298530	0.941973
DHFR	dihydrofolate reductase	Hs.83765	0.92837
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	Hs.119597	0.907834
KYNU	kynureninase (L-kynurenine hydrolase)	Hs.444471	0.890839
THBS1	thrombospondin 1	Hs.164226	0.882066
MDM2	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	Hs.212217	0.877956
SDC2	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)	Hs.1501	0.845833
CTSB	cathepsin B	Hs.135226	0.836647
MAPK1	mitogen-activated protein kinase 1	Hs.324473	0.830008
VEGF	vascular endothelial growth factor	Hs.73793	0.81609
EPOR	erythropoietin receptor	Hs.127826	0.811253

Table 4-1A: Pattern A-Genes with differential expression during melanoma development (Upregulated during benign to malignant transition)

Table 4-1A continued

Gene	Description	Unigene ID	Score(d)
CDK5	cyclin-dependent kinase 5	Hs.166071	0.791784
NOV	nephroblastoma overexpressed gene	Hs.235935	0.791717
MTX1	metaxin 1	Hs.247551	0.776363
S100B	S100 calcium binding protein, beta (neural)	Hs.422181	0.772773
HCAP-G	chromosome condensation protein G	Hs.443468	0.771527
DKFZp566C0424	putative MAPK activating protein PM20,PM21	Hs.252967	0.771213
STAT3	signal transducer and activator of transcription 3 (acute-phase response factor)	Hs.421342	0.755746
CCNA1	cyclin A1	Hs.417050	0.752108
MCM5	MCM5 minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	Hs.77171	0.750909
HSPCB	heat shock 90kDa protein 1, beta	Hs.74335	0.737687
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	Hs.789	0.717871
ARHD	ras homolog gene family, member D	Hs.15114	0.715483
MAGEA3	melanoma antigen, family A, 3	Hs.417816	0.702232
CCNB1	cyclin B1	Hs.23960	0.694339
CTSD	cathepsin D (lysosomal aspartyl protease)	Hs.343475	0.683029
AKT1	v-akt murine thymoma viral oncogene homolog 1	Hs.368861	0.676928
MCAM	**melanoma cell adhesion molecule**	**Hs.511397**	**0.671687**
CCNB2	cyclin B2	Hs.194698	0.654283
PTOV1	prostate tumor overexpressed gene 1	Hs.227429	0.644979
CALM1	calmodulin 1 (phosphorylase kinase, delta)	Hs.282410	0.644444
ANAPC5	anaphase promoting complex subunit 5	Hs.7101	0.639346
CDC2	cell division cycle 2, G1 to S and G2 to M	Hs.334562	0.638058
MCM4	MCM4 minichromosome maintenance deficient 4 (S. cerevisiae)	Hs.460184	0.634996
SNRPD1	small nuclear ribonucleoprotein D1 polypeptide 16kDa	Hs.86948	0.631476
VIM	vimentin	Hs.435800	0.612012
ELK1	ELK1, member of ETS oncogene family	Hs.181128	0.605782
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	Hs.6906	0.603939
ANGPTL6	angiopoietin-like 6	Hs.306971	0.584266
TUFM	Tu translation elongation factor, mitochondrial	Hs.12084	0.583913
SIP	Siah-interacting protein	Hs.27258	0.570864
MAGEA12	melanoma antigen, family A, 12	Hs.169246	0.56047
RRAS	related RAS viral (r-ras) oncogene homolog	Hs.9651	0.547366
RUNX1	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	Hs.410774	0.544528
RCN2	reticulocalbin 2, EF-hand calcium binding domain	Hs.79088	0.535974

Table 4-1A continued

Gene	Description	Unigene ID	Score(d)
FGF1	fibroblast growth factor 1 (acidic)	Hs.278954	0.532307
FYN	FYN oncogene related to SRC, FGR, YES	Hs.390567	0.514778
MET	**met proto-oncogene (hepatocyte growth factor receptor)**	**Hs.419124**	**0.511836**
TPR	**translocated promoter region (to activated MET oncogene)**	**Hs.170472**	**0.501261**
MAGEA5	melanoma antigen, family A, 5	Hs.37108	0.489863
CD63	CD63 antigen (melanoma 1 antigen)	Hs.445570	0.47859
MAGEA10	melanoma antigen, family A, 10	Hs.18048	0.473667
CSPG4	chondroitin sulfate proteoglycan 4 (melanoma-associated)	Hs.436301	0.473657
MLANA	melan-A	Hs.154069	0.457391
JUN	v-jun sarcoma virus 17 oncogene homolog (avian)	Hs.78465	0.437808
MEL	mel transforming oncogene (derived from cell line NK14)-RAB8 homolog	Hs.5947	0.437565
PIM1	pim-1 oncogene	Hs.81170	0.431887
CCNE2	cyclin E2	Hs.408658	0.397038
JUND	jun D proto-oncogene	Hs.2780	0.371873
DEK	DEK oncogene (DNA binding)	Hs.110713	0.365788
FZD7	frizzled homolog 7 (Drosophila)	Hs.173859	0.350576
CPR8	cell cycle progression 8 protein	Hs.259326	0.347389
IF2	translation initiation factor IF2	Hs.158688	0.347308
EIF4G3	eukaryotic translation initiation factor 4 gamma, 3	Hs.402697	0.345669
GSTM5	glutathione S-transferase M5	Hs.75652	0.341597
ADSS	adenylosuccinate synthase	Hs.90011	0.340727
MGC16824	esophageal cancer associated protein	Hs.300404	0.340465

Gene	Description	Unigene	Score(d)	Fold change
NXP2	nuclear matrix protein NXP2	Hs.421150	2.537191	18.84069
PDIP38	polymerase delta interacting protein 38	Hs.241543	1.742249	6.34651
OAZ1	ornithine decarboxylase antizyme 1	Hs.446427	1.679052	4.36699
SRRM2	serine/arginine repetitive matrix 2	Hs.433343	1.554538	1.68956
LMNA	lamin A/C	Hs.436441	1.447401	3.82169
YES1	v-yes-1 Yamaguchi sarcoma viral oncogene homolog 1	Hs.194148	1.380769	2.89408
HRB2	HIV-1 rev binding protein 2	Hs.269857	1.376746	4.11076
SOAT1	sterol O-acyltransferase (acyl-Coenzyme A: cholesterol acyltransferase) 1	Hs.446331	1.375063	0.50817
KIAA0644	KIAA0644 gene product	Hs.21572	1.356151	2.33422
GBE1	glucan (1,4-alpha-), branching enzyme 1(glycogen branching enzyme)	Hs.511986	1.352833	1.25952
HSPC111	hypothetical protein HSPC111	Hs.424552	1.351426	2.19891

Table 4-1B: Pattern B-Genes with differential expression during melanoma development (downregulated during benign to malignant transition).

Gene	Description	Unigene	Score(d)
SPON1	spondin 1, (f-spondin) extracellular matrix protein	Hs.5378	0.505191
F2RL1	coagulation factor II (thrombin) receptor-like 1	Hs.154299	0.503865
KRT15	keratin 15	Hs.80342	0.465565
DF	D component of complement (adipsin)	Hs.155597	0.412603
FGFR3	fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)	Hs.1420	0.399166
FLJ13841	hypothetical protein FLJ13841	Hs.99607	0.396787
IL18	interleukin 18 (interferon-gamma-inducing factor)	Hs.83077	0.396636
EPN3	epsin 3	Hs.165904	0.38154
MEOX2	mesenchyme homeo box 2 (growth arrest-specific homeo box)	Hs.77858	0.369276
RPIB9	Rap2 binding protein 9	Hs.411488	0.364838
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	Hs.76224	0.347931
PKP1	plakophilin 1 (ectodermal dysplasia/skin fragility syndrome)	Hs.313068	0.345121
HOP	homeodomain-only protein	Hs.13775	0.344845
ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	Hs.58351	0.341928

Table 4-1C: Pattern C-Genes with differential expression during melanoma development and progression (Benign to malignant to inasive transition

4.2 Quantitative real-time RT-PCR confirmation of gene array findings

4.2.1 Validation of gene expression in different stages of the cutaneous melanoma tumor progression model

qReal-time-PCR using TaqMan® Gene Expression Assay (Applied Biosystems Inc., Foster City, CA, USA) was performed to confirm the expression profiles. Four genes (*ASK/Dbf4*, *Tpr*, *MCAM/MUC18* and *c-MET)* representing diverse functional categories were assessed in an additional set of compound, junctional or congenital nevi (n=9), primary cutaneous melanoma (n=18), cutaneous melanoma metastasis (n=15), with NHM as calibrator. Some identical RNA samples from the microarray study (e.g. P61, P84, P85, P171, and P172) were included in this sample set as correlation controls.

4.2.1.1 Confirmation of *ASK/Dbf4* mRNA expression

In this confirmatory study *ASK/Dbf4* showed a median overexpression of 17.9-fold and 13.2-fold relative to NHM in primary cutaneous melanoma and cutaneous melanoma metastasis, respectively, while the majority of nevi expressed similar or lower levels than NHM (Figure 4-4). *ASK/Dbf4*, transcripts were highly overexpressed in most investigated melanoma cell lines and were not significantly overexpressed in non-melanoma skin cancers. To ensure that the observed expression of *ASK/Dbf4* was indeed from melanoma cells, it was confirmed that the basal transcript levels in normal human keratinocytes (NHK) were not significantly higher than in NHM. The fold change of NHK/NHM for *ASK/Dbf4* was -2.2

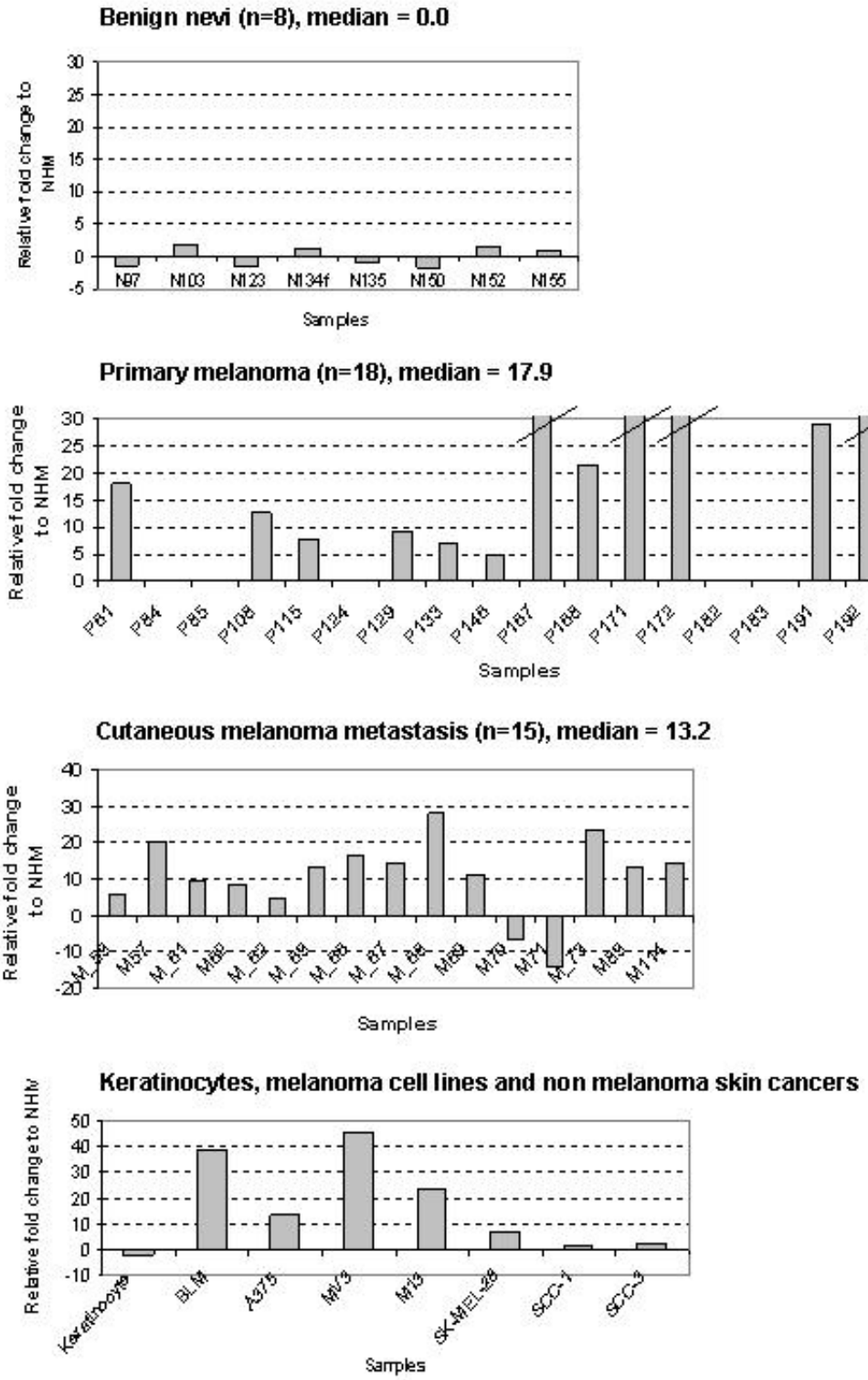


Figure 4-4: Upregulation of ASK/Dbf4 in primary cutaneous melanoma, cutaneous melanoma metastasis and melanoma cell lines in comparison to benign nevi, keratinocytes and non-melanoma skin cancers. NHM served as calibrator.

4.2.1.2 Confirmation of *MCAM/MUC18* mRNA expression

MCAM/MUC18 showed a median overexpression of 1.4-fold relative to NHM in primary cutaneous melanoma while the majority of nevi showed a much lower expression (- 6.2 fold less) than NHM (Figure 4-5). *MCAM*/*MUC18*, transcripts were highly overexpressed in most investigated melanoma cell lines and were not significantly overexpressed in non-melanoma skin cancers. To ensure that the observed expression of *MCAM*/*MUC18* was indeed from melanoma cells, it was confirmed that the basal transcript levels in normal human keratinocytes (NHK) were not significantly higher than in NHM. *MCAM*/*MUC18* transcripts were undetectable in NHK.

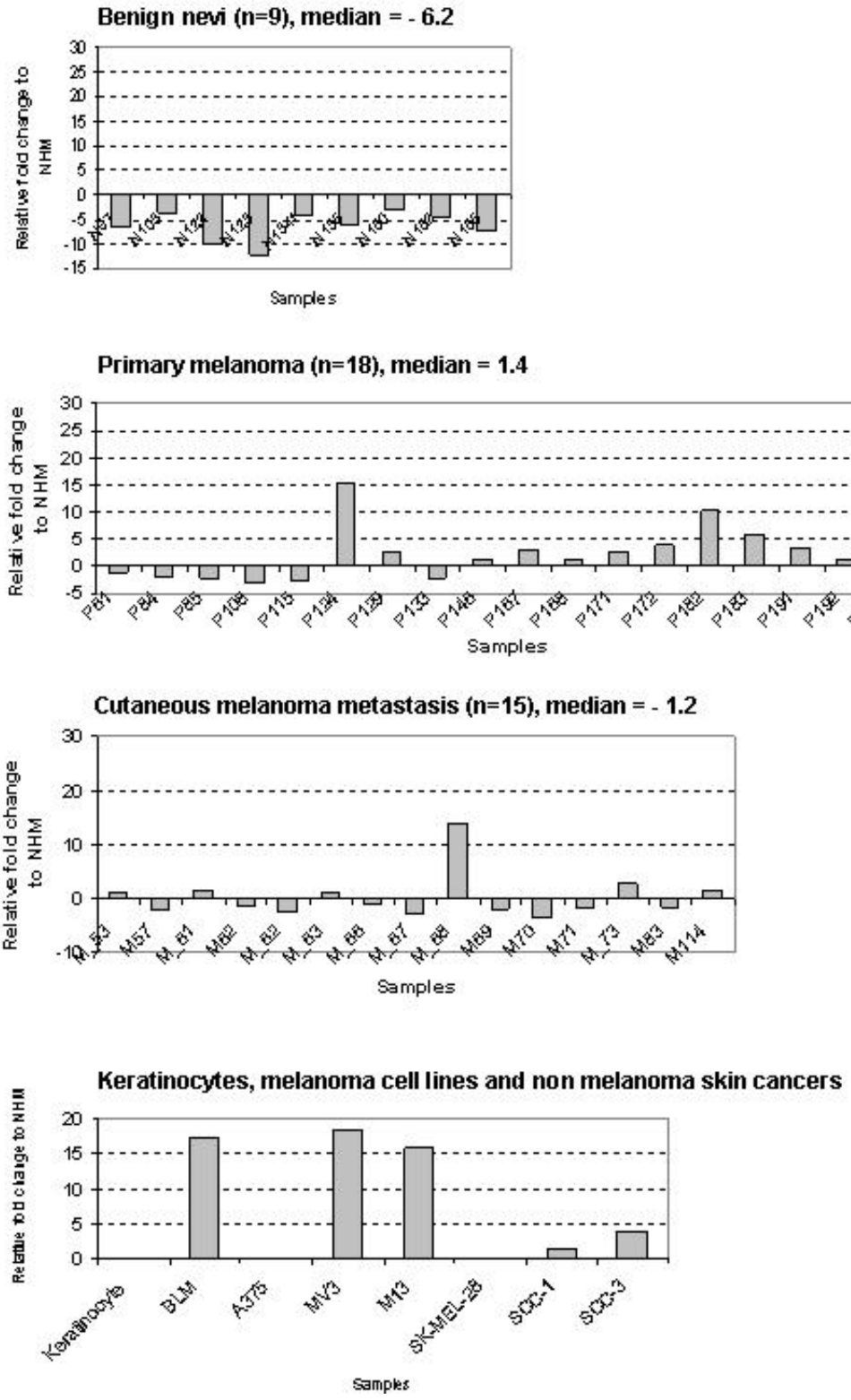


Figure 4-5: Upregulation of *MCAM*/*MUC18* in primary cutaneous melanoma cutaneous melanoma metastasis and melanoma cell lines in comparison to benign nevi, keratinocytes and non melanoma skin cancers. NHM served as calibrator.

4.2.1.3 Confirmation of *c-MET* mRNA expression

c-MET showed a median overexpression of 1.5-fold and 2.3-fold in primary cutaneous melanoma and cutaneous melanoma metastases, respectively, while the majority of nevi expressed lower levels (2.5-fold less) than NHM (Figure 4-6). *c-MET*, transcripts were highly overexpressed in most investigated melanoma cell lines and were not significantly overexpressed in non-melanoma skin cancers. To ensure that the observed expression of *c-MET* was indeed from melanoma cells, it was confirmed that the basal transcript levels in normal human keratinocytes (NHK) were not significantly higher than in NHM. *c-MET* transcripts were undetectable in NHK.

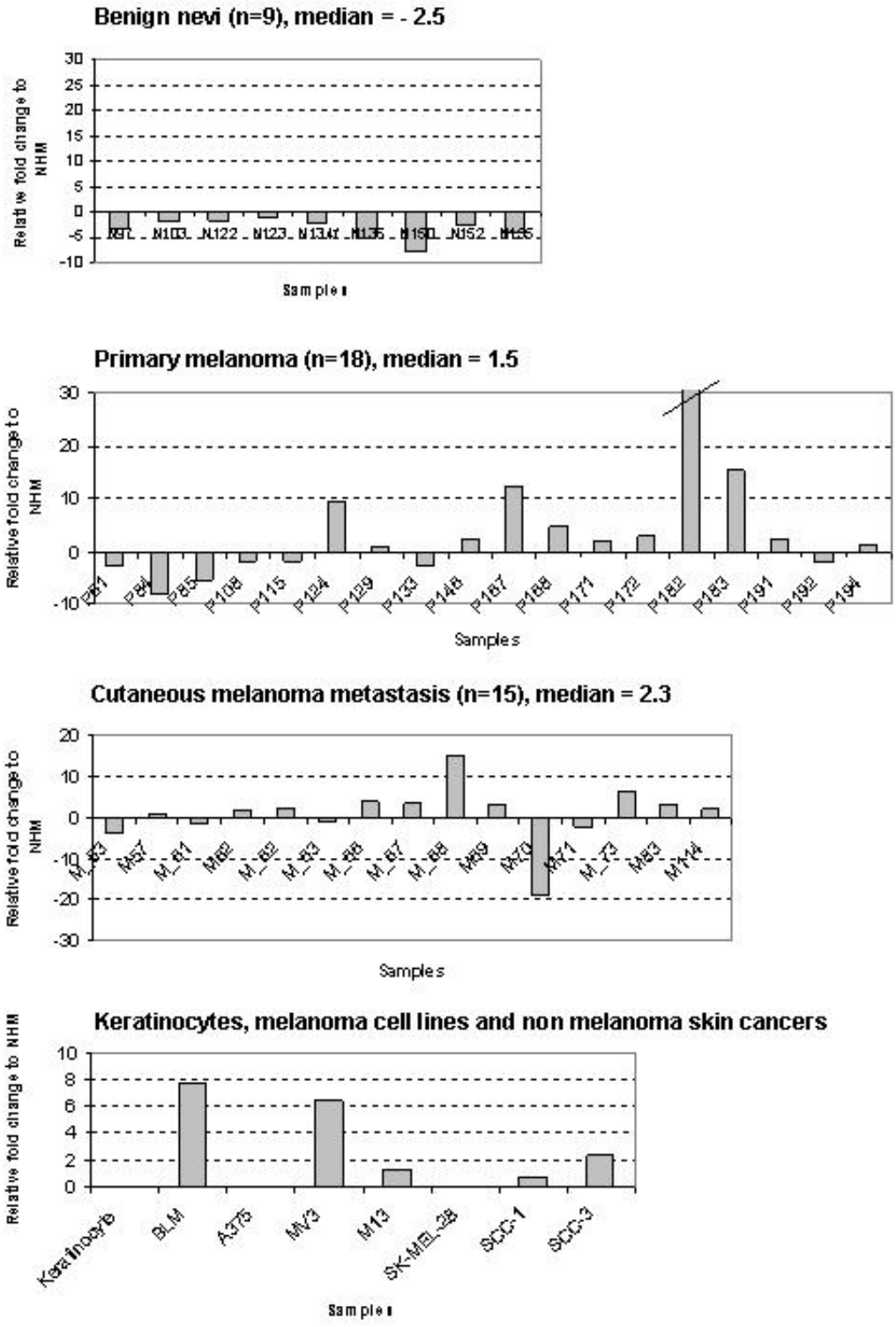


Figure 4-6: Upregulation of c-*MET* in primary cutaneous melanoma, cutaneous melanoma metastasis and melanoma cell lines in comparison to benign nevi, keratinocytes and non melanoma skin cancers. NHM served as calibrator.

4.2.1.4 Confirmation of *Tpr* mRNA expression

Tpr gene was described not as an independent gene but as a rearranged fusion hybrid with *c-MET*. The *Tpr-MET* rearrangement (henceforth referred to as *Tpr-MET* hybrid) produces a *Tpr-MET* transcript translated into a constitutively active c-MET receptor initially reported in gastric cancer and its precursor lesions [Soman et al., 1991]. Unlike gastric cancer, in melanoma no previous study has investigated the presence of the *Tpr-MET* hybrid transcripts, as *Tpr* overexpression has not been reported to date, although c-MET activation through autocrine or paracrine loops has been known [Otsuka et al., 1998; Yu et al., 2002]. As the microarray data showed overexpression of both, *Tpr* and *c-MET*, in cutaneous melanoma, it was imperative to discriminate whether the observed overexpression was of *Tpr* only or of *Tpr-MET* (leading to the activation of c-MET). Therefore the confirmation of *Tpr* expression involved investigating the prevalence of *Tpr-MET*, and *Tpr* in a series of primary and metastatic melanoma patients was performed. A TaqMan® MGB assay directed towards the junction of *Tpr* and *c-MET* in the *Tpr-MET* hybrid transcript and a TaqMan® MGB assay for *Tpr* (exon 21-22 present only in *Tpr* and not in *Tpr-MET*) was used for this purpose (Figure 4-7)..

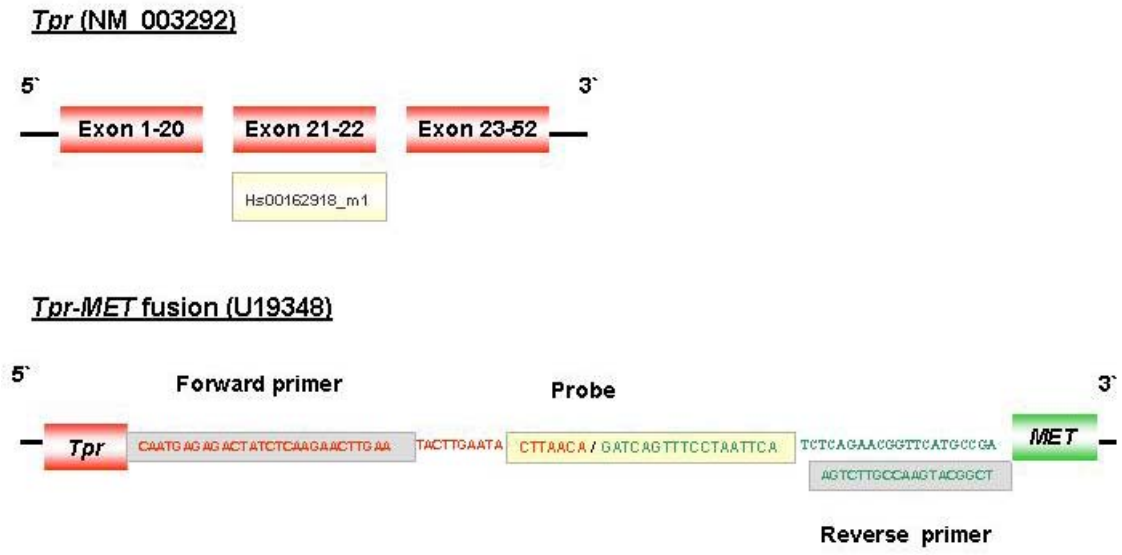


Figure 4-7: Schematic representation of *Tpr* and *Tpr-MET* TaqMan® MGB assay location.

Moreover the expression of *Tpr* (exon 21-22 present only in *Tpr* and not in *Tpr-MET*) and expression of *Tpr-MET* was determined at the mRNA level in the same series of samples. To ensure that the sensitivity of the assay was at the same

level, *Tpr and Tpr-MET* were investigated from the same reverse transcribed RNA for all specimens.

Tpr (exon 21-22 present only in *Tpr* and not in *Tpr-MET*) showed a median overexpression of 29.5-fold and 69.1-fold in primary cutaneous melanoma and cutaneous melanoma metastases, respectively, while the majority of nevi expressed similar to or slightly higher levels than NHM (Figure 4-8). *Tpr*, transcripts were highly overexpressed in most investigated melanoma cell lines and were not significantly overexpressed in non-melanoma skin cancers. To ensure that the observed expression of *Tpr* was indeed from melanoma cells, it was confirmed that the basal transcript levels in normal human keratinocytes (NHK) were not significantly higher than in NHM. The fold change of NHK/NHM for *Tpr* was 1.2

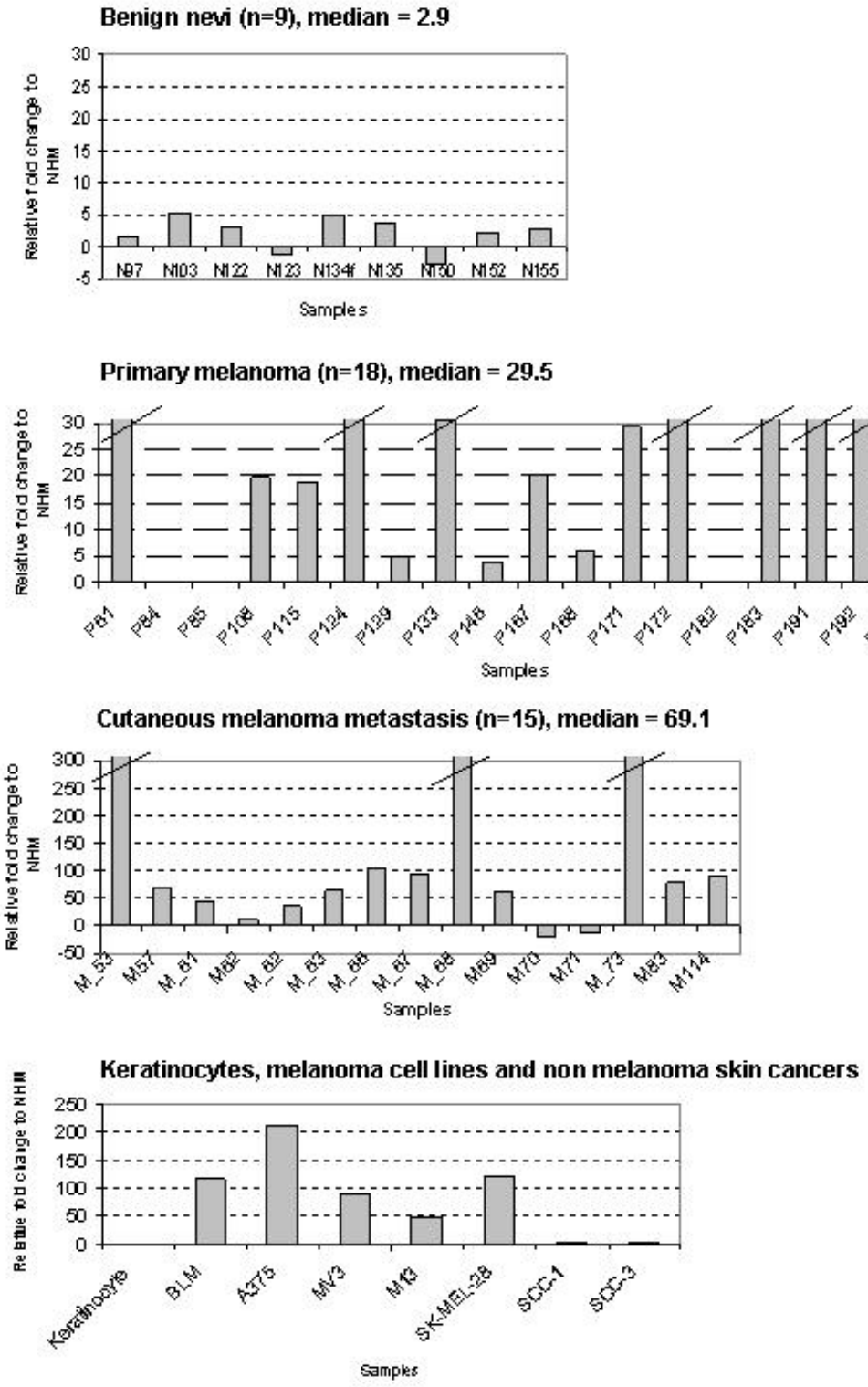


Figure 4-8: Upregulation of *Tpr* (exon 21-22) in primary cutaneous, melanoma cutaneous melanoma metastasis and melanoma cell lines in comparison to benign nevi, keratinocytes and non melanoma skin cancers. NHM served as calibrator.

Tpr-MET hybrid transcript was undetectable in the same reverse transcribed RNA for all primary melanoma and melanoma metastasis specimens (Table 4-2A, 4-2B).

Primary melanoma	Gender	Age	*CDKN2a* status	Clark level	Breslow index (mm)	*Tpr* (exon 21-22)	c-*MET*	*18s* control	*Tpr-MET* fusion
P61	F	85	M	IV	1.6	102.1	-2.6	+	-
P84	F	57	-	IV	6.5	ND	-8.1	+	-
P85	M	70	-	IV	1.65	ND	-5.4	+	-
P108	M	65	-	II	0.85	19.7	-2.0	+	-
P115	M	64	+	IV	1.2	19.0	-1.9	+	-
P124	M		+	IV	2.2	1570.2	9.5	+	-
P129	M	81	-	IV	2.5	4.6	1.0	+	-
P133	F	51	-	IV	0.8	30.4	-2.6	+	-
P146	F	82	-			3.9	2.3	+	-
P167	F	74	-		3.2	20.1	12.3	+	-
P168	F	74	-		3.2	5.7	4.8	+	-
P171	M	74	-	IV	0.55	29.5	1.7	+	-
P172	M	49	+	IV	1.12	132.3	2.8	+	-
P182	M	25	+	IV	2.25	ND	170.3	+	-
P183	F	46	+	III-IV	0.8	1452.4	15.3	+	-
P191	F	67	-	IV	4.23	74.6	2.0	+	-
P192	M	62	-	IV	1.6	32.6	-2.0	+	-
P194	M	45	+	II-III	0.8	8.2	1.2	+	-
Median						29.5	1.5	Present	Absent

Table 4-2A: Upregulation of *Tpr* (exon 21-22) and absence of *Tpr-MET* hybrid transcript in primary cutaneous melanoma. For *CDKN2a* status "-" or "+" denotes lower and higher expression in relation to normal skin, respectively. "M" equals marginal levels. For *18S* and *Tpr-MET* "-" or "+" denotes present or absent.

Melanoma metastasis	Gender	Age	*CDKN2a* status	*Tpr* (exon 21-22)	c-*MET*	*18s* control	*Tpr-MET* fusion
M53	M	68	-	1442.4	-3.7	+	-
M57	M	79	+	69.1	1.2	+	-
M61			-	45.5	-1.4	+	-
M62	M	79		10.2	1.9	+	-
M62	M	75	+	37.7	2.7	+	-
M63	M		-	65.0	-1.1	+	-
M66	F	69	+	103.0	4.0	+	-
M67	F	69	+	92.0	3.5	+	-
M68	F	69	+	780.4	15.2	+	-
M69	M	64	M	63.3	3.1	+	-
M70	M	69	+	-18.9	-18.9	+	-
M71	M	73	-	-14.1	-2.3	+	-
M73	F	69	+	321.6	6.3	+	-
M83	M	64	M	78.6	3.1	+	-
M114	F	91	+	89.7	2.3	+	-
Median				69.1	2.3	Present	Absent

Table 4-2B: Upregulation of *Tpr* (exon 21-22) and absence of *Tpr-MET* hybrid transcript in primary cutaneous melanoma. For *CDKN2a* status "-" or "+" denotes lower and higher expression in relation to normal skin, respectively. "M" equals marginal levels. For *18S* and *Tpr-MET* "-" or "+" denotes present or absent.

Thus, the expression of *Tpr* (exon 21-22 present only in *Tpr* and not in *Tpr-MET*) was confirmed but the junction of *Tpr* and *c-MET* in the *Tpr-MET* hybrid transcript was undetectable in all investigated primary melanoma and cutaneous melanoma metastasis specimens.

Additionally a spearman correlation was performed with expression levels *of c-MET* Vs *Tpr* levels in 29 melanoma patients. The analysis revealed a coefficient of determination or r^2 to be 0 .1880 indicating that the correlation is weak (Figure 4-9).

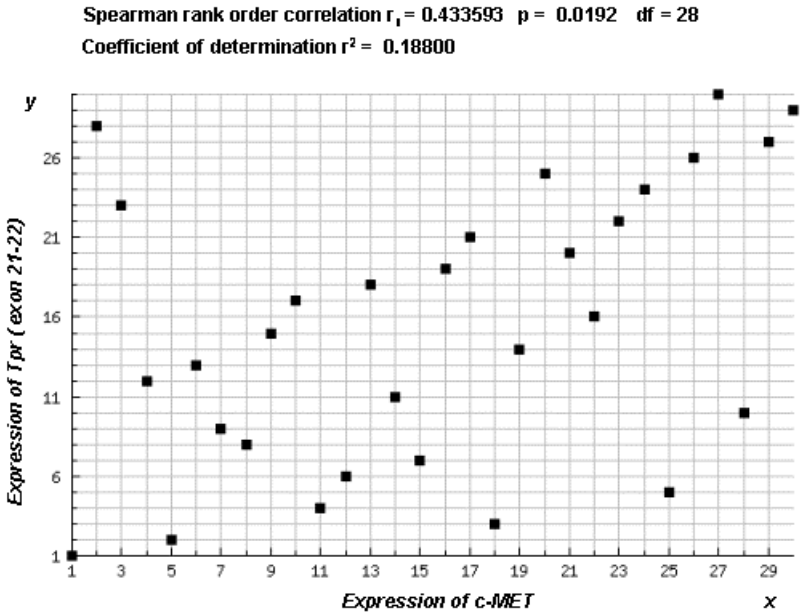


Figure 4-9: Spearman rank order correlation of *c-MET* Vs *Tpr* transcript levels in 30 melanoma patients. Coefficient of determination or $r^2 = (0.433)^2 = 0.1880$. We can therefore conclude that the correlation is weak with only 18.80% of the variation in *c-MET* explained by the variation in *Tpr* amongst melanoma patients.

Collectively these data not only provided a direct evidence that *Tpr* per se was being up regulated in cutaneous melanomas but also that there was no relation between over expression of *Tpr* and *c-MET* in melanoma

4.3 Patterns in the individual expression of the four validated genes across the 3 classes

Next the pattern in the expression of the four validated genes (*ASK*, *MCAM/MUC18*,*c-MET* and *Tpr*) across the 3 classes of specimens i.e., benign nevi, primary cutaneous melanoma and cutaneous melanoma metastasis were investigated. Sixty seven percent of primary cutaneous melanoma and 86.7% cutaneous melanoma metastases overexpressed *ASK/Dbf4* more than 2-fold higher as NHM (Table 4-3). Fourty four percent of primary cutaneous melanoma and 13.3% cutaneous melanoma metastases overexpressed *MCAM*/*MUC18* more than 2-fold higher as NHM (Table 4-3). Fourty four percent of primary cutaneous melanoma and 53.3% cutaneous melanoma metastases overexpressed *c-MET* more than 2-fold higher as NHM (Table 4-3). Eighty three percent of primary

cutaneous melanoma and 86.7% cutaneous melanoma metastases overexpressed *Tpr* more than 2-fold higher as NHM (Table 4-3).

Percentage of cutaneous melanoma samples > 2 fold to NHM

	Primary melanoma	Cutaneous melanoma metastasis
ASK	66.7	86.7
Tpr	83.3	86.7
c-MET	44.4	53.3
MCAM/MUC18	44.4	13.3

Table 4-3: Percentages of individual expression of the four validated genes in primary melanoima and cutaneous melanoma metastasis

All the four genes were upregulated in primary melanoma and melanoma metastasis in comparison with benign nevi. However the pattern of change between primary melanoma and cutaneous melanoma metastasis were different for the individual genes (Figure 4-10)

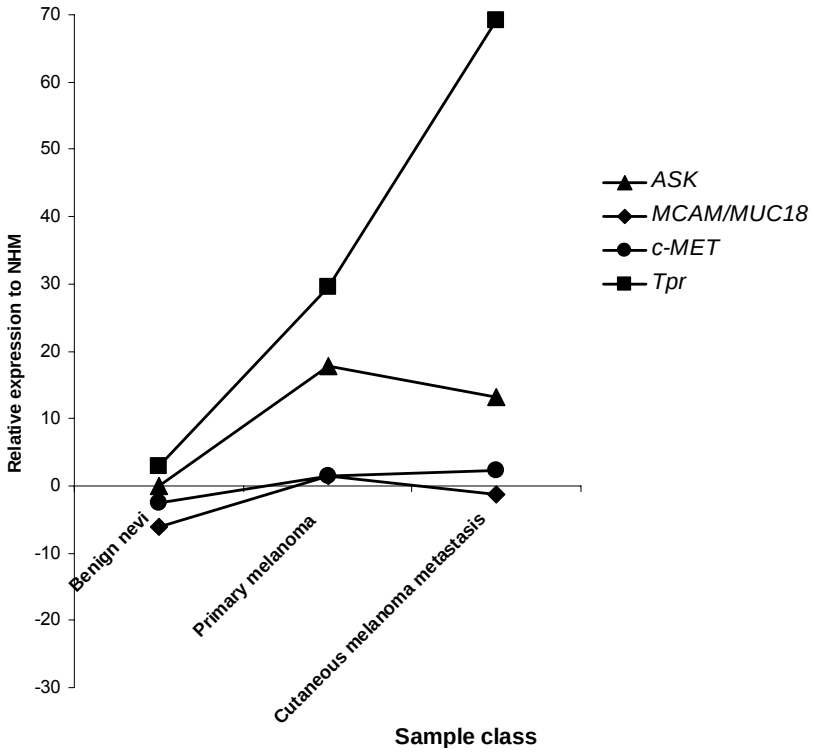


Figure 4-10: Patterns in the expression of 4 validated genes across the 3 classes

4.4 Comparative evaluation of stage-specific properties using a 4-gene signature as a novel molecular discriminator

Cancers in general and melanoma in particular is a multi-genic event. Combinatorial trends of relative gene expressions from multiple genes therefore have better potential to define stages or transitions more accurately than individual genes. Towards this the potential of the combined transcriptional information of the 4 confirmed genes to distinguish benign nevi and cutaneous malignant melanoma was investigated. To understand the combinatorial behavior/trend from these 4 genes, the summation of their relative expression was determined for all analyzed samples. This summation was then evaluated across the 3 classes of specimens i.e., benign nevi, primary cutaneous melanoma and cutaneous melanoma metastasis. It was observed that the combined relative expression of all 4 genes yielded a negative integer in 87.5% (7/8) of benign nevi and a positive integer in 100 % (13/13) and 86.7% (13/15) of primary cutaneous melanoma and cutaneous melanoma metastases, respectively (Figure 4-11A,B).

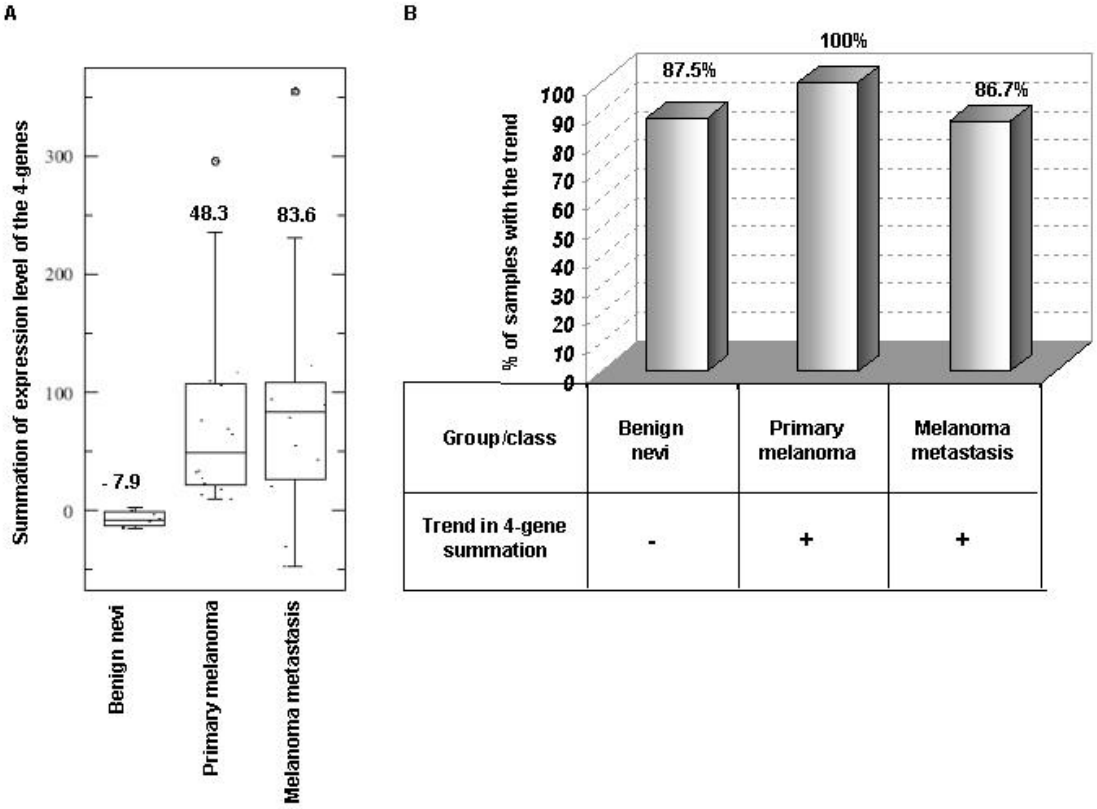


Figure 4-11: Comparitive evaluation of stage specific properties of a 4-gene signature as a molecular discriminant distinguishing cutaneous melanoma. (A) Box plot of summation of expression level of the 4-genes across benign nevi, primary melanoma and melanoma metastasis elucidating a differential trend (B) Bar graph showing percentage of samples in each class with the trend

Thus, the summation of the relative expression of the 4 genes *ASK/Dbf4 Tpr*, *c-MET* and *MCAM/MUC18* to obtain a 4-gene discriminatory signature not only served as a in distinguishing benign nevi to cutaneous melanoma but proved highly consistent within the respective classes.

4.5 Functional characterization of *ASK/Dbf4* upregulation in cutaneous melanoma

4.5.1 Western blot and immunohistochemistry analysis of ASK/Dbf4 protein expression in cutaneous melanoma development

To confirm the identified upregulation of ASK/Dbf4 at the protein level, ASK/Dbf4 expression was investigated in a series of normal skin, NHM and primary cutaneous melanoma, cutaneous melanoma metastasis and 4 melanoma cell lines (A375, BLM, MV3 and M13) by Western blot analysis of frozen specimens using anti-human ASK/Dbf4 polyclonal antibody raised against the amino acids 336-349 (C-FDFVEYEKDTPKKK-amide) in rabbit (BioGenes, Germany) in conjugation with carrier protein LPH. The pre-immune sera from the same animals were used as negative controls for Western blot analysis.

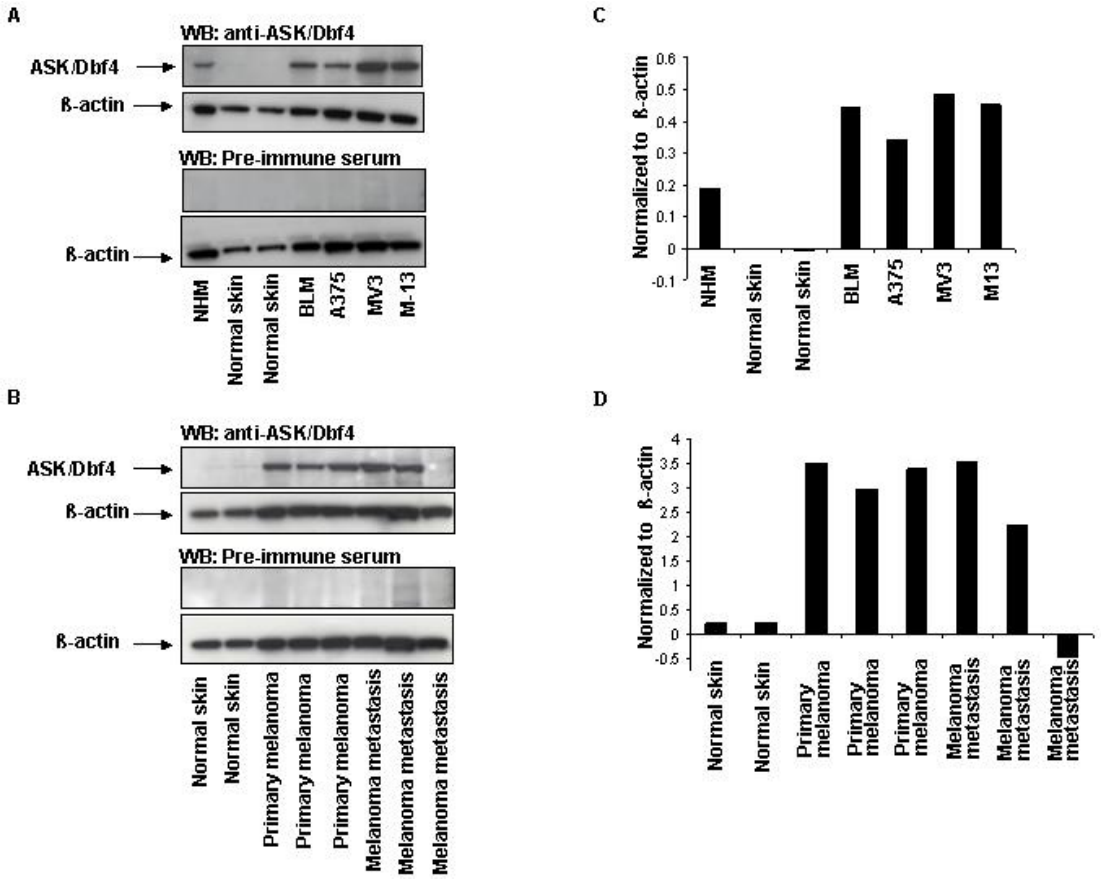


Figure 4-12: (A) Western blot analysis of ASK/Dbf4 in melanoma cell lines and NHM (B) Western blot analysis of ASK/Dbf4 in normal skin and several cutaneous melanoma samples (C) Quantitative representation of beta actin normalized levels of ASK/Dbf4 in melanoma cell lines and NHM (D) Quantitative representation of beta actin normalized levels of ASK/Dbf4 in normal skin and several cutaneous melanoma samples.

In NHM and normal skin, ASK/Dbf4 immunostaining was very weak, while it was strong in several melanoma cell lines and all melanoma specimens analyzed (Figure 4-12A, B, C, D). The pre-immune sera from the same animals were used as negative controls for Western blot analysis.

Immunonohistochemistry analysis of normal skin, benign nevi, primary melanoma, melanoma metastasis specimens and melanoma cell lines revealed that in normal skin and benign nevi ASK/Dbf4 immunostaining was weak, while it was strong in primary melanoma, melanoma metastasis (Figure 4-13) and melanoma cell lines (Figure 4-14).

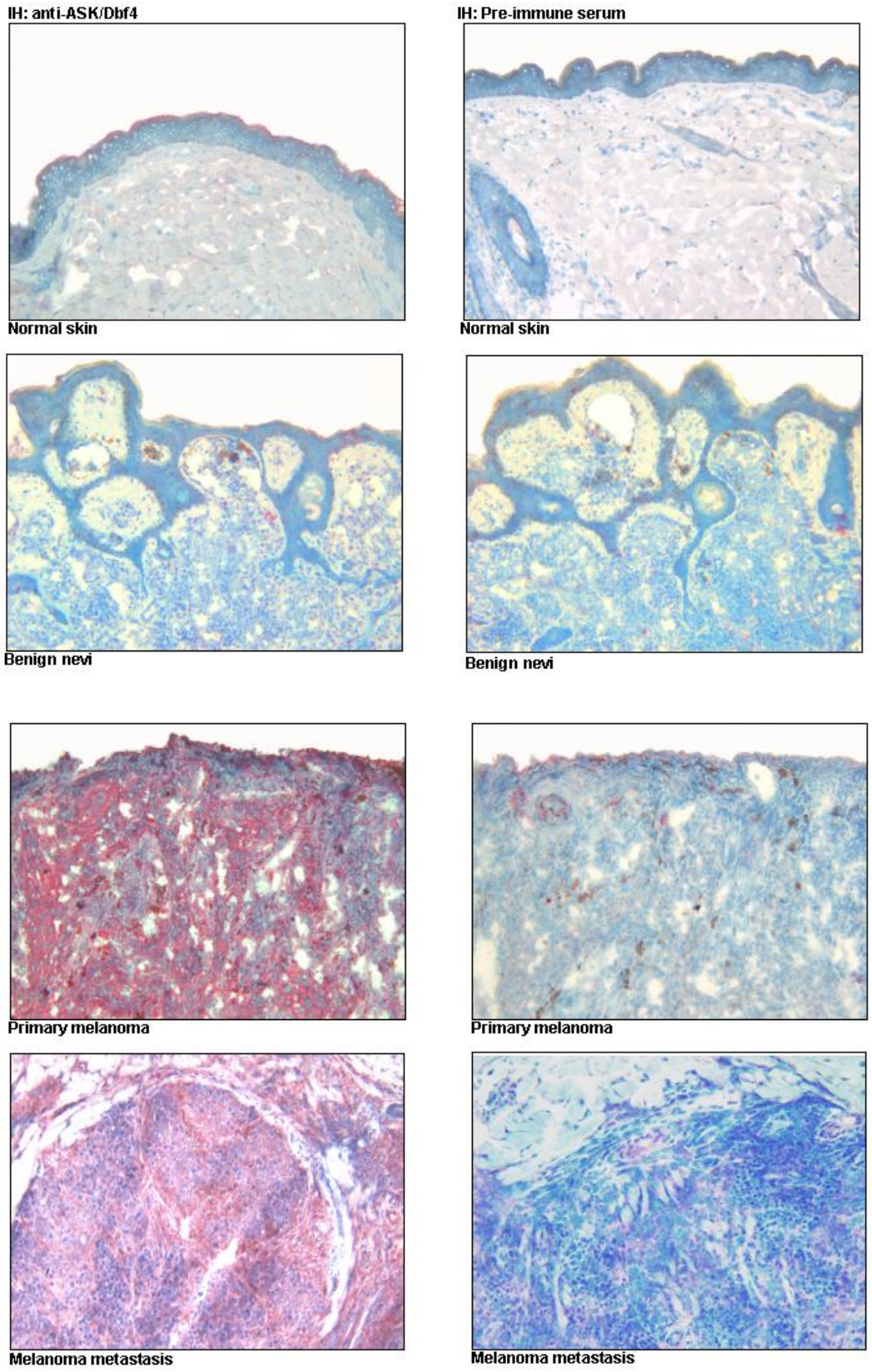


Figure 4-13: Representative images of immunohistochemical staining of ASK/Dbf4 in normal skin, benign nevi, primary melanoma and melanoma metastasis (magnification 200x).

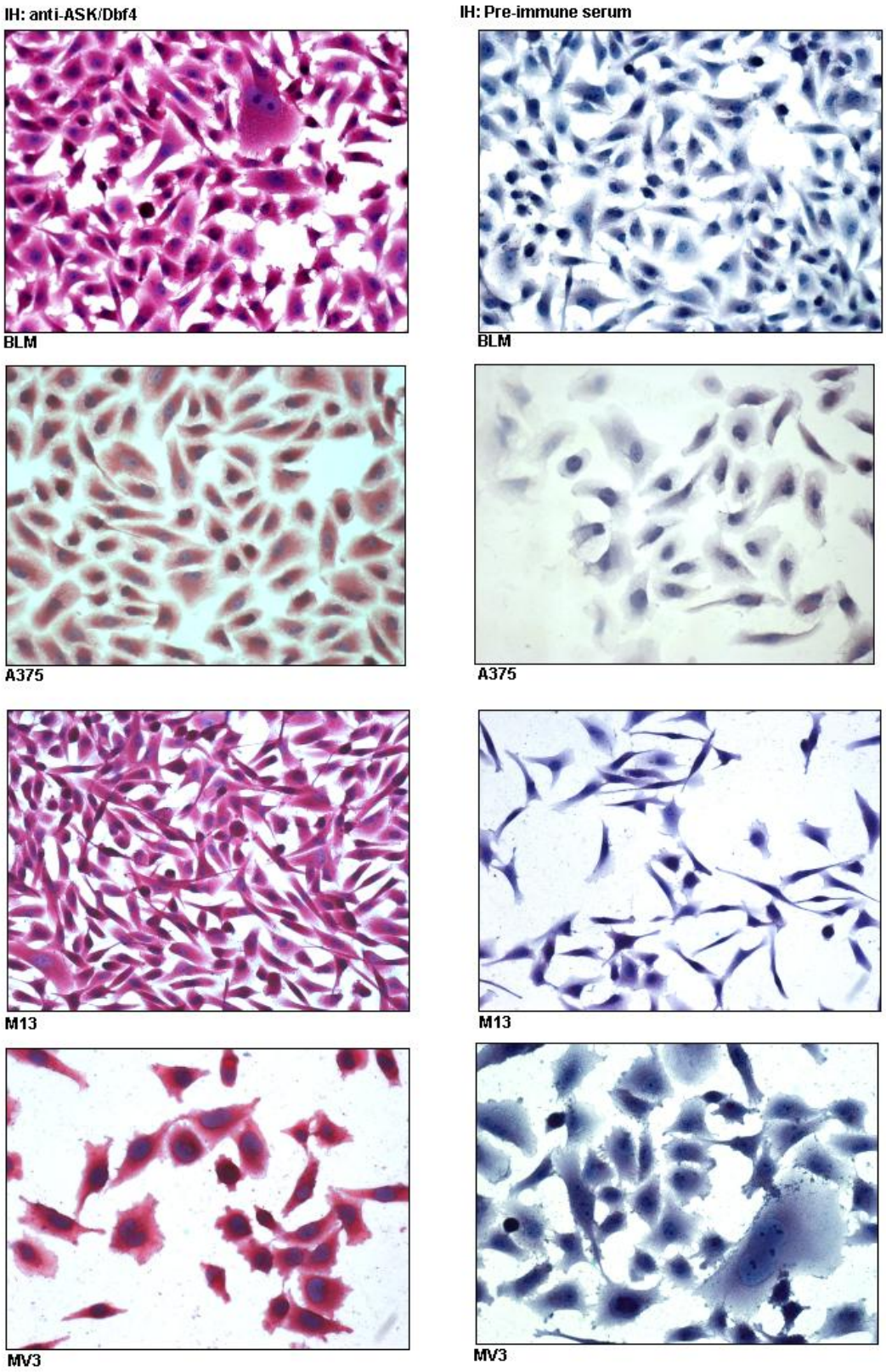
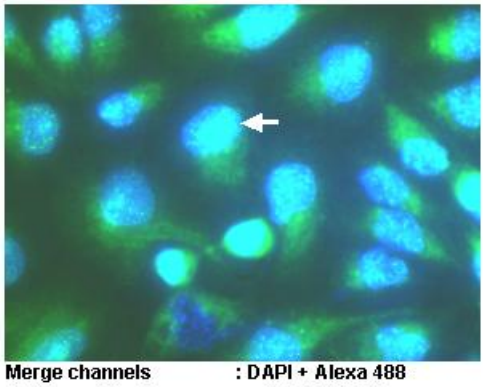


Figure 4-14: Representative images of immunohistochemical staining of ASK/Dbf4 in melanoma cell lines.

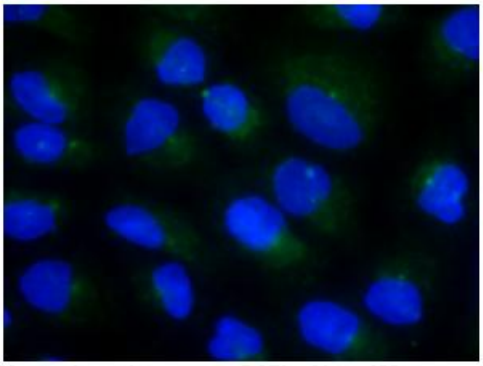
4.5.2 Association of ASK/Dbf4 and human Cdc7 in human melanoma

Cdc7 is a serine /threonine kinase which is required for the initiation of DNA replication, and is conserved from yeasts to human [Sclafani et al., 2000; Kim et al., 2002]. Although the level of Cdc7 is constant throughout the cell cycle, Cdc7 kinase activity peaks at late G1 through S phase [Yoon et al., 1993; Jackson et al., 1993]. This cyclic control, which is observed in yeasts and humans reflects changes in the abundance of its regulatory subunit ASK/Dbf4 [Cheng et al., 1999; Jiang et 1999., Oshira et al., 1999]. Sato *et al*. has described two stretches of amino acid sequences adjacent to the C-terminus of motif-C (NLS1: P346KKKRIK) and to the N-terminus of motif-M (NLS2: K201RVGSGAQKTRTGRLKK) of ASK/Dbf4 [Sato et al., 2003]. Therefore to further characterize the expression of *ASK*, its subcellular distribution in human melanoma cells was examined. Immunofluorescence with ASK-specific antibody in A375 melanoma cells detected the endogenous ASK protein predominantly in the nuclei as bright speckles (Figure 4-15A). Similarly, huCdc7 was detected in both cytoplasm and nuclei under the same conditions (Figure 4-15B). Further a co-immunofluorescence with both ASK-specific antibody and anti-Cdc7 antibody in A375 cells suggested that the huCdc7 and ASK proteins are co-localized in the nuclei of melanoma cells (Figure 4-15C), although it is not known whether they are present at the same subnuclear localization.

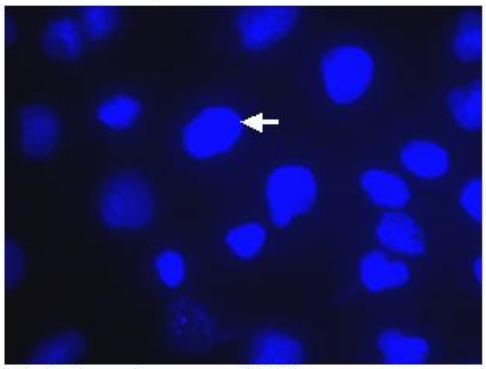
A



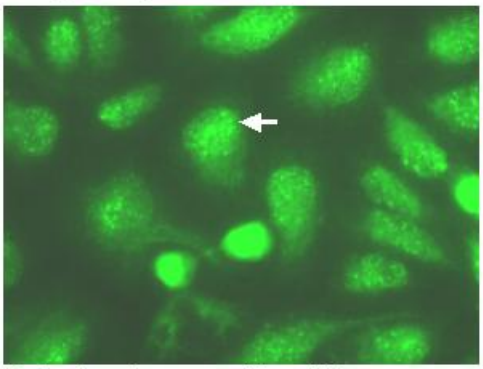
Merge channels : DAPI + Alexa 488
Primary antibody : anti- ASK/Dbf4



Merge channels : DAPI + Alexa 488
Primary antibody : Pre-immue serum

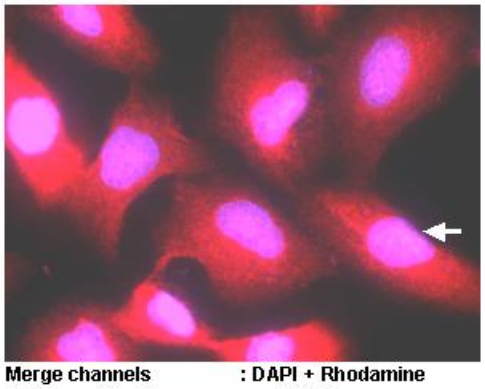


Single channel : DAPI
Primary antibody : anti-ASK/Dbf4

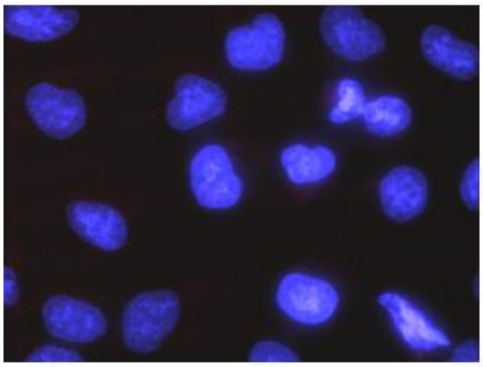


Single channel : Alexa 488
Primary antibody : anti-ASK/Dbf4

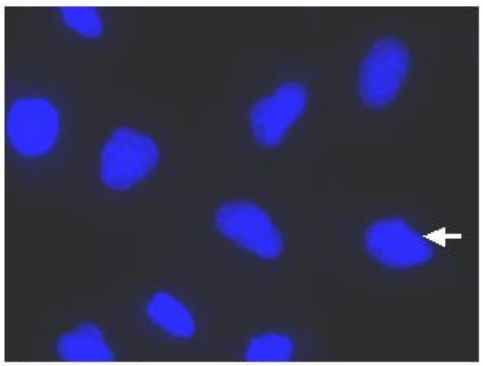
B



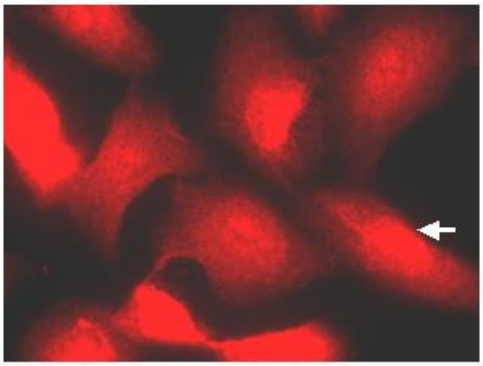
Merge channels : DAPI + Rhodamine
Primary antibody : anti-Cdc7



Merge channels : DAPI + Rhodamine
Primary antibody : none



Single channel : DAPI
Primary antibody : anti-Cdc7



Single channel : Rhodamine
Primary antibody : anti-Cdc7

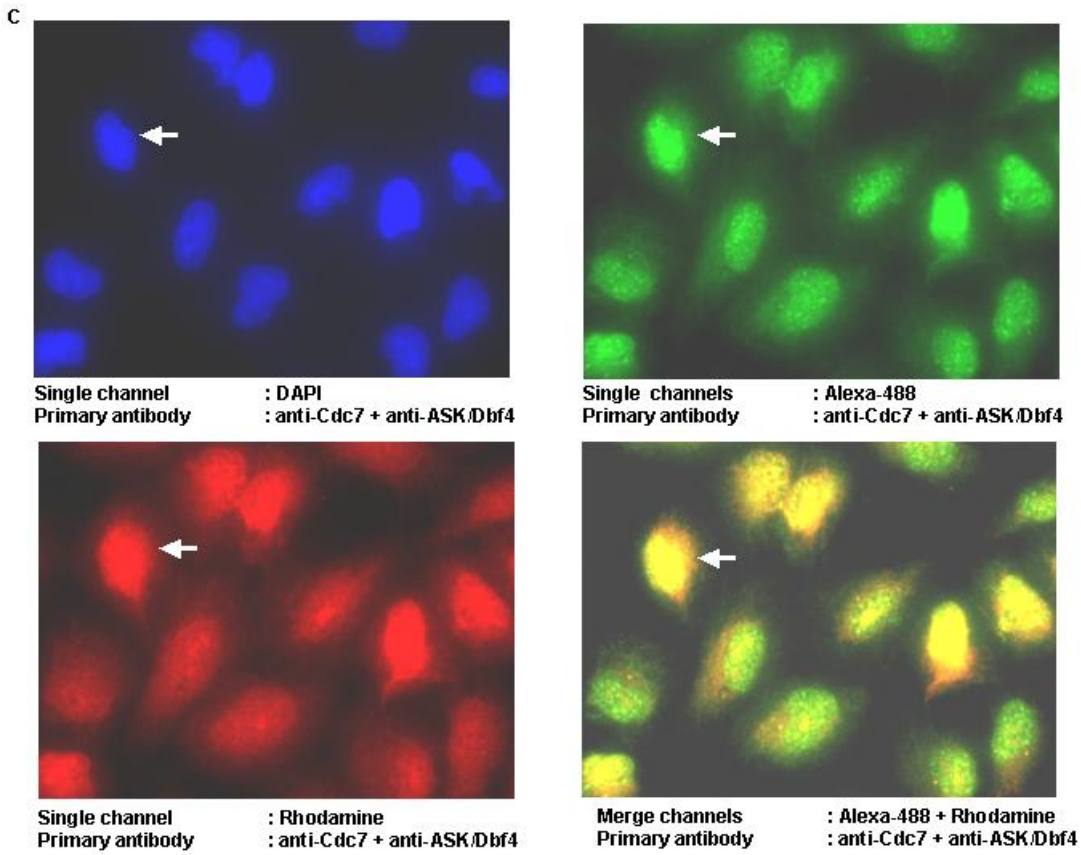


Figure 4-15: Immunoflourescence based subcellular localization of (A) ASK/Dbf4 (B) Cdc7 (C) ASK/Dbf4 and Cdc7 in A375 melanoma cells.

To further investigate whether the ASK/Dbf4 in cutaneous melanoma was indeed associated with human Cdc7, the expression of Cdc7 in the melanoma cell lines BLM, A375, MV3 and M-13 ws examined. While detectable in all melanoma cell lines studies, human Cdc7 was highly abundant in MV3 and M-13 cells (Figure 4-16A). Next, to examine the ability of ASK/Dbf4 to bind to human Cdc7, immunoprecipitation studies with anti-Cdc7 antibodies were performed from melanoma cell extracts, showing the presence of ASK/Dbf4 protein in human Cdc7 immunocomplexes in 3 (A375, MV3 and M-13) of a total of 4 investigated melanoma cell lines (Figure 4-16B).

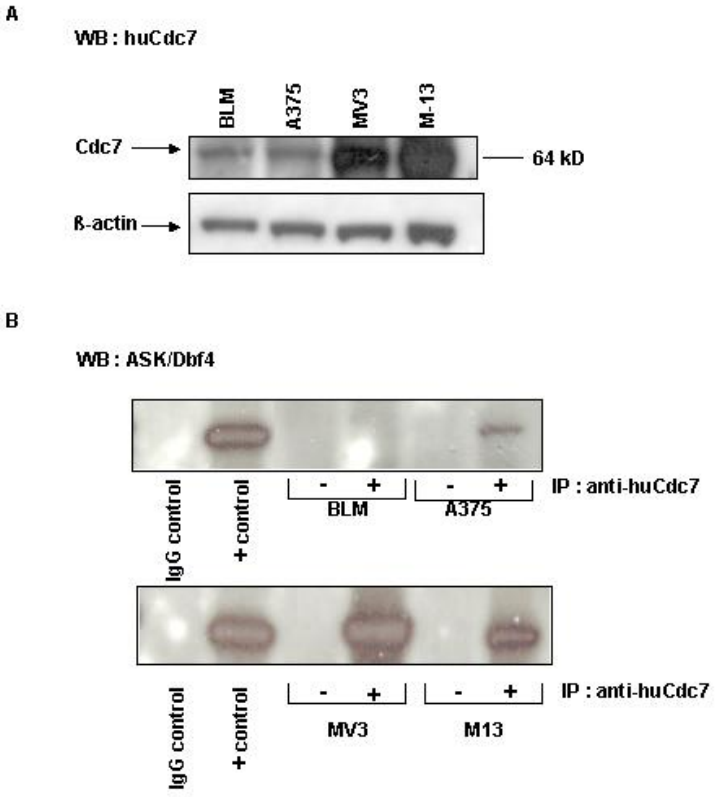


Figure 4-16: (A) Western blot analysis of human Cdc7 in melanoma cell lines. (B) Immunoprecipitation with anti-human Cdc7 followed by Western blot analysis of ASK/Dbf4 in several melanoma cell lines.

These results clearly demonstrate that human Cdc7 and ASK/Dbf4 form a functional complex in cutaneous melanoma.

4.5.3 SiRNA mediated knockdown of *ASK/Dbf4*

To elucidate the role of ASK/Dbf4 in human melanoma, the effect of reducing the level of ASK/Dbf4 in melanoma cell line MV3 was examined. Fifty thousand MV3 cells were synchronized by 24 hours serum deprivation and were transfected with 100 nM *ASK/Dbf4* specific siRNA (Ambion). For siRNA treatments greater than 24 hours, the treatment was repeated every 24 hours. Total proteins were extracted at 24, 48 and 72 hours and were subjected to Western blot analysis using the anti-ASK/Dbf4 rabbit polyclonal antibody. The protein level of ASK/Dbf4 was estimated as level of ASK/Dbf4 normalized to the respective beta actin (Figure 4-17A, B). The ASK/Dbf4 level after siRNA-*ASK* treatment at 24, 48 and 72 hours were compared to that of respective siRNA scrambled controls within each time point. At 48 hours ASK/Dbf4 protein levels decreased partially by 55.2% in siRNA-*ASK* treatment as compared to siRNA-scrambled. Where as by 72 hours there was a

near to complete loss of ASK/Dbf4 protein level (decreased by 83.7%) in siRNA-*ASK* treatment as compared to siRNA-scrambled (Figure 4-17A, B).

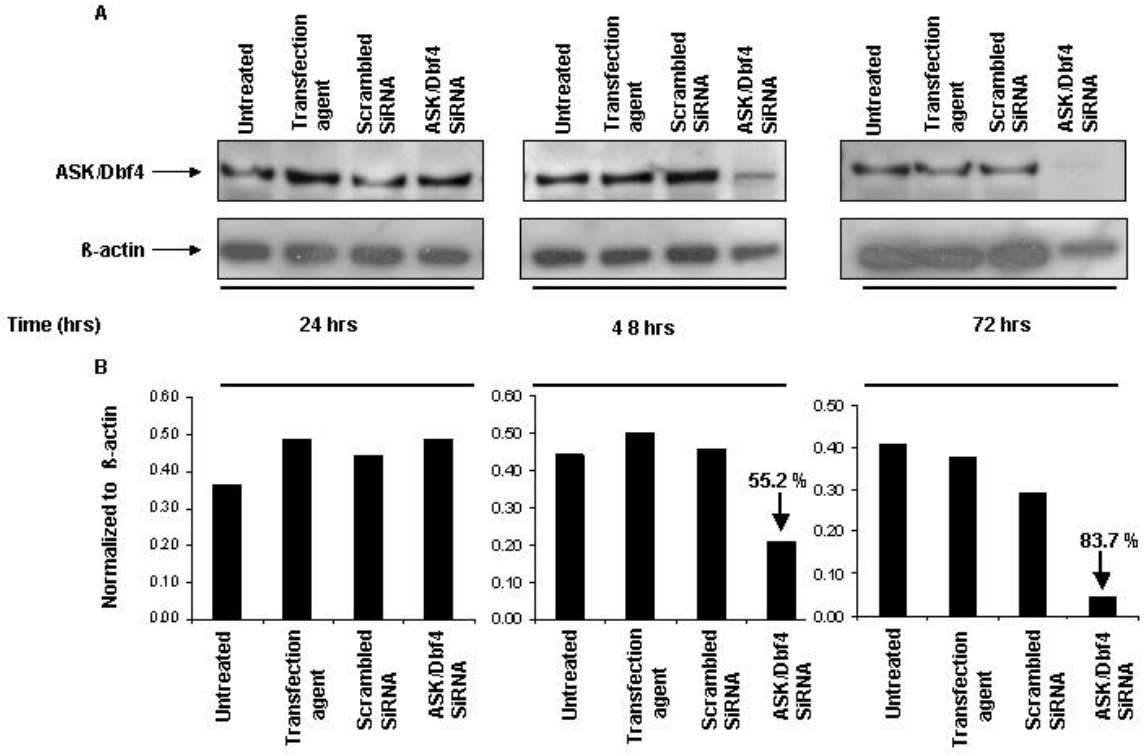
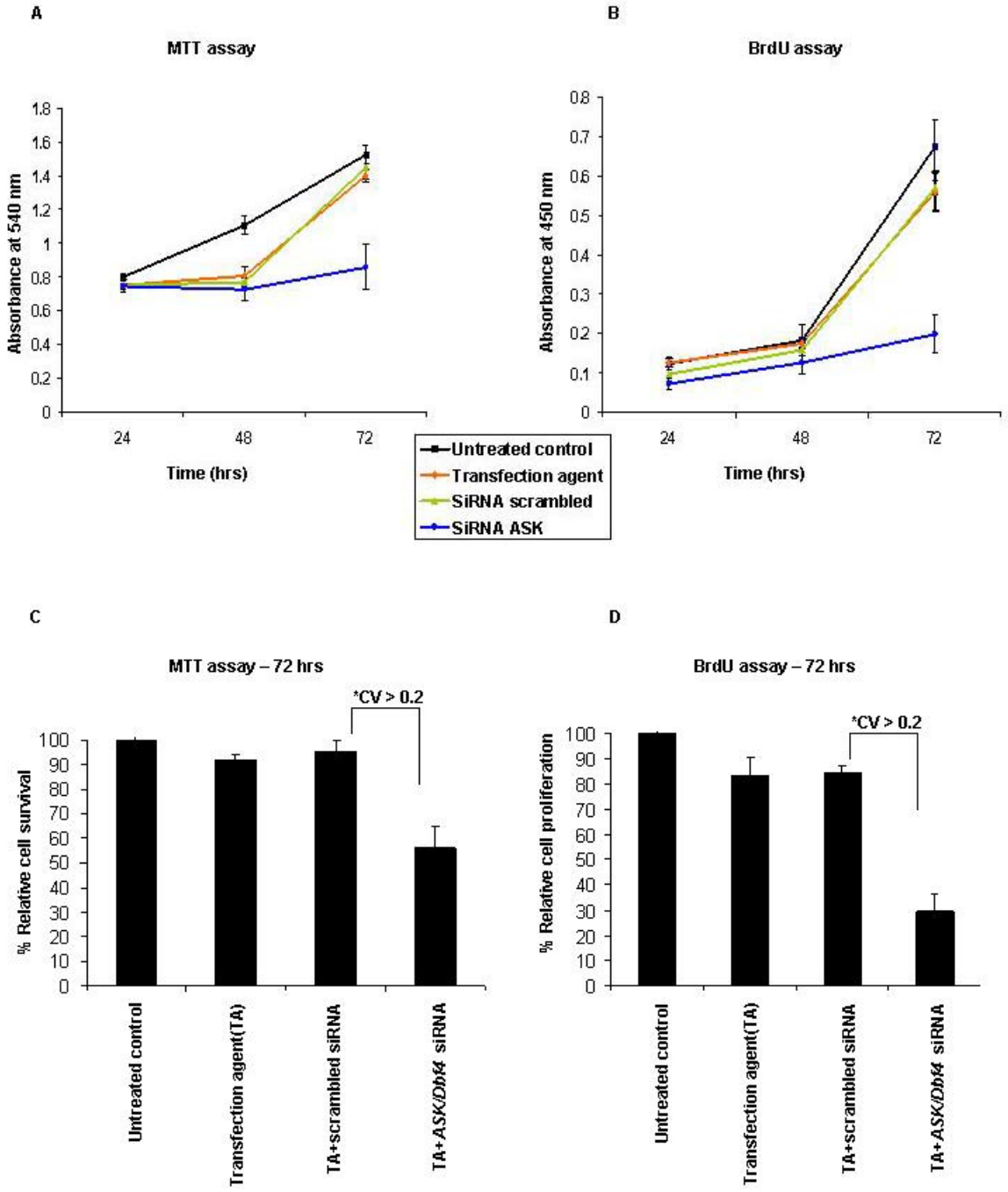


Figure 4-17: (A) SiRNA treatment of MV3 cells followed by Western blot analysis of ASK/Dbf4 (B) Quantitative representation of beta actin normalized levels of ASK/Dbf4 Western blot.

4.5.4 Measurement of cell viability, proliferation and apoptosis post siRNA mediated knockdown of ASK/Dbf4

Differentiating cause-and-effect of dysregulated genes is central in understanding their role in cancer. To elucidate a cause-and-effect association between loss of endogenous ASK/Dbf4 and melanoma cell growth, kinetics of (1) cell survival, as measured by conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (MTT assay) (2) cell proliferation/cells in S phase, as measured by (BrdU) 5-bromo2'-deoxy-uridine incorporation, at various times after SiRNA treatment, was investigated (Figure 4-18A, B). At 48 hrs when the decrease in ASK/Dbf4 was partial there was no significant impact on cell survival or proliferation. However the near to complete loss of ASK/Dbf4 at 72 hours in siRNA-*ASK* treated cells correlated with a significant reduction in both cell survival and cell proliferation without any significant change in apoptosis

(measured by annexin V positive cells) in comparison to the respective siRNA-scrambled control (Figure 4-18C, D, E). Therefore endogenous *ASK/Dbf4* confers proliferative advantage to melanoma cells. Collectively these results strongly indicate that the function of ASK/Dbf4, and therefore most likely that of the huCdc7-ASK/Dbf4 complex, is for cell proliferation and DNA synthesis in melanoma cells.



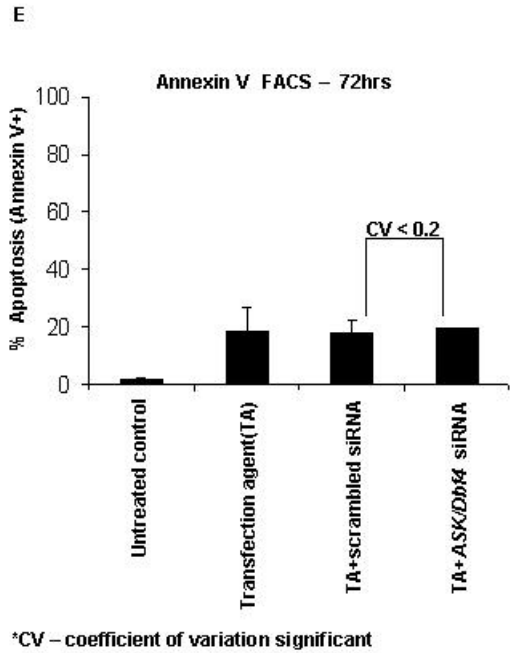


Figure 4-18: (A) Kinetics of cell survival post SiRNA treatment of MV3 melanoma cells (MTT assay at 24, 48 and 72 hours cells) (B) Kinetics of cell proliferation post SiRNA treatment of MV3 melanoma cells (Brdu incorporation at 24, 48 and 72 hours) (C). MTT assay 72 hours post SiRNA treatment of MV3 melanoma cells (D) Brdu assay 72 hours post SiRNA treatment of MV3 melanoma cells (D) Percentage of Annexin V positive cells by FACS, 72 hours post SiRNA treatment of MV3 melanoma cells.* Coefficient of variation(CV) significant.

4.5.5 E2F1 binds to the ASK/Dbf4 minimal promoter in melanoma through a non-canonical E2F1 site

Genes may be essential, but the pivotal role that cis- and trans-acting factors play in human disease is increasingly being recognized [Pennisi et al., 2004]. E2F1 is one trans-acting factor with tremendous molecular importance within the complex transcription regulation network. E2F1 can act both, as an oncogene and a tumor suppressor. A 63-base pair *ASK/Dbf4* promoter segment, called *ASK* minimal promoter (MP), serves as a template for E2F1 binding that is sufficient to stimulate growth (Figure 4-19). This *ASK* MP segment containing a Sp1 site, but no canonical E2F site, can be activated by ectopic E2F expression [Yamada et al., 2002].

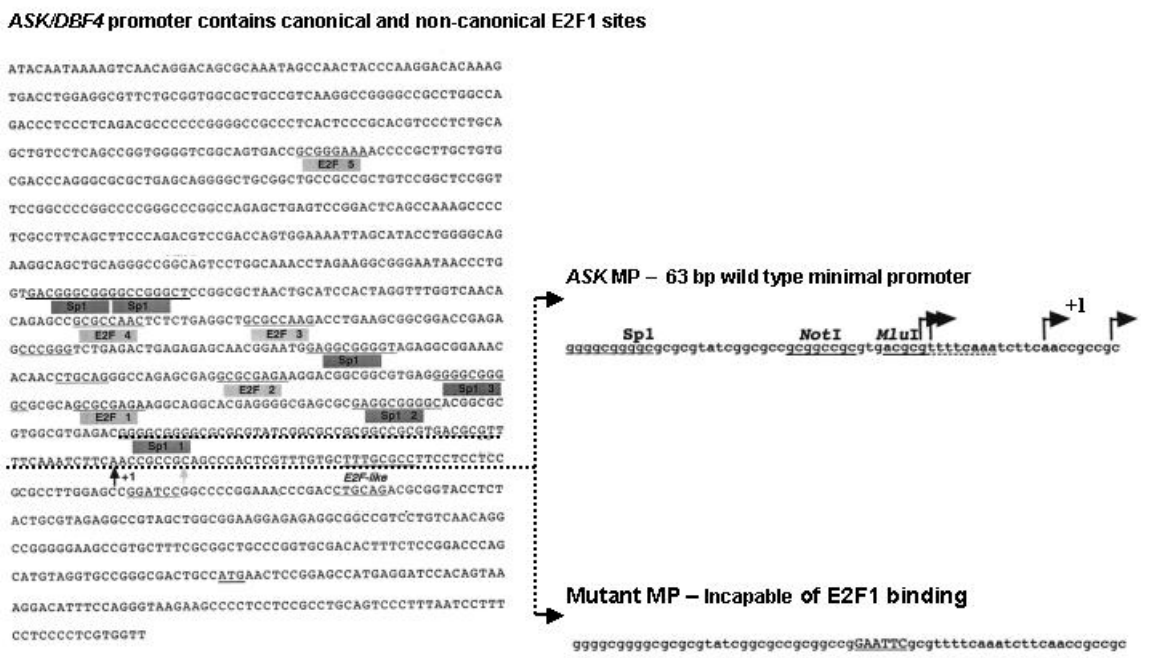


Figure 4-19: *ASK/Dbf4* promoter showing E2Fbinding sites described by [Yamada et al., 2002]. A 63 bp minimal promoter region (*ASK* MP) that binds to E2F1 is highlighted. Mutant MP refers to the same region sequence modified to abrogate E2F1 binding.

Therefore, to clarify if this transcriptional regulatory mechanism was conserved in melanoma and whether overexpression of *ASK/Dbf4* in melanoma could indeed be explained through this atypical mode of recognition, gel mobility shift assays (Figure 4-20A, B) were performed. *ASK* MP and its mutant derivative Mutant MP (MPM4), that is incapable of binding E2F1 for these studies [Yamada et al., 2002] were used. A specific band in melanoma cell lines BLM, M13, MV3, A375 and SK-MEL-28 was observed following the incubation of nuclear extracts with the ^{32}P-labeled *ASK* MP sequence (Figure 4-20A, lanes 1, 3, 5, 7 and 9), whereas a corresponding band was not detectable when the same nuclear extracts were incubated with the ^{32}P-labeled mutant MP (Figure 4-20A, lanes 2, 4, 6, 8 and 10). To further determine specificity, we incubated the nuclear extracts with an anti-E2F1 antibody prior to the addition of the *ASK* MP. The *ASK* MP band was abolished by the addition of unlabelled competitor (Figure 4-20A, lane 11). Addition of the anti-E2F1 antibody retarded the mobility of the observed band in BLM cells (Figure 4-20B, compare supershifted band in lane 1 to the non retarded bands in lanes 2 and 13), demonstrating the presence of E2F1 in this complex. This supershifted band was also observed in SK-MEL-28, MV3, M13 and A375

cells (Figure 4-20B, compare supershifted bands in lanes 5, 7, 9 and 11 to the non retarded band in lane 2). Also, the *ASK* MP band was abolished by the addition of unlabelled competitor (Figure 4-20B, lanes 2 and 15). These results indicated that protein-DNA complexes formed on the *ASK* MP contained the E2F1 protein. As cisplatin is frequently used in polychemotherapy of stage IV melanoma, we also investigated the effect of cisplatin-mediated abrogation of E2F1 binding to the *ASK* MP with regard to the transcription of *ASK/Dbf4*. Twenty-four hours following cisplatin treatment (100 µM final concentration) of BLM cells, E2F1 binding to the *ASK* MP was abrogated (Figure 4-20B, lanes 2 and 4). This was accompanied by a decrease in *ASK/Dbf4* transcript levels (% coefficient of variation = 22.7) (Figure 4-20C).

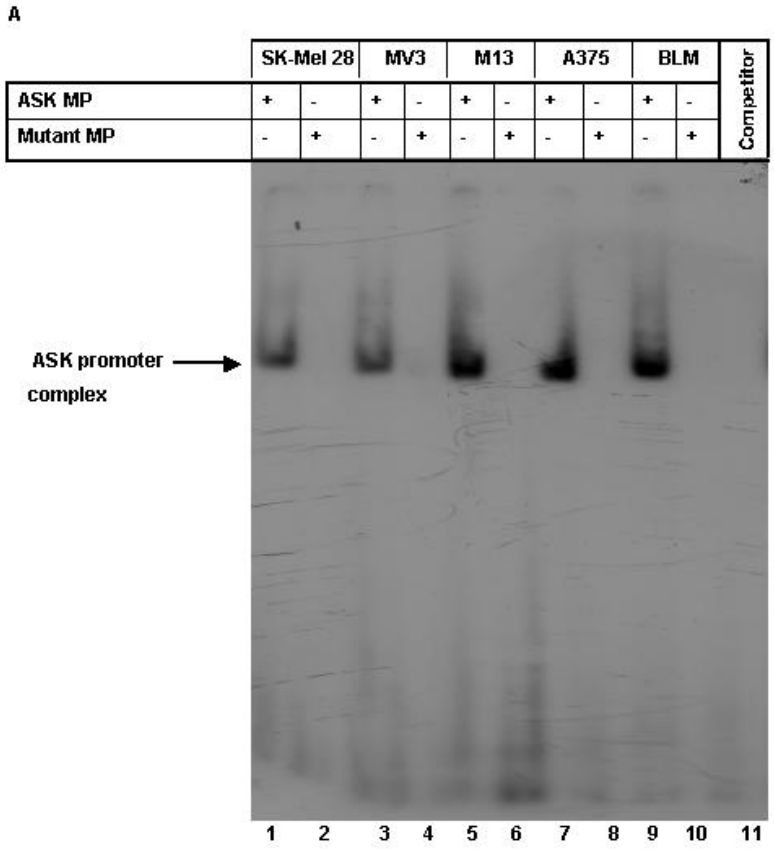
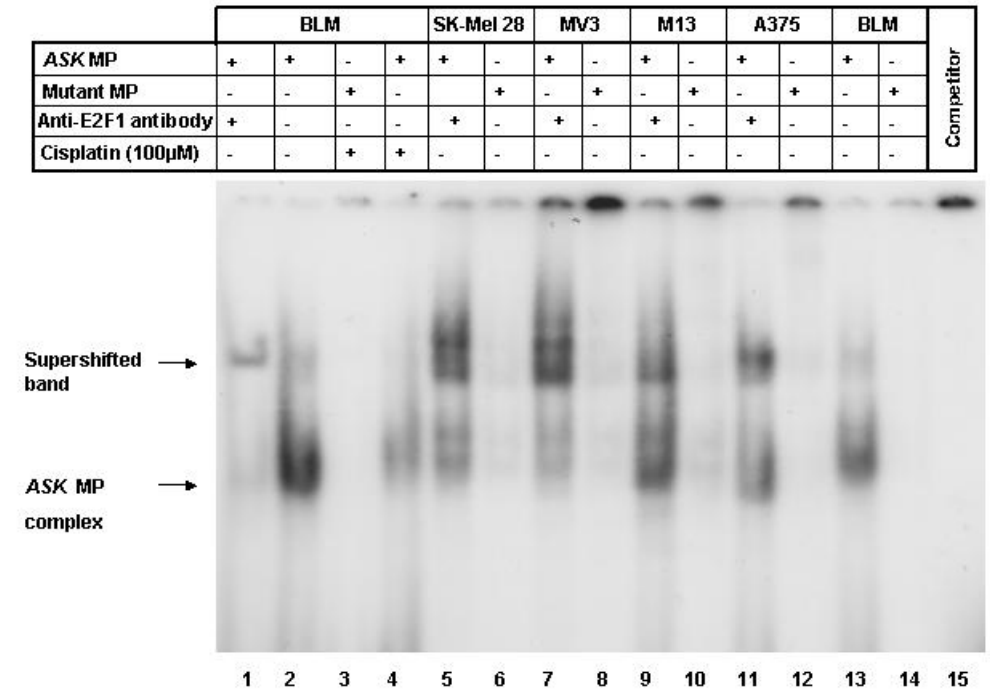


Figure 4-20: (A) Gel shift assay with ^{32}P-*ASK* MP and ^{32}P-Mutant MP incubated with nuclear extracts from melanoma cell lines SK-MEL-28, MV3, M13, A375 and BLM, respectively. Nuclear extracts incubated with the ^{32}P-labeled *ASK* MP sequence (lanes 1, 3, 5, 7 and 9) and ^{32}P-Mutant MP (lanes 2, 4, 6, 8 and 10).

B



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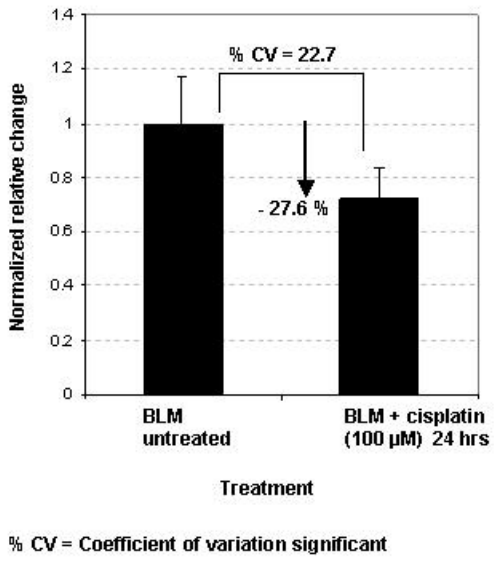

% CV = Coefficient of variation significant

Figure 4-20: (B) Supershift assay with anti-E2F1 antibody visualized supershifted bands in lanes 5, 7, 9 and 11 and the non retarded band in lane 2. Cisplatin-treatment abrogated E2F1 binding to the *ASK* MP (compare lanes 2 and 4). (C) qReal-time-PCR based detection of *ASK* transcription 24 hours post cisplatin treatment of the melanoma cell line BLM.

These data provided direct evidence that an atypical mode of recognition of *ASK* MP by E2F1 was present in melanoma cells. However, a complete or near

complete abrogation of E2F1 binding to the *ASK* MP in BLM cells corresponded to a transcriptional decrease of *ASK/Dbf4* of only 27.6 %, suggesting the presence/contribution of additional mechanisms.

4.5.6 *ASK/Dbf4* expression is refractory to the melanoma risk factor UVB

UV radiation is a major environmental carcinogen for melanoma. UV exposure at the B (UV-B; 260 to 320 nm) range is the most biologically relevant factor for sporadic melanoma and is responsible for the transformation of melanocytes into malignant melanoma along with other pre disposability factors such as genetic factors and the tumor microenvironment [Van Schanke et al., 2005, Hengge et al., 2006]. Therefore it was necessary to investigate if the melanoma risk factor UVB had a role in inducing the expression of *ASK/Dbf4* in NHM. NHM were treated with 200mJ/ cm^2 of UVB and protein extracts were prepared after 15 min, 1, 6 and 24 hours. As p53 is induced after UVB treatment, and increase in p53 level was used as a measure for the effectiveness of the UVB treatment .

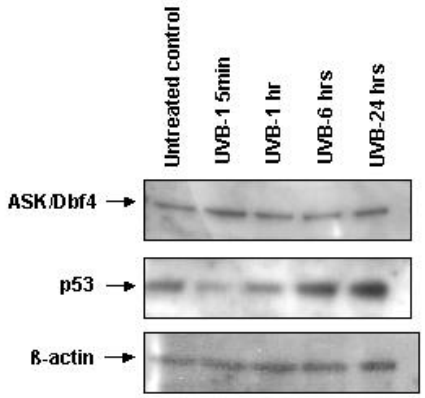

Figure 4-21: Western blot of p53 and ASK/Dbf4 after UVB 200mJ/cm^2 treatment of normal human melanocytes.

It was observed that ASK/Dbf4 remained constant even after 24 hours of UVB treatment (Figure 4-21) suggesting that the melanoma risk factor UVB may not have a role in inducing the expression of ASK/Dbf4.

5 DISCUSSION

5.1 Distinct gene expression profiles can distinguish benign to malignant transition

Melanoma is the most life-threatening neoplasm of the skin and its incidence and mortality have been increased worldwide [Geller et al., 2002; MacKie et al., 2002]. The development of melanoma is a classical example of a neoplasm progressing through discrete stages that have well-known clinical and histologic features. However, the key underlying molecular events have not been clearly elucidated, which may explain why no targeted therapy has been developed and why almost no clinical benefit from new therapies has been demonstrated in patients with melanoma since the late 1970s. Results of several expression studies designed to investigate the molecular mechanisms associated with melanoma progression have recently been reported [Bittner et al., 2000; Carr et al., 2003; Clark et al., 2000; Hoek et al., 2004; McDonald et al., 2004; Pavey et al., 2004; Wang et al., 2002: de Wit et al., 2005; Busam et al., 2005; Vogl et al., 2005; Haqq et al., 2005]; however, these studies used melanoma cell lines or metastatic tissue samples. No large gene expression profiling study of primary human melanomas from patients with long clinical follow-up has yet been reported because of limitations in the availability of primary human melanoma tissue. In fact, the entire primary tumor is conventionally fixed and embedded in paraffin for histologic evaluation, while frozen tissue is usually only available from metastatic melanomas. Thus, gene expression profiling data for human primary cutaneous melanomas are scarce, and data with prognostic implication are entirely lacking. Therefore in this study, the gene expression profiles of a series of benign congenital nevi, primary cutaneous melanomas, and cutaneous melanoma metastases were investigated using the human genome Affymetrix™ GeneChip system HG-U133A microarrays.

The study, yielded a general separation of three patterns (Pattern A, B, C) using unsupervised hierarchical clustering [Eisen et al., 1998] based on their expression profiles. Patterns A represented genes upregulated during benign to malignant transition and whose expression distinguished benign nevi from malignant melanomas. This included among others, activator of S-phase kinase (*ASK/Dbf4*) and translocated promoter region (*Tpr*), in addition to known markers such as

melanoma cell adhesion molecule (*MCAM*/*MUC18*) and hepatocyte growth factor receptor (*c-MET)*. *ASK*, a mammalian homolog of Dbf4 of budding yeast [Yoon et al., 1993], is an activation subunit of Cdc7 kinase and is required for S phase initiation in yeasts [Sclafani et al., 2000; Jackson et al., 1993].*Tpr* also known as translocated promoter region to activated *MET* oncogene is a coiled-coil protein localized to intranuclear filaments of the nuclear pore complex (NPC) mediating export of macromolecules out of the nucleus [Bangs et al., 1998].The c-*MET* proto-oncogene is the high affinity receptor for hepatocyte growth factor/scatter factor (HGF/SF), a multifunctional cytokine and is overexpressed in many types of tumors, including cutaneous melanoma and cancers of the kidney, thyroid, pancreas and osteosarcoma [Bottaro et al., 1991; Weidnee et al., 1993., Nambiar et al., 2005., McGill et al., 2006]. The melanoma-associated glycoprotein MUC18 / melanoma adhesion molecule (MCAM), a member of the immunoglobulin superfamily and homologue of several cell adhesion molecules, is associated with tumor progression and the development of metastasis in human melanoma [Sers et al., 1993; Sers et al., 1994]. Pattern B represented genes down regulated during benign to malignant transition and whose expression also distinguished benign nevi from malignant melanomas. This included among others spondin 1 (*SPON1*) and interleukin-18 (*IL-18*). SPON1*,* a secreted signaling molecule implicated in neuronal development and repair, binds to the conserved central extracellular domain of amyloid-beta precursor protein (APP) and inhibits beta-secretase cleavage of APP and FE65-dependent transactivation of the chromosome-remodeling factor TIP60 [Ho, 2004]. IL-18 is a new member of the IL-1 family that plays a central role in the immune response by acting on Th1 cell differentiation, cell-mediated cytotoxicity, and inflammation and it has been reported that constitutive or genetically manipulated production of IL-18 can lead to enhanced antitumor response and improved survival [Osaki, 1999; Tanaka, 2002]. Pattern C represented genes upregulated during benign to malignant transition along with a quantitative difference in the level of upregulation between primary melanoma and melanoma metastasis and hence not only distinguished primary tumors from nevi but also primary tumors from cutaneous metastases. LMNA is a structural protein component of the nuclear lamina that determines nuclear shape and size and in complex with LAP2α is capable of anchoring hypophosphorylated Rb in the nucleus [Markeiwicz, 2002].

Analysis of the collated data showed that genes reported to be overexpressed in this study were also overexpressed in earlier non-array based studies like melanoma antigen family A 12 *(MAGE A12),* melanoma antigen family A 5 *(MAGE A5)*, melanoma adhesion molecule *(MCAM/MUC18)*, MET proto-oncogene/hepatocyte growth factor receptor (*c-MET*) were also highly expressed in most of our primary melanoma and metastasis patients, but were significantly lower in most congenital nevi (Figure 4-1; Table 4-1A). Likewise, some of the upregulated genes such as *CXCL1* (melanoma growth-stimulating activity), minichromosome maintenance deficient 4 (*MCM4*), cell division cycle 2 (*Cdc2*), reticulocalbin 2 (*RCN2*), dihydrofolate reductase (*DHFR*), cathepsin B (*CTSB*) were found to distinguish melanoma from benign nevi in our study (Table 4-1A) and another recent cDNA array-based study [Haqq et al., 2005]. In addition, the PCA based evaluation of the data revealed that the molecular changes between benign nevi and primary cutaneous melanoma were distinct and discernible. In contrast between primary cutaneous melanoma and metastasis our data revealed no absolute distinction. It is likely that a considerable overlap of expressed genes between cutaneous melanoma and metastasis as seen in this study reflects the close biological relationship between them by describing gradual changes. Nevertheless genes such as *NXP2* and *LMNA*, were differentially expressed in primary melanoma *vs* metastasis. These findings suggest that these might be more effective therapeutic targets in metastasis than in primary melanoma

In summary through this study several post genomic candidates for generating profiles have became available to generate and optimize a prediction models for diagnostic purposes.

5.2 A novel qRT-PCR based 4-gene signature discriminate benign nevi from malignant melanoma

High thoroughput strategies like the microarray platform are increasingly being used to explore biological causes as well as to diagnose and categorize similar diseases. Yet, accurate diagnostic and prognostic markers have not been defined and the confirmation of genetic patterns is still pending. This is due to several

reasons. First, the data generated using multiple commercial microarrays showed considerable divergence [Tan et al., 2003]**.** Second, reproducibility for most platforms within a given laboratory was typically good, but reproducibility between platforms and across laboratories was generally poor [Bammler et al., 2005]. Despite emerging standards like Minimum Information About a Microarray Experiment (MIAME) to unify the technology at various levels, significant discordance remains [Marshall et al., 2003]. Third, there are several ways of data analysis that may influence the correlation between platforms [Jarvinen et al., 2006]. Eventhough, dendrograms generated from microarray results have been used to define signatures, these results are generally obtained from relatively small and selected groups of patients, thereby requiring confirmation in an independent series of samples. Even in the case of larger series, experts agree that profiles must be validated in independent series before their widespread use [Dobbin et al., 2005; Michiels et al, 2005]. Fourth, the predictive value of these profiles should be further improved before they are can be accepted as cancer subtype signatures and classifiers. This is because these profiles are mainly generated by cluster analysis, and hence are unstable i.e, there is no operational definition or no prespecified rules or cutoffs for specific gene profiles. Finally, even tumors with similar histological background may acquire completely different clinical courses based on their particular expression profiles. For these reasons, it may be more meaningful and reliable to generate patterns from qRT-PCR confirmed lists of selected genes.

Using qRT-PCR validated signature, has some advantages. qRT-PCR as a technique requires smaller quantities of valuable tumor tissue and provides accurate, reproducible, and quantitative results [Endoh et al., 2004; Lossos et al., 2004]. Recent reports suggest the possibility to quantify gene expression with the use of sections of routinely prepared blocks of fixed, paraffin-embedded tumor tissue, which represent the most abundant source of tissue specimens associated with clinical records [Paik et al., 2004; Ma et al., 2004]. Second, because the gene expressiom profiles are generated from previous original microarray study, the possibility of an association by chance is low. Espinosa *et al.,* successfully used qRT-PCR to reproduce the results obtained with a 70-gene prognostic signature in patients with early breast cancer [Espinosa et al., 2005] based on an original gene signature described using microarray technology [Vijver et al., 2002], In Europe,

researchers have already investigated the usefulness of two different groups, or "panels", of genes in studies on early stage breast tumors from node negative patients. One is a 70-gene panel, and another is a 76-gene panel [Van t'Veer et al., 2002; Wang et al., 2005]. Only three genes are common to both panels, while all the others are unique to one test or the other. In spite of this difference, both panels have yielded results that appear to be promising [Foekens et al., 2006; Paik et al., 2004], and a commercial test based on the 70-gene panel is already available in the United States under the name Mammaprint®. Gene expression profiling using RT-PCR has enabled studies in the United States to investigate several other panels of genes that produce too much or too little protein in early stage, node negative, estrogen-receptor positive breast cancer cells. One of these is a 21-gene panel known commercially as Oncotype DX [Paik et al., 2004]. Similarly a qRT-PCR validated 5 gene prediction model is now available for non-small cell lung cancer [Chen et al., 2007]. However similar qRT-PCR based multi-gene prediction models remain un attempted in cutaneous melanoma.

Therefore this study undertook to generate patterns from qRT-PCR confirmed lists of selected genes. Based on the qRT-PCR data obtained from the present study the transcriptional information of the 4 validated genes (*ASK/Dbf4 Tpr*, *c-MET* and *MCAM/MUC18*) could be combined to distinguish benign nevi from cutaneous malignant melanoma. Interestingly it has been observed that the combined relative expression of all 4 genes yielded a negative integer in benign nevi and a positive integer in primary cutaneous melanoma and cutaneous melanoma metastases, respectively. This 4-gene discriminatory signature not only served in distinguishing benign nevi and cutaneous melanoma but also proved to be highly consistent within the respective classes. The potential of this signature was further strengthened from the observation that benign nevus sample N134f, the only specimen that revealed a positive integer among all analyzed nevi, interestingly was obtained from a patient who also suffered from cerebral melanoma metastasis.

In addition, we also observed that these 4 genes were over expressed in most melanoma cell lines in comparison to NHM and hence these cell lines can serve as ideal invitro models for further studies on these candidate genes. Also NHK/NHM ratios for these genes were low, suggesting that melanocytes were the major source of these transcripts within normal skin.

5.3 Absence of *Tpr-MET* fusion transcript and independent upregulation of *Tpr* and *c-MET* in cutaneous melanoma

Tpr also known as translocated promoter region to activated *MET* oncogene is located on chromosome 1q25, contains 51-52 exons spanning about 63 kb and encodes a 2349 amino acid, coiled-coil protein localized to intranuclear filaments of the nuclear pore complex (NPC). Ectopic expression of *Tpr* caused a dramatic accumulation of poly $(A)^+$ RNA within the nucleus as *Tpr* is tethered to intranuclear filaments of the NPC by its coiled-coil domain leaving the acidic COOH terminus free to interact with soluble transport factors and to mediate export of macromolecules out of the nucleus [Bangs et al., 1998]. Initially however the *Tpr* gene was described not as an independent gene but as a fusion partner with *c-MET*, a proto- oncogene through a rearrangement in a cell line rendered tumorigenic N-methyl-N-prime-nitrosoguanidine (MNNG). This *Tpr-MET* rearrangement (*Tpr-MET* hybrid) produces a *Tpr-MET* transcript translated in to a Tpr-MET oncoprotein. This fusion protein contains the constitutive promoter and first 424 coding nucleotides (142 amino acids) of Tpr, and the tyrosine kinase domain of the *c-MET* protooncogene. As a consequence of this rearrangement the Tpr-MET fusion oncoprotein is localized to the cytoplasm and forms a constitutively activated kinase initially reported in gastric cancer and its precursor lesions [Soman et al., 1991]. Hence the presence of *Tpr-MET* fusion transcripts in addition to only *Tpr* or *c-MET* can be used as a direct indicator for the existence of Tpr mediated activation of c-MET. Unlike gastric cancer, in melanoma no previous study had investigated the presence of the *Tpr-MET* hybrid transcripts, as a unique *Tpr* overexpression has not been reported to date, although c-MET activation through autocrine or paracrine loops has been known [Otsuka et al., 1998; Yu et al., 2002]. Results obtained from this present study revealed that: - first, the *Tp*r exon 21-22 present only in *Tpr* and not in *Tpr-MET* was detectable but the junction of *Tpr* and *c-MET* in the *Tpr-MET* hybrid transcript was undetectable in all melanoma specimens, Second *Tpr* and *c-MET* were both upregulated between nevi and melanoma in different ranges of expression. Finally the Spearman correlation analysis between expression of *Tpr* and *c-MET* revealed a coefficient of determination (r^2) to be 0.1880 indicating that the correlation is weak with only 18.89% of the variation in *c-Met* explained by the variation in *Tpr* amongst cutaneous melanoma patients. Collectively these data provided direct

evidence for the absence of *Tpr-MET* fusion transcript and independent upregulation of *Tpr* and *c-MET* in cutaneous melanoma.

5.4 *ASK/Dbf4*, one of the components of the 4-gene signature is overexpressed in cutaneous melanoma both at mRNA and protein level.

The accumulation of multiple genetic alterations, dysregulation of various cellular molecules and alterations in their functional role underlies the complexity of cancer. Even though, multi-gene signatures like the one described in this study (4-gene signature with *ASK/Dbf4*, *Tpr*, *c-MET MCAM/MUC18*) is useful to discriminate benign to malignant phases in models of tumor development and progression, subsequently the question needs to be addressed whether individual components in such emerging signatures of dysregulated genes are the cause or consequence of the neoplastic state. Differentiating such cause-effect relationships may not have a direct impact on using these signatures for diagnostic purposes but would have profound implications in understanding the biological mechanism of melanoma and for evaluating potential targets for specific therapy. The study therefore further focused on functional characterization of the role of *ASK/Dbf4*, one of the components in the 4-gene signature. Towards this up-regulation of ASK/Dbf4 was initially confirmed at protein level by Western blot analysis to be upregulated in primary melanoma, cutaneous melanoma metastasis and melanoma cell lines in comparison to normal skin and normal human melanocytes. Subsequently immunohistochemical staining revealed the protein to be specifically over expressed in melanoma cells over normal skin and benign nevi.

5.5 *ASK/Dbf4* is a novel cell survival gene in melanoma conferring proliferative advantage

Eukaryotic chromosomal replication is a tightly regulated process, which must be strictly coordinated with other cell-cycle events, such as cell division, to ensure that the daughter cells maintain the same ploidy as the parental cell. Replication of each segment of DNA occurs once and once only during the S phase of the cell cycle. Genetic and biochemical evidence has now emerged to support a model in

which the initiation of DNA replication is regulated through two mutually exclusive cellular states [Dutta and Bell et al., 1997]. The first occurs in the G_1 phase of the cell cycle and involves the regulated assembly of prereplication complexes (pre-RCs) at DNA replication origins. Assembly of the pre-RCs is sequential, with the origin recognition complex (ORC) recruiting the Cdc6 protein, which in turn promotes loading of minichromosome maintenance (MCM) proteins [Coleman et al., 1996; Donovan et al., 1997; Tanaka et al., 1997]. Although necessary, the assembly of pre-RCs during G_1 is not sufficient to initiate DNA replication. During the G_1−S transition, cells must enter the second state in which DNA replication can be initiated. This process requires the activation of two S-phase-promoting kinases: cyclin-dependent kinases, Cdks, and the ASK/Dbf4-dependent kinase, Cdc7 [Stillman et al., 1996; Dutta and Bell et al., 1997]. This activation of Cdc7 kinase activity is specifically achieved by its own regulatory subunit, the ASK/Dbf4 protein [Sclafani et al., 2000]. However such functional roles of cellular molecules implied from lower organisms or a generalized mammalian model may not always represent its functional role within the perspective of tumor development and progression For example the oncogenic activity of cyclin E does not exclusively rely on its ability as a positive regulator of G_1 progression rather, cyclin E harbors other functions, independent of Cdk2 activation and $p27^{Kip1}$ binding, that contribute significantly to its oncogenic activity [Geisen et al., 2002]. Recent data obtained in cyclin E1 and cyclin E2 knockout mice [Geng et al., 2003] indicate that cyclin E is dispensable for proliferation of normal cells and that LMW cyclin E forms in cutaneous melanoma functions to generate angiogenic tumors with prominent perineural invasion [Bales et al., 2005]. The biological significance of these findings together with absence of any previous report of upregulation of *ASK/Dbf4* in melanoma prompted the study to focus whether the pro-proliferative role of *ASK /Dbf4* inferred from lower organisms and general mammalian models [Yoon et al., 1993; Jackson et al., 1993] is valid within the context of cutaneous melanoma development. Therefore the ability of ASK/Dbf4 to associate with human Cdc7 was investigated in melanoma cells. Immunofluorescence based experiments revealed ASK/Dbf4 and Cdc7 were co-localized in the nuclei of melanoma cells. Subsequently immunoprecipitation studies in melanoma cells demonstrated the presence of ASK/Dbf4 protein in human Cdc7 immunocomplexes. Hence the

observed overexpression of ASK/Dbf4 in cutaneous melanoma was indeed for associating with human Cdc7

A variety of techniques can be used to study gene function. One such class of technique being loss-of-function (LOF) studies such as gene disruption, antisense, small-molecule inhibitors, antibody microinjection, dominant-negative mutants and ribozymes. The most recent addition to the arsenal of loss-of-function (LOF) techniques is RNA interference (RNAi), which allows the selective ablation of gene expression in almost any cellular setting that is amenable to the delivery or expression of small interfering RNAs (siRNAs). RNA interference (RNAi) has emerged as one of the standard techniques to study gene function in diverse experimental systems. The Introduction of double-stranded RNA (dsRNA) into a cell decreases the level of the complementary mRNAs producing a knockdown of the corresponding protein. The current model of the RNAi mechanism proposes that the silencing "trigger" is processed by Dicer into small RNAs of 21-22 nucleotides in length. These become incorporated into an RNA-induced silencing complex with endonuclease activity (RISC), which, in turn, identifies and cleaves homologous mRNAs [Zamore et al., 2001; Hannon et al., 2002]. Thus in order to investigate the cause-effect relationship of *ASK/Dbf4* in cutaneous melanoma we employed repetitive siRNA-mediated specific depletion of *ASK/Dbf4* in melanoma cell line MV3 for 24, 48 and 72 hours and measured cell survival, proliferation and apoptosis using MTT, BrdU assays and annexin-V based FACS respectively. The specific depletion of ASK/Dbf4 protein in MV3 cells at 72 hours post siRNA treatment was found to be associated with a significant retadation in cell survival and proliferation without any significant difference in apoptosis. These results demonstrated that the essential role of *ASK/Dbf4* in cell proliferation and DNA synthesis is conserved in cutaneous melanoma cells Interestingly, it was also observed that a near to complete loss of ASK/Dbf4 protein at 72 hours post siRNA-ASK treatment was associated with only 40.7% and 65% decrease in cell survival and proliferation respectively as compared to siRNA-scrambled. This inability to produce a complete loss of cell survival and proliferation following near to complete loss of ASK/Dbf4 protein may be explained by the redundancy of pro-proliferative pathways in melanoma. Alternatively it is also possible that in the absence of ASK/Dbf4, its homologue Drf1/ASKL1 may have a role in driving basal cell proliferation [Yoshizawa-Sugata et al., 2005].

In addition to its role in activating Cdc7 in mammalian cells, ASK/Dbf4 has also been shown to interact with the tumor suppressor menin in multiple endocrine neoplasia type I (MEN1), a hereditary tumor syndrome [Schnepp et al., 2004]. Recently Tenca *et al* demonstrated that genotoxic agents do not down regulate Cdc7·ASK/Dbf4 complexes. Thus suggesting that they are correctly positioned to regulate proteins required for replication of the genome. However sustained inhibition of Cdc7 in the presence of genotoxic drugs increased cell death supporting the notion that the Cdc7-ASK/Dbf4 maybe playing a role in maintaining cell viability during replication stress [Tenca et al., 2007]. Therefore a combination of specific Cdc7 or ASK/Dbf4 inhibitors along with drugs that impair the elongation reaction of DNA synthesis may be required as a viable strategy to tackle tumor cells

5.6 Transcription regulation of *ASK/Dbf4* in cutaneous melanoma is mediated by E2F1 through a non-canonical E2F1 site

ASK is essential for DNA replication and as an activating partner of Cdc7, a critical component of the pre-RC generated at each replication origin. Therefore, its activity needs to be strictly regulated so that origins are fired with the correct timing and in a coordinated manner. Because Cdc7 kinase activity in mammals totally depends on availability of ASK protein, the regulation of *ASK* expression is critical for control of initiation by Cdc7 kinase. In *S.cerevisiae*, transcription of *DBF4* gene is likely to be regulated by the transcription factor, MBF (MCB binding factor), since the promoter region of *DBF4* contains a consensus motif called MCB (*Mlu*I cell cycle box) to which MBF is known to bind in a cell cycle-dependent manner [Toyn et al., 1995; Andrews et al., 1990]. *ASK* gene expression is also growth- and cell cycle-regulated and appears to be up-regulated in many transformed cell lines [Kumagai et al., 1999]. Therefore, analysis of transcriptional regulation of the *ASK* gene should contribute not only to understanding the mechanism of regulation of origin firing but also in determining the correlation between deregulated *ASK* gene activation and tumorigenesis. However, there are no canonical E2F binding sites within the 63-bp minimal promoter segment of *ASK* and E2F1 was found to recognize a non-canonical sequence on *ASK* MP through a domain distinct from its known DNA binding domain. Interestingly, Weinmann *et al.* has recently

identified novel E2F target sites using chromatin immunoprecipitation assays, among them, ChET4 (chromatin-precipitated E2F target 4) and ChET8 were strongly bound by E2Fs *in vivo* despite the absence of consensus E2F binding site [Weinmann et al., 1999]. Cyclin E promoter was also onservedto be regulated by E2F by a non-consensus E2F site [Le Cam et al., 1999]. Thus, recruitment of E2F onto promoters lacking consensus E2F site seems not to be unique to the *ASK* promoter alone. Further studies on *ASK* promoter activation will clarify the nature of E2F-mediated transcriptional activation on those promoters without apparent E2F binding sites.

The E2F1 regulation of the *ASK* promoter has two important significance. First, is its link with the p16-Cdk4-Rb pathway. The p16-Cdk4-Rb pathway, which plays a critical role in regulation of G_1/S transition, is frequently mutated in tumors. E2F is the most important downstream target of Rb protein. Yamada et al demonstrated that overexpression of E2F caused up-regulation of the *ASK* promoter activity [Yamada et al., 2002]. The present study has further elucidated that this E2F binding to *ASK* promoter is still conserved within a tumor scenario as in the case of melanoma and that cisplatin mediated abrogation of E2F binding to *ASK* promoter decreased transcription of *ASK*, suggesting that the overexpression of ASK protein can be the result of a disruption of the Rb-E2F pathway. The second significance of E2F1 regulation of the *ASK* promoter arises due to ASK/Dbf4 being the regulatory subunit of Cdc7 kinase. Cdc7 kinase, is the critical determinant regulating the firing of individual replication origins. Deregulated Cdc7 activity resulting from overexpression of ASK protein within a neoplastic scenario may lead to overfiring of origins causing hyper-proliferation or loss of ordered activation of origins thereby increasing the chances of gene amplification, gene deletion, chromosomal instability, or polyploidy.

Interestingly, the current study also revealed that a complete or near complete abrogation of E2F1 binding to the *ASK* MP in BLM cells corresponded to a transcriptional decrease of *ASK/Dbf4* of only 27.6 %, suggesting the presence/contribution of additional mechanisms. Similarly It should be noted, that, at present, contribution of an E2F-activated second factor to *ASK* promoter activation cannot also be formally ruled out. Yamada *et al* have shown that a putative Sp1 site in the *ASK* MP plays a crucial role in its basal promoter activity.

Sp1 was previously reported to form a complex with E2F1, 2,and 3 [Karlseder et al., 1996; Lin et al., 1996]. Therefore, a possibility that Sp1 may be one of the factors in the E2F complex generated on *ASK* MP, facilitating transcription in the basal state as well as after stimulation is to be considered. Such an interaction between E2F and Sp1 within the neoplastic context can acheive sustained expression of *ASK* through S to G_2 phase. This is because Cdk2-cyclin A kinase complex is known to bind and phosphorylate E2F1 in S phase and down-regulate its DNA binding activity [Xu et al., 1994; Kitagawa et al., 1995], therefore an E2F-Sp1 complex can be sequestered away from phosphorylation and consequently from inactivation, permitting continued *ASK/Dbf4* expression throughout S phase.

5.7 Melanoma risk factor UVB may not have a role in inducing the expression of *ASK/Dbf4*

Photocarcinogenesis is known to be one of the possible consequences of ultraviolet (UV) irradiation of living tissues [Black et al., 1997] and epidemiologic studies have shown that sun exposure is a risk factor for melanoma development. Once a rare cancer, melanoma frequency is increasing in the Caucasian white population of developed countries [Elwood., 1996; Green et al, 1999]. Individual genetic predisposition and exposure to UV from sunlight combine together and result in tumoral transformation of melanocytes. Intermittent acute sun exposure rather than total dose of exposures seems to play a major role in induction of melanocarcinogenesis [Elwood., 1996]. In addition, UVB radiation is also the primary inducer of sunburn, and epidemiologic studies suggest that an intense sun exposure leading to sunburn in the early years of life is the first step that triggers changes in melanocytes towards possible malignant transformation [Elwood., 1996; Doré et al., 2001]. At the molecular level, the effects of UVB stress on melanocytes are still poorly understood, as most studies in various cell types have focused on the mutagenic, DNA-damaging and cell-cycle-disturbing actions of UV. More than 50 genes are presently known to participate in UV stress response in mammalian cells [Trautinger et al., 1996; Piepkorn., 2000] and some of them may be specific to melanocytes [Ablett et al., 1998]. Obviously, not all the known genes modulated by UVB irradiation are involved in carcinogenesis, but many of them reflect a partly transformed phenotype [Friedberg et al., 1995], including malignant

melanoma [Friedberg et al., 1995; Muijen., et al, 1995]. Other UV-sensitive genes may participate in cell homeostasis, such as NF-κB, which plays a pivotal role in immune, inflammatory, cell migratory, and growth responses [Benoliel et al., 1997; Legrand-Poels., et al, 1998]. Valery *et al*, compared UV-modulated genes selected by microarray to published gene expression profiles in melanoma and showed that many of them undergo modification of their expression in the disease [Valery et al, 2001]. Their study revealed an overlap between UV-modulated genes melanoma gene profiles from Bittner *et al*, which employed a similar methodology and DNA microarray platform. Fifty-nine percent of known genes modulated by UV had their expression modified in the same way in melanoma samples. Therefore considering that *ASK/Dbf4* is a melanoma modulated gene and has an essential role in melanoma cell proliferation, its UVB inducibilty was examined in the present study. The study seemed to suggest that *ASK/Dbf4* is refractory to melanoma risk factor UVB and thereby not an early event in melanomagenesis. However in this study cultured cells were irradiated once at a fluence of 200 mJ per cm^2 with lamps, which provided mainly UVB radiation. Obviously, these conditions do not match exactly the *in vivo* situation, especially with regard to sun exposure modalities and spectral irradiances in humans. A careful interpretation of data from studies using artificial UV sources is required [Gasparro and Brown., 2000]. In this investigation, a single UV irradiation of NHM in culture was chosen to approximate such minimum step. A common difficulty with cultured cells is to take into account the notion of skin phototype and related (just perceptible) minimal erythema dose (MED) [Farr and Diffey et al., 1985]. In practice, the biologically efficient dose (BED) is substituted for MED in the absence of vasculature [Bernerd and Asselineau, 1997]. Young *et al* have evaluated for several wavelengths a median MED (just perceptible minimal erythema dose) in patient populations of skin phototypes I and II. MED were 250 and 2400 mJ per cm^2 at UVB wavelengths 310 and 320 nm, respectively [Young., et al 1998]. In this investigation, the choice of 200 mJ per cm^2 fluence at 313 nm was based on such photodermatology and *in vitro* cutaneous cell biology reports,as well as by assessing NHM viability with UV dose-response curves. A fluence of 200 mJ per cm^2 UV at 312 nm allowed >75% NHM viability 15 h after UV irradiation, demonstrating that it was not drastically harmful. However, Inspite of these limitations in simulating *in vitro* , the sun exposure modalities and spectral irradiances in humans, such studies provide a

basis for understanding the role of genes in ultraviolet-induced melanoma and provide novel insights into developing new molecular-based strategies for risk prediction in patients.

6 SUMMARY

The molecular and genetic events that contribute to the development and progression of malignant melanoma are only partly understood. To identify novel determinants for tumor development and progression, oligonucleotide microarray-based comparison of gene expression profiles of a series of nevi primary cutaneous melanomas, cutaneous melanoma metastases and melanoma cell lines with normal human melanocytes (NHM) as calibrator, was performed. Based on this data, molecular changes between benign nevi and primary cutaneous melanoma were distinct and discernible. In contrast between primary cutaneous melanoma and metastasis the data revealed no absolute distinction. Presumably a considerable overlap of expressed genes between cutaneous melanoma and metastasis as seen in this study reflects the close biological relationship between them by describing gradual changes. Since qRT-PCR based multi-gene prediction models was un attempted in melanoma, this study was the first to generate a multi-gene signature containing 4 genes namely *ASK/Dbf4, Tpr, c-MET and MCAM/MUC18* that were able to differentiate nevi from melanoma. This study then further focused on functional characterization of the role of *ASK/Dbf4*, one of the components in this 4-gene signature.

In keeping with its expected role as a cyclin-like regulatory subunit of mammalian Cdc7, the data suggested that upregulated ASK is localized to the nucleus and binds to human Cdc7 to form Cdc7-ASK/Dbf4 complexes in melanoma cells. Subsequently, this study also demonstrated that ASK has an essential function in melanoma cell growth by showing that siRNA-mediated specific depletion of *ASK* retarded cell survival and proliferation. Furthermore, the current study also explained that *ASK/Dbf4* is a transcriptional target of the important cell cycle regulator E2F1 in melanoma and that it is a melanoma modulated gene refractory to melanoma risk factor UVB and hence its expression may not be an early event in melanomagenesis. Thus in summary, this study identified and characterized, a novel cell survival gene in melanoma, namely *ASK/Dbf4*.

ZUSAMMENFASSUNG

Die molekularen und genetischen Vorgänge, die zu einer Bildung und der Progression von malignen Melanomen führen, sind noch nicht vollständig aufgeklärt. Zur Identifizierung neuer Determinanten für die Entstehung und Progression von Melanomen wurden auf Oligonukleotid-Mikroarrays basierende Vergleiche der Genexpressionsprofile von Nevi, primären kutanen Melanomen, Metastasen von kutanen Melanomen, sowie Melanom-Zelllinien, mit normalen, humanen Melanozyten (NHM) als Kalibrator, durchgeführt. Die molekularen Veränderungen zwischen Nevi und primären kutanen Melanomen waren aufgrund der Ergebnisse distinkt und eindeutig. Im Gegensatz dazu konnten keine absolut eindeutigen Veränderungen zwischen primären kutanen Melanomen und Metastasen ausfindig gemacht werden. Es wird angenommen, dass die erhebliche Überlappung in den exprimierten Genen zwischen kutanen und metastasierten Melanomen in dieser Studie die enge biologische Verwandtschaft zwischen diesen aufzeigt und nur graduelle Veränderungen vorliegen. Da qRT-PCR basierende Multi-Gen Modelle in Melanomen noch nicht angewandt wurden, konnte mit Hilfe dieser Studie zum ersten Mal eine Multi-Gen-Signatur, welche die Gene *ASK/Dbf4*, *Tpr*, *c-Met* und *MCAM/MUC18* enthält, generiert werden, die eine Unterscheidung von Nevi und primären Melanomen möglich macht. Diese Studie konzentrierte sich dann auf die funktionelle Charakterisierung der Rolle von *ASK/Dbf4,* einer der Komponenten dieser 4-Gen-Signatur.

Es konnte gezeigt werden, dass hochreguliertes ASK im Nukleus lokalisiert ist und an humanes Cdc7 bindet, um einen Cdc7-ASK/Dbf4-Komplex in Melanomzellen zu bilden. Außerdem konnte in dieser Studie gezeigt werden, dass ASK eine essentielle Rolle im Zellwachstum von Melanomen hat, da eine durch siRNA verursachte Verminderung von *ASK,* Zellüberleben und -proliferation verzögerte. Die Studie zeigte ebenfalls, dass *ASK/Dbf4* ein transkriptionelles Ziel des wichtigen Zellzyklus-Regulators E2F1 in Melanomen ist und das es einen melanom-modulierten Schutz von Genen gegen den Risikofaktor UVB darstellt und daher seine Expression möglicherweise kein früher Vorgang in der Melanomgenese ist.

Zusammenfassend, wurde in dieser Studie ein neues Gen, welches das Überleben von Zellen in Melanomen sichert, *ASK/Dbf4*, identifiziert und charakterisiert.

7 LITERATURE

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8 APPENDIX

ID	RT/MA	Gender	Age	Histopathologic diagnosis	Localization
N97	RT/MA	F	41	Atypic congenital compound nevus	Posterior trunk
N103	RT/MA	F	12	Congenital compound nevus	Posterior trunk
N122	RT	M	62	Congenital compound nevus	-
N123	RT/MA	M	35	Atypic Congenital Compound Nevus	Abdomen
N134f	RT	M	38	Nevus	Right lower leg
N135	RT	F	73	Dysplastic nevus from total Compound nevus	Right 2./3.toe
N150	RT	F	15	Congenital compound nevus	-
N152	RT/MA	M	67	Congenital compound nevus	Abdomen
N155	RT	F	13	Congenital compound nevus	Back
N174	MA	M	45	Atypic Congenital Compound Nevus	Posterior trunk
N212	MA	F	17	Congenital compound nevus	Posterior trunk
N213	MA	F	9	Atypic Congenital Compound Nevus	Ear right
N250	MA	F	12	Congenital compound nevus	-
N258	MA	M	12	Atypic Congenital Compound Nevus	-
N269	MA	M	1	Congenital compound nevus	-
N269(2)*	MA	M	1	Congenital compound nevus	-

Table 8-1: Details of nevi samples used in the study. RT denotes that sample was used for qRT-PCR, MA denotes that sample was used for the microarray study. * denotes technical replicate.

ID	RT/MA	Gender	Age	Histopathologic diagnosis	Clark level	Breslow (mm)	Localization
P61	RT/MA	F	83	Ulcerated superficial spreading melanoma (SSM)	IV	1,6	Posterior trunk
P84	RT/MA	F	55	Ulcerated nodular melanoma	IV	6,5	Right ear
P85	RT/MA	M	68	SSM	IV	1,65	Decollete
P108	RT	M	65	SSM	II	0,85	Right abdomen
P113	MA	M	44	SSM	IV	1,23	Posterior trunk
P115	RT	M	64	SSM	IV	1,2	Back
P117	MA	M	62	Primary melanoma	IV	5,58	Posterior trunk
P124	RT	M		Primary Melanoma	IV	2,2	Chest
P129	RT	M	81	SSM,secondary nodular melanoma	IV	2,5	Scalp
P133	RT	F	51	Primary melanoma	IV	0,8	Lower right leg
P146	RT	F	82	Primary melanoma	-	-	Right shoulder
P167	RT	F	74	Ulcerated primary melanomam	-	3,2	-Abdomen
P168	RT	F	74	Ulucerated primary melanoma	-	3,2	-Lower back
P171	RT/MA	M	73	Primary melanoma	IV	0,55	Right abdomen
P172	RT/MA	M	48	Primary melanoma	IV	1,12	Posterior trunk
P182	RT	M	25	Spitzoide(Angular) primary melanoma	IV	2,25	Right lowe leg
P183	RT	F	46	SSM	III-IV	0,8	Left arm
P191	RT	F	67	Primary melanoma	IV	4,23	Lower right leg
P192	RT	M	62	Primary melanoma	IV	1,6	Right foot
P194	RT	M	45	SSM	II-III	0,8	Back
P221	MA	F	58	Primary melanoma with spitzoid differentiation	III-IV	2,23	Scalp
P254	MA	F	65	SSM,secondary nodular	III-IV	1,8	Lower leg
P254(2)*	MA	F	65	SSM,secondary nodular	III-IV	1,8	Lower leg

Table 8-2: Details of primary melanoma samples used in the study. RT denotes that sample was used for qRT-PCR, MA denotes that sample was used for the microarray study. * denotes technical replicate.

ID	RT/MA	Gender	Age	Histopathologic diagnosis	Localization
M53	RT	M	68	Cutaneous melanoma metastasis	-Back
M61	RT			Cutaneous melanoma metastasis	-Abdomen
M62	RT	M	79	Cutaneous melanoma metastasis	-Right arm
M62	RT	M	75	Cutaneous melanoma metastasis	-Neck
M63	RT	M		Cutaneous melanoma metastasis	-Face
M66	RT	F	69	Cutaneous melanoma metastasis	-Left leg
M67	RT	F	69	Cutaneous melanoma metastasis	-Scalp
M68	RT	F	69	Cutaneous melanoma metastasis	-Left leg
M69	RT/MA	M	62	Cutaneous melanoma metastasis	Right ear
M70	RT	M	69	Cutaneous melanoma metastasis	Scalp
M71	RT	M	73	Cutaneous melanoma metastasis	Scalp
M73	RT	F	69	Cutaneous melanoma metastasis	Right arm
M83	RT/MA	M	62	Cutaneous melanoma metastasis	Right ear helix
M114	RT/MA	F	89	Cutaneous melanoma metastasis	Left shank
M145	MA	F	53	Cutaneous melanoma metastasis	Lower right leg
M175	MA	M	80	Lymphnode melanoma Metastasis	Right neck
M185a	MA	F	89	Cutaneous melanoma metastasis	Left shank
M190	MA	F	48	Cutaneous melanoma metastasis	Right leg
M201	MA	F	48	Cutaneous melanoma metastasis	Lower left leg
M226	MA	F	81	Cutaneous melanoma metastasis	Left arm
M56(2)	MA	M	53	Cutaneous melanoma metastasis	Left arm
M57(2)	RT/MA	M	77	Ulcerated cutaneous melanoma metastasis	Right thigh

Table 8-3: Details of metastatic melanoma samples used in the study. RT denotes that sample was used for qRT-PCR, MA denotes that sample was used for the microarray study

9 CURRICULUM VITAE

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Publications

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Book Chapters

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Research Grants

Forschungskommission der Medizinischen Fakultat, University of Duesseldorf, Grant period 2007-2008, Grant 9772313 "Molekulare Detektion der Melanomenstehung - Ein Ansatz basierend auf "microfluidic cards."

Poster presentations

Nambiar, S., Mirmohammadsadegh, A., Hassan, M., Marini, A., Alaoui, A., Hegemann, J.H., Hengge, U.R. "Upregulation of *ASK* and *Tpr* expression in human cutaneous melanomas: two novel discriminative determinants in melanoma development." XXXIII. Jahrestagung der Arbeitsgemeinschaft Dermatologische Forschung (ADF), Aachen, 23-25 March, 2006.

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Nambiar, S., Mirmohammadsadegh, A., Doroudi, R., Gustrau, A., Marini, A., Roeder, G., Ruzicka, T., Hengge, U.R. "Signaling Networks in Cutaneous Melanoma Metastasis Identified by cDNA Microarrays." 35th meeting of European Society for Dermatological Reseach (*ESDR*), Tübingen, 22-24 September, 2005.

Oral presentations

"Molecular profiling of cutaneous melanoma and functional characterization of *ASK/Dbf4* upregulation." 44. Deutsche Dermatologischen Gesellschaft (DDG) Tagung, Dresden, 25-28 April, 2007.

"Molecular profiling of cutaneous melanoma and functional characterization of *ASK/Dbf4* upregulation." 36th meeting of European Society for Dermatological Reseach (*ESDR*), Paris, 07-09 September, 2006.

"Microrray platform : When the chips are done." XIII. Jahrestagung Deutsche Gesellschaft für Gentherapie, Düsseldorf, 12-14 July, 2006.

EIDESSTATTLICHE ERKLÄRUNG

Hiermit versichere ich, dass die vorliegende Arbeit von mir selbst verfasst wurde und dass ich keine anderen als die von mir angegebenen Hilfsmittel verwendet habe. Alle Stellen, die aus anderen Werken im Wortlaut oder dem Sinn entsprechend übernommen wurden, habe ich mit Quellenangaben kenntlich gemacht.

Düsseldorf, 30. April 2007 ----------

(Sandeep Nambiar)