

**STUDIES ON REGULATION AND BIOLOGICAL FUNCTION OF
MAPK SIGNALING IN DEVELOPMENT AND PROGRESSION OF
MALIGNANT MELANOMA**

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Oslo, September 2008

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2. ABBREVIATIONS

ABC	Avidin-biotin-complex
AEC	3-amino-9-ethylcarbazole
AIF	Apoptosis inducing factor
Akt	Proteine kinase Akt
AP-1	Activator protein 1
APAF-1	Apoptosis protease activationg factor-1
APC	Adenomatous polyposis coli
ARF	Alternative reading frame
ATF1 and 2	Activating transcription factor 1 and 2
Bak	Bcl-2 antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell leukemia/lymphoma-2
Bid	BH3 interacting domain death agonist
Bim	Bcl-2 like 11
B-RAF	V-raf murine sarcoma viral oncogene homolog B1
cAMP	Cyclic adenosine monophosphate
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CDKN2A	Cyclin dependent kinase inhibitor 2A
c-fos	Cellular fos proto-oncogene
c-jun	Cellular jun proto-oncogene
c-myc	Cellular myc proto-oncogene
CREB	cAMP responsive element binding protein
DAB	Diaminobenzidine tetrahydrochloride
DISC	Death-inducing signal complex
DNA	Deoxyribonucleic acid
DPC4	Deleted in pancreatic cancer, locus 4/SMAD family member 4
E2F	E2F transcription factor
ECM	Extracellular matrix
EGF	Epidermal growth factor

EGR1	Early growth response 1
EGFR	Epidermal growth factor receptor
Elk1	ELK1, member of ETS oncogene family
ERK	Extracellular signal-regulated kinases
Ets1	v-ets erythroblastosis virus E26 oncogene homolog 1
FABP	Fatty acid binding protein
FAK	Focal adhesion kinase
FADD	Fas-associated death domain protein
Fas	TNF receptor superfamily, member 6
FasL	Fas ligand
FGFR	Fibroblast growth factor receptor
GSK3- β	Glycogen synthase kinase-3 beta
IAP	Inhibitor of apoptosis
ICAT	beta-catenin-interacting protein
ILK	Integrin-linked kinase
INK4	Inhibitor of cyclin dependent kinase 4
JNK	c-jun N-terminal kinase
kDa	kilo dalton
KIT	v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog
LEF1	Lymphoid enhancer binding factor-1
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia sequence 1
MDM2	Mouse double minute 2
MEF2A	Myocyte enhancer factor 2A
MITF	Microphthalmia-associated transcriptional factor
MMP	matrix metalloproteinase
MNK1/2	MAPK interacting serine/threonine kinase 1 and 2
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NFAT4	Nuclear factor of activated T-cells 4
NF κ -B	Nuclear factor kappa-B
NGF	Nerve growth factor
H-RAS	Human homolog to Harvey rat viral sarcoma oncogene, encoding RAS
N-RAS	Neuroblastoma RAS viral (v-ras) oncogene homolog

PDGFR	Platelet-derived growth factor receptor
PK1 and 2	Phosphoinositide-(3,4,5)-triphosphate-dependent-kinases 1 and 2
PI3K	Phosphoinositide 3 kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2
PMA	Phorbol-12-myristate-13-acetate
PP2	4-Amino-5-(4-chlorophenyl)-7-(t-butyl)(3,4-d) pyrimidine
PPAR	peroxisome proliferators-activated receptor
PTEN	Phosphatase and tensin homolog deleted from chromosome 10
RACK1	Receptor for activated kinase C 1
RB	Retinoblastoma
RTK	Receptor tyrosine kinase
RGF	Radial growth phase
SA- β -GAL	Senescence associated acidic β -galactosidase
Sap-1a	Serum response factor (SRF) associated protein 1
Shc	Src homology 2 domain containing
siRNA	Small interfering RNA
Smac/Diablo	Second mitochondria-derived activator of caspases/Direct IAP-binding protein
SMAD4	Mothers against decapentaplegic homolog 4
Src	Human homolog to the avian v-Src gene of the Rous Sarcoma virus
STAT1/3	Signal transducer and activator of transcription 1 and 3
Tau	Microtubule-associated protein Tau
TPA	12-O-tetradecanoyl phorbol 13-acetate
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase end labeling
UV	Ultra violet
VEGFR	Vascular endothelial growth factor receptor
VGf	Vertical growth phase
Wnt	Wingless-type MMTV integration site family

3. AIMS OF THE STUDY

Malignant melanoma is a severe cancer form, and the death risk among patients with an advanced stage of this cancer is high. So far, cancer therapy has had little effect on survival, and the need for identifying factors involved in development and progression of malignant melanoma is urgent. Mapping of cell signaling mechanisms involved in oncogenesis is of importance, and relevant signaling pathways may be potential targets for treatment.

The aims of this thesis were;

- I. To investigate the expression of MAP kinases in malignant melanoma specimens, and analyze the relationship to clinicopathological parameters and patient survival.
- II. To study the regulation of the ERK1/2 pathway by PKC and elucidate the molecular mechanisms responsible for this regulation

4. LIST OF PAPERS

- I. K. Jørgensen, R. Holm, G.M. Mælandsmo, V.A. Flørenes: **Expression of activated ERK1/2 in malignant melanomas. Relationship to clinical outcome.** *Clin. Cancer. Res.* 9 (14): 5325-5331, 2003
- II. K. Jørgensen, B. Davidson, V.A. Flørenes: **Activation of c-Jun N-Terminal Kinase (JNK) is associated with cell proliferation and shorter relapse-free period in superficial spreading malignant melanoma.** *Mod. Pathol.* 19: 1446-1455, 2006
- III. K. Jørgensen, M. Skrede, V. Cruciani, S.O. Mikalsen, A. Slipicevic, V.A. Flørenes: **Phorbol ester phorbol -12-myristate-13 acetate promotes anchorage-independent growth and survival of melanomas through MEK-independent activation of ERK1/2.** *Biochem. Biophys. Res. Commun.* 329 (1): 266-274, 2005
- IV. A. Slipicevic, K. Jørgensen, M. Skrede, A.K. Ree Rosnes, G. Trøen, B. Davidson, V.A. Flørenes: **The fatty acid binding protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells.** *BMC Cancer* 8(1):276, 2008

5. GENERAL INTRODUCTION

5. 1. Cancer

Homeostasis, the finely tuned balance between cell proliferation and cell death, is essential for maintaining the organism. When homeostasis is disturbed, either by increased cell proliferation or by a decrease in cell death, a neoplasm (neoplasm = new growth) may develop (1). In order to maintain homeostasis, genes that positively and negatively regulate cell growth and proliferation are required, and these genes are called proto-oncogenes and tumor suppressor genes, respectively (2). Moreover, series of repair genes contributes to keeping genetic alterations to a minimum. Molecular changes in several of these genes at the same time may give growth advantages to the cell and eventually lead to cancer development and progression (3).

The development of cancer is a multi-step process. One of the hallmarks is increased cell proliferation. Cancer cells eventually become self-sufficient with growth signals, allowing the cells to proliferate in absence of mitotic signals. Tumor cells are also insensitive to anti-growth signals, are able to avoid apoptosis, and have limitless replicative potential. Together, these events contribute to increased cell proliferation. Sustained angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is another cancer hallmark. Blood vessels supply tissue with nutrients and oxygen, and all cells in the body reside close to a capillary blood vessel. During cancer progression, tumor cells move out from their origin and invade adjacent tissue where blood and lymph vessels allow them to travel to distant sites where they establish a metastasis (Figure 1.) (4).

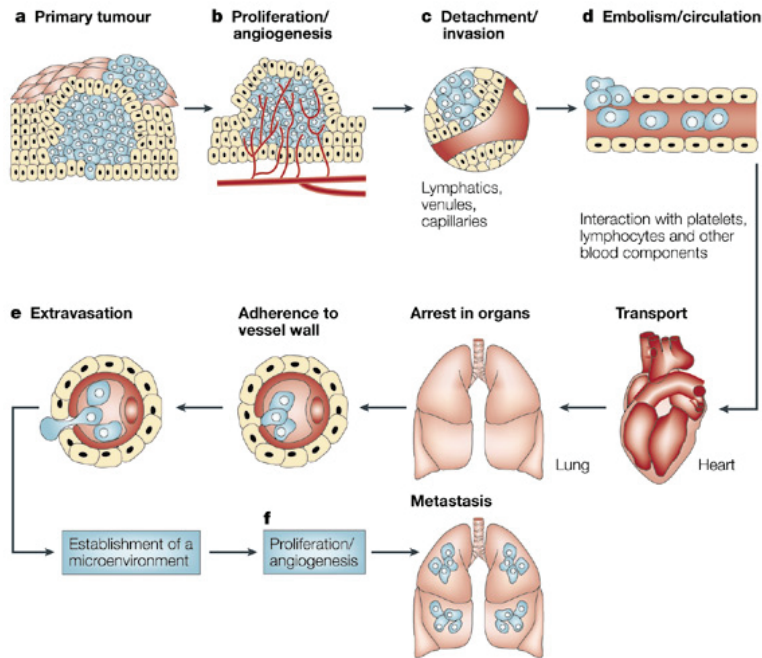


Figure 1. The main steps in the metastatic process. Reprinted by permission from Macmillan Publishers Ltd : ref. (5), © 2003 .

5.2. Cell cycle regulation

In order to divide, cells go through a cycle divided into four phases, G1, S, G2 and M. The periods associated with DNA synthesis (S-phase) and mitosis (M-phase) are separated by gaps of varying length, G1 and G2. However, most cells pause in their progress around the cell cycle, and rest in a state called G0 (6). Progression through the G1 phase is highly dependent on extra-cellular mitotic stimuli, like growth factors, stimulating the cells to overcome the so-called restriction point in late G1 phase. Two main cell cycle checkpoints control the order and timing of cell cycle transitions (G1–S and G2–M) and ensure that critical events such as DNA replication and chromosome segregation are completed correctly before allowing the cells to progress further through the cycle (7-9). After the G1 restriction point, the cells can complete their cycle independently of external stimuli (6). Progression through the cell cycle is driven by the

actions of cyclin dependent kinases (CDKs) and their activating cyclin subunits (Figure 2). CDK-cyclin complexes phosphorylate and inactivate the retinoblastoma protein, pRb, which then releases the E2F transcription factor, allowing the cells to express genes essential for cell cycle progression (10).

CDK activity is suppressed through interactions with two main families of inhibitory proteins (CDK inhibitors or CDKIs); The INK4 family and the CIP/KIP family. The INK4 family includes p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d} (ARF) and specifically binds and inhibits CDK4 and CDK6. p19^{ARF} shares two exons with p16^{INK4a} as a result of a spliced *CDKN2A* gene and is also known as an activator of p53 through blocking of the function of MDM2 (11). The Cip/Kip family comprises p21^{Cip1/WAF1}, p27^{Kip1}, and p57^{Kip2}. The Cip/Kip proteins act as negative regulators of cyclin E/A-Cdk2 complexes and the cyclin B-Cdk1 complex as well as positive regulators of cyclin D-Cdk4/6 complexes (12).

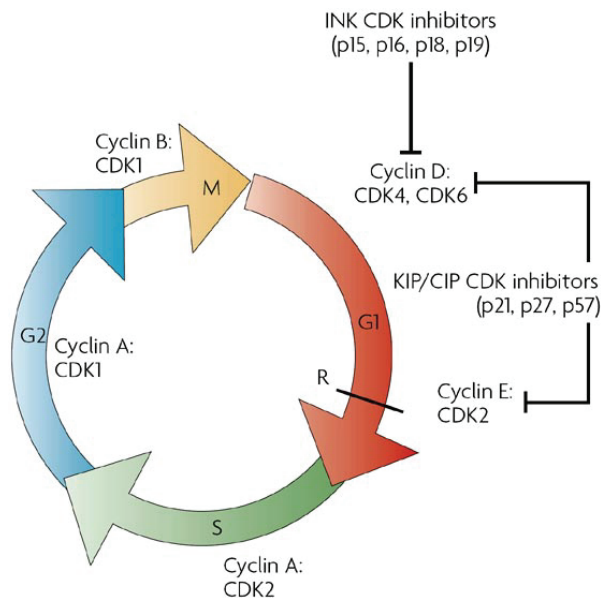


Figure 2. The cell cycle. Reprinted by permission from Macmillan Publishers Ltd.: ref. (13), © (2007)

5.3. Apoptosis

The process of self-destruction of cells that are genetically programmed to have a limited life span or are damaged is called apoptosis (14). The mechanisms of programmed cell death are of great importance, being involved in development, differentiation, proliferation, homeostasis, regulation and function of the immune system and in the removal of defective cells (15). The morphological characteristics of apoptosis are chromatin condensation (pyknosis), nuclear fragmentation, plasma membrane blebbing and cell shrinkage (16). Apoptosis can be induced either by a stimulus, such as irradiation or toxic drugs, or by removal of a repressor agent, leading the cells to disintegrate into membrane-bound particles that are eliminated by phagocytosis (17).

The major executors of the apoptotic machinery are the caspases, a family of cysteine-dependent aspartate-specific proteases that cleaves substrates after aspartic acid (asp) residues (18). The caspases are divided into the initiator caspases (caspase-2, -8, -9 and 10) and effector caspases (caspase-3, -6, and -7) and they are activated through either an extrinsic or an intrinsic pathway (Figure 3).

The extrinsic pathway is initiated through stimulation of transmembrane death receptors by ligands such as Fas, TNF- α and TRAIL (19). For example, binding of FasL to Fas receptors on the target cell will trigger the aggregation of multiple receptors on the surface. Following this aggregation, an adaptor protein known as Fas-associated death domain protein (FADD) is recruited in the cytoplasm. FADD, in turn, activates caspase-8 to form the death-inducing signal complex (DISC). Through the recruitment of caspase-8 to DISC, caspase-8 will be activated and then directly activate caspase-3 to initiate degradation of the cell. Active caspase-8 can also cleave the Bid protein to tBid, which acts as a signal on the membrane of mitochondria to facilitate the release of cytochrome c in the intrinsic pathway (20).

The intrinsic pathway, also referred to as the mitochondrial pathway, is initiated through the release of signaling factors by mitochondria within the cell (17). The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by

factors such as DNA damage and heat shock (21), leading to a permeability change in the mitochondrial membrane. The pro-apoptotic proteins, Bax and Bid, residing in the cytoplasm, as well as Bak within the mitochondria, are now allowed to promote the release of cytochrome c from the mitochondria and into the cytosol. Following this release, cytochrome c forms a complex in the cytoplasm with ATP and Apaf-1, which will activate caspase-9. Activated caspase-9 will together with the complex of cytochrome c, ATP and Apaf-1 form an apoptosome, which in turn activates caspase-3, the effector protein that initiates degradation. In addition to cytochrome c, the released intramembrane content also contains apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/DIABLO proteins to inhibit inhibitor of apoptosis (IAP) proteins (22).

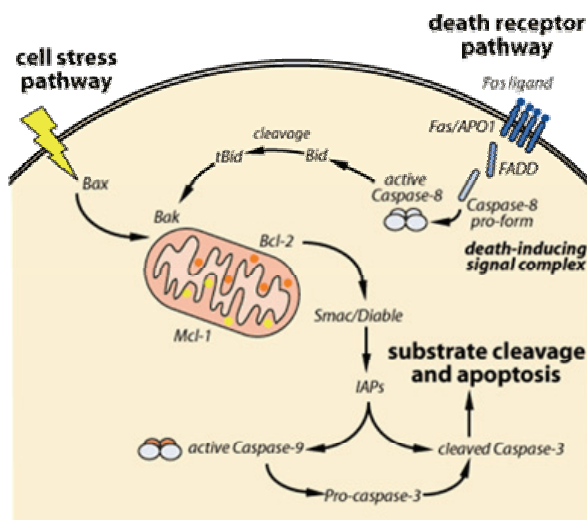


Figure 3. The intrinsic and extrinsic pathways leading to apoptosis. Reprinted by permission from The Science Creative Quarterly.

5.3.1. Anoikis

Adhesion to extracellular matrix (ECM) is essential for survival of most normal cells, and loss of adhesion induces a special form of anchorage-deprived apoptosis termed “anoikis”, a Greek word for homelessness (23). Anoikis is of great importance *in vivo*, because disruption of this life-and death control enhances tumor metastasis, allowing cells to survive in totally inappropriate ECM environments (24). Adhesion of cells to ECM is mainly mediated by integrins. These are transmembrane cell surface heterodimers composed of an α -chain and a β -chain. In mammals, 18 α -subunits and 8 β -subunits have been identified, which can combine into 24 different heterodimers, each with specific recognition and affinities for various ECM components and other adhesion molecules (25). Integrins mediate their signals via integrin-associated non-receptor kinases such as focal adhesion kinase (FAK), integrin-linked kinase (ILK) and Shc, leading to activation of the PI3K, MAPK/ERK1/2 and JNK pathways (25;26). Over-expression of FAK and ILK has been shown to inhibit anoikis *in vitro* (27;28). During anoikis, cells undergo the same phenotypic changes as an apoptotic cell.

A number of studies have implicated members of the Bcl-2 family in playing important roles in anoikis (23). Depending on cell type, anoikis has been accompanied by changes in protein expression levels or phosphorylation/activation status in the pro-apoptotic proteins Bad, Bax and Bak, as well as in the anti-apoptotic protein Mcl-1 (29-31). Moreover, the pro-apoptotic protein Bim has been shown to promote anoikis in epithelial cells (32).

5.3.2 Entosis

In the absence of apoptosis/anoikis, cells still manage to convert into a necrotic state, but the roles of necrosis as well as other possible mechanisms for cell elimination in cancer development are poorly understood. In 2007, Overholtzer *et al.* (33) described a new mode of non-apoptotic cell elimination called “entosis” (a greek word for “within”). In short, this mechanism, that resembles cell cannibalism, is provoked by loss of attachment to matrix. A single, live cell invades another live cell, and will during its internalization be degraded by lysosomal enzymes. This death process does not involve apoptotic signals and is therefore distinct from the phagocytic ingestion of dying cells

observed during apoptosis. Moreover, not all internalized cells are degraded. A small percentage actually divides within the host cell and is eventually released. The invasion of cells is carried out through signaling via the small GTPase Rho, which mediates actomyosin-mediated contraction. Before invasion, the two cells establish E-cadherin- β -catenin-mediated adherens junctions, whose compaction appears to directly mediate the entosis process. This cell-in-cell phenotype is associated with human tumors, and it has been suggested that entosis functions as a tumor suppressing mechanism which eliminates detached cells.

5.4. Signaling pathways

The cellular homeostasis is under control of different protein kinases that can transduce signals from extracellular matrix (ECM) to the nucleus where gene expression is triggered. The most prominent signal transduction pathways in this regard are the mitogen-activated protein kinase (MAPK) cascades and the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway.

5.4.1. *The MAPK pathways*

The MAPKs comprise a family of protein serine-threonine kinases which, depending on the context, have been correlated to different responses like proliferation, cell growth, differentiation and cell survival (34). In mammalian cells, three major MAPK families have been characterized; the extracellular signal-regulated kinases 1 and 2 (ERK1/2), C-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 kinase. Each of these cascades consists of three enzymes that are activated in series: a MAPK kinase kinase (MAPKKK or MEKK), a MAPK kinase (MAPKK or MEK) and a MAP kinase (MAPK). At least 14 MAPKKKs, 7 MEKs and 12 MAPKs have been identified in mammalian cells (35).

5.4.1.1. *RAF*s

The receptor tyrosine kinase effector RAF (Rapidly Accelerated Fibrosarcoma) belongs to a family of serine/threonine protein kinases. There are three mammalian RAF isoforms identified; A-RAF B-RAF and C-RAF/RAF-1, all translated from distinct genes on different chromosomes and expressed in various tissues (36;37). RAF plays an important role in tumorigenesis, both as a signal transducer from its upstream mediators, but also as an oncogene itself. Both A-RAF and C-RAF requires two mutated spots for oncogenic transformation, thus, oncogenic mutations in human cancers are rare. However, B-RAF requires only one mutational event, and activating mutations in B-RAF have been observed in many human cancers like malignant melanoma, ovarian cancer, colorectal cancer and non-small-cell lung cancer (36). The most common mutation in B-RAF is a valine to glutamic acid substitution at residue 600 which is followed by constitutive activation of the MEK-ERK1/2 pathway (38). In addition to its high mutational frequency, B-RAF is also the isoform with the highest basal kinase activity as well as the one that interacts best with its upstream mediator, RAS (36).

5.4.1.2. *The ERK1/2 pathway*

The best characterized MAPK signaling pathway is the one that proceeds through the extracellular signal-regulated kinases 1 and 2 (ERK1/2). ERK1 and ERK 2 have the size of 44 kDa and 42 kDa, respectively, and share 83 % homology, but most of the differences reside outside their kinase core (39;40). Several experiments suggest that ERK1 and ERK2 are functionally equivalent, and the two ERK isoforms are usually co-expressed (39). ERK1/2 is expressed in all tissue, and upon activation ERK1/2 plays a central role in several processes, including proliferation, differentiation and development, as well as cell survival, migration, apoptosis, morphology determination and oncogenic transformation (41). The ERK1/2 signaling cascade is initiated by extracellular signals, such as growth factors or hormones, that bind to a receptor tyrosine kinase (RTK) in the cell membrane which then activates the GTP-bound protein, RAS. A variety of RTKs interacts with RAS, including the epidermal growth factor receptor (EGFR), v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT), platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor (FGFR). Activated RAS will in

turn activate the first MAPKKK (RAF), which again phosphorylates and activates MEK1/2. ERK1/2 is then subsequently activated by dual phosphorylation of MEK1/2 (40-42). Following ERK1/2 phosphorylation, its downstream cytoplasmic targets pp90^{RSK}, EGFR, RAF-1, STAT1/3 and MEK1/2 are phosphorylated (43-46), and following translocation to the nucleus, ERK1/2 phosphorylates and activates transcription factors such as c-jun, c-myc (47), elk1/p62TCF (48) and RNA polymerase II (49) (Figure 4).

MEK1 and MEK2 are activated by phosphorylation of two serine/threonine residues, each partially increasing the activity of MEK1/2 (42;50). However, the activity is regulated by additional phosphorylation/dephosphorylation processes as well. Activated ERK1/2, for instance, will be able to phosphorylate MEK1 at specific threonine residues, thereby leading to a negative feedback control on the ERK1/2 signaling cascade (41). The ERK1/2 signaling cascade plays an important role in cell cycle progression by inducing transcription of the cyclin D1 gene (51). On the other hand, recent evidence suggest a role for the ERK1/2 pathway in senescence and growth arrest, indicating a tumor suppressive effect as well (52). The ability of ERK1/2 to cause different and even opposing effects in the same cells has been questioned. Several studies have reported different effects based on the strength and duration of ERK1/2 activation. In the first model described, PC12 cells were stimulated with epidermal growth factor (EGF) and nerve growth factor (NGF), known to promote proliferation and differentiation, respectively. It was shown that while EGF caused strong, transient, activation of ERK1/2, NGF caused strong, sustained activation of ERK1/2. Interestingly, it was also shown that artificially prolonged ERK1/2 activity induced differentiation instead of proliferation in the same cells. Moreover, it has been demonstrated that ERK1/2 signal duration is likely to influence the transcription and activity of immediate early genes, such as the transcription factors c-fos, c-jun, c-myc and EGR1 (41).

ERK1/2 is an important component in oncogenesis. Increased ERK1/2 activity, associated with disease, has been demonstrated in prostate, breast, pancreatic and lung cancers (52), and constitutive activation of ERK1/2 has been shown to be present in malignant melanoma (53;54). Thus, this signaling pathway represents a major target for cancer therapy.

5.4.1.3. The SAPK/JNK and p38 pathways

SAPK/JNK and p38 are called stress-activated protein kinases and are activated by cellular stress (eg, UV irradiation, osmotic stress, heat shock, protein synthesis inhibitors), inflammatory cytokines (eg, tumor necrosis factor- α (TNF- α) and interleukin-1) and G-protein coupled agonists (eg, thrombin). Like ERK1/2, both JNK and p38 are dually phosphorylated on tyrosine and threonine residues by MAPKKs. JNK is selectively phosphorylated by MKK4 and MKK7 and p38 is phosphorylated by MKK3 and MKK6 (34) (Figure 4).

JNK and p38 regulate the expression of a number of cytokines, transcription factors and cell cycle regulators. The major outcome of JNK activation is phosphorylation of the c-jun transcription factor, which upon activation leads to increased expression of genes with AP-1 (Activator protein-1) sites in their promoters (55). Other JNK targets include the transcription factors ATF2, Elk1, c-myc, p53, DPC4, Sap1a and NFAT4, all positive regulators of the transcription factor c-fos, further increasing the AP-1 level (51;56). On the other hand, activation of p38 is followed by phosphorylation of phospholipase A2, the microtubule-associated protein Tau, ATF1 and -2, MEF2A, Sap1, Elk1, NF- κ B, Ets1, and p53 amongst others (57) (Figure 4).

Constitutive activation of the MAPK cascades has been observed in various *in vitro* tumor cell lines (58;59) and has been associated with carcinogenesis and metastatic potential of human cancers (60;61). Until recently it has been anticipated that the MAPK/ERK1/2 signaling pathway is involved in cell proliferation and survival because of its response to mitotic signals and proliferative cytokines, whereas activated JNK and p38 have been suggested to act as pro-apoptotic tumor suppressors in response to stress-mediated signals. Recent discoveries have, however, revealed that several MAPKs can phosphorylate the same substrates and also affect each other through cross-talk reactions and feedback mechanisms (52). For instance, several studies have demonstrated that MAPKKs, believed to be specific for the JNK and p38 signaling pathway, directly phosphorylate the ERK1/2 specific MEK1/2 (62) (Figure 4).

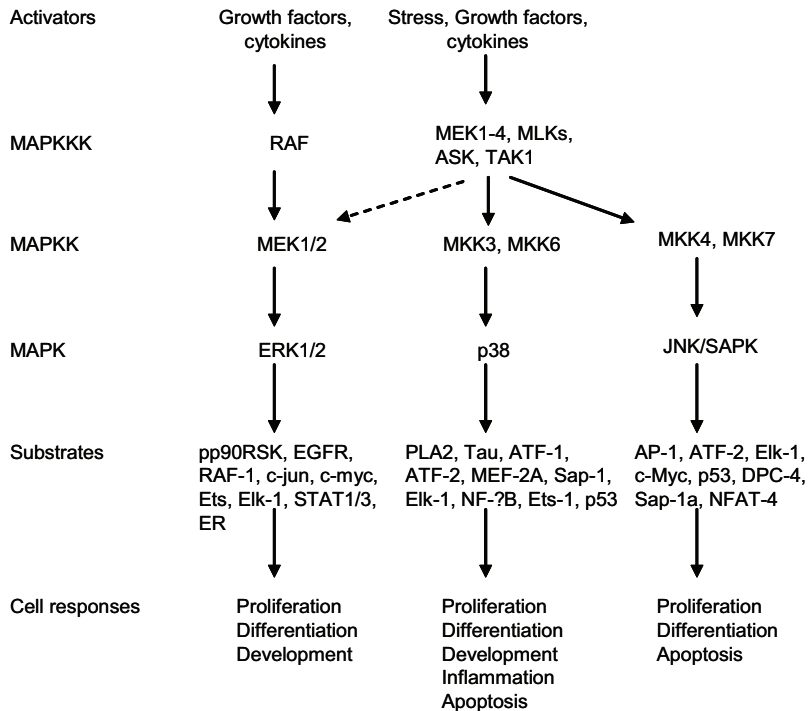


Figure 4. Major MAPK signaling cascades in mammalian cells. Adapted from (51).

5.4.1.4. The PI3K pathway

Phosphoinositide 3-kinases (PI3Ks) generate specific inositol lipids that have been implicated in regulation of cell growth, proliferation, survival, differentiation and cytoskeletal changes (63). The PI3K family is divided into three classes (I, II and III), but only class I PI3Ks are coupled to external stimuli and involved in oncogenesis (63;64). Class I PI3Ks are further classified into two functionally and structurally different subgroups, 1A and 1B, which transmit signals from receptor tyrosine kinases (RTK) and G-protein coupled receptors, like RAS, respectively (63). PI3K catalyzes phosphorylation of inositol-containing lipids, known as phosphatidylinositols (PtdIns) (65). The primary substrate of PI3K *in vivo* is phosphatidylinositol-4,5-bisphosphate

(PIP2) which is converted to phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylation (66).

The mechanism by which PI3K promotes cell survival involves downstream activation of the protein kinase Akt (PKB). PIP2 and PIP3 lipids produced by PI3K are able to bind Akt, recruiting Akt to the plasma membrane where the phosphoinositide-(3,4,5)-triphosphate-dependent-kinases, PDK1 and PDK2, phosphorylate it to its active form. Activation of Akt promotes multiple effects on cell cycle regulation through phosphorylation and inactivation of the two cell cycle regulators, p27^{KIP1} and p21^{Cip1/WAF1}. Akt activation also prevents degradation of cyclin D1 by inactivation of glycogen synthase kinase 3 β (GSK-3 β) (67-69). Moreover, activated Akt targets proteins involved in cell death, including members of the Bcl-2 family and caspase-9, protecting cells from apoptosis. In addition, PI3K activation is able to increase the expression of members of the Inhibitors of Apoptosis family such as the c-IAPs (70).

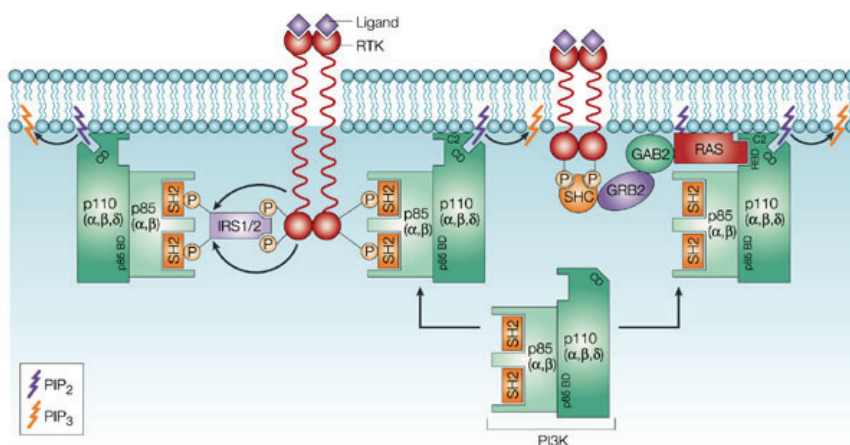


Figure 5. The PI3-kinase/Akt pathway. Reprinted by permission from Macmillan Publishers Ltd : ref. (66), © (2002).

The PI3K pathway is regulated by the tumor suppressor protein PTEN (Phosphatase and tensin homologue deleted on chromosome 10), which dephosphorylates PIP3 (71). Inactivation of PTEN due to mutation or deletion leads to increased activation of Akt

and thereby amplified effect of PI3K signalling (72). Several components of the PI3K pathway, such as Akt and mTor, are often deregulated in cancer, making this pathway an important therapeutic target (66).

In a simplified version, the ERK1/2 pathway and the PI3K pathway act in parallel to promote survival. However, cross-talk between the two pathways has been demonstrated in several cell lines. The balance between ERK1/2 signalling and PI3K signalling depends on the nature of the agonist and the cellular background (73-75).

5.4.1.5. PKC

The protein kinase C (PKC) family is a family of serine/threonine kinases that plays key regulatory roles in a variety of cellular processes, such as proliferation, gene expression, differentiation, apoptosis, malignant transformation and metastasis (76;77), and represents one of the most important mechanisms for signal transduction induced by extracellular stimuli, such as growth factors, hormones and neurotransmitters (78). Once activated, PKC translocates from the cytosol to the cell membrane, where it binds to anchoring molecules called receptors for activated C kinases (RACKs) which localize PKC close to its protein substrates (79).

The PKC family involves 12 isoforms which differ in expression pattern, function, and response to extracellular stimuli (80). Depending on their biochemical properties, co-factor requirements and sequence homology, the PKCs have been divided into three subclasses; the classical PKCs (α , β I, β II, and γ) are Ca^{2+} - and diacylglycerol (DAG) dependent whereas the novel PKCs (δ , ϵ , η , θ , ν , and μ) are Ca^{2+} -independent but respond to DAG. The atypical PKCs (ζ , λ) are insensitive to both Ca^{2+} and DAG (81;82).

Being a target for growth factors as well as a receptor for tumor promoting agents, PKC plays a major role in tumorigenesis (83). The PKC-isoenzymes are ubiquitously expressed, with the classical PKCs as the most abundantly represented. Among these, PKC- α is the most thoroughly studied. Abnormal levels of PKC- α have been found in several transformed cell lines as well as in a variety of human tumor tissues, including malignant melanoma (84). Even though there are conflicting results, the vast majority of

the studies report an association between PKC- α activation and increased motility and invasion of cells, and favour the role of PKC- α as an inducer of proliferation and a suppressor of apoptosis (84). Activation of PKC has been demonstrated to affect both the MAPK/ERK1/2 and the PI3-K pathways, as well as GSK-3 β and nuclear factor kappa B (NF κ B) (83;85;86).

Chemical tumor promoters have been identified as specific PKC activators. Phorbol 12-myristate 13-acetate (PMA) is a tumor promoting phorbol ester which binds to the DAG-binding site of PKC and causes its activation followed by malignant transformation. Tobacco smoke contains PKC activating substances, such as catechols and hydroquinone, considered to act in a tumor promoting way. Moreover, high consumption of dietary lipids may be followed by cancer initiation carried out partly via PKC activation (83).

5.5. FABP7

Fatty acid binding protein 7 (FABP7), also known as brain lipid binding protein (BLBP), belongs to a family of structurally related proteins showing tissue specific patterns of expression. The FABP family is divided into two main groups: those associated with the plasma membrane (FABP_{PM}) and those associated with the cytoplasm (FABP_C) (87). Nine tissue-specific cytoplasmic FABPs have been identified so far; FABP1 - FABP9, mainly expressed in liver, intestine, heart, adipose tissue, epidermis, ileum, brain, peripheral nervous system and testis, respectively (88). FABP7 is highly expressed in radial and Bergmann glia cells throughout the developing nervous system (89;90) and is involved in lipid metabolism, including uptake and intracellular trafficking of fatty acids and retinoids. In addition, the FABP7 protein has been shown to play a role in gene regulation, cell signalling, cell growth and differentiation (88;91). Several members of the FABP family have been reported to be differentially expressed in cancer. The FABP1 level has been shown to decrease with progression of colon cancer (92) while loss of expression of FABP4 was reported in bladder cancer (93). On the other hand, both FABP1 and FABP2 are over-expressed in prostate and breast cancers (94-97), and high FABP7 expression in glioblastomas is related to poor prognosis (98). Moreover, the FABP5 expression level was recently demonstrated to be

increased in prostate cancer as compared to normal tissue and prostatic hyperplasia, and was further associated with poor prognosis (94;99). Two recent studies have addressed the expression of FABP7 in melanoma tissue and cell lines. deWit *et al.* demonstrated that FABP7 was down-regulated in primary and metastatic malignant melanomas compared to benign nevi (100), while Goto *et al.* found FABP7 to be frequently expressed in malignant melanomas and involved in proliferation and invasion of melanoma cells. Hence, it has been suggested that FABP7 plays a role in malignant melanoma tumor progression (101).

5.6. Malignant melanoma

Melanoma is a malignant tumor originating from melanocytes, the pigment-forming cells of the skin. Melanocytes are derived from the neural crest (ectoderm), and during embryogenesis these cells migrate to different body areas, such as the skin, uvea (eye), and mucous membranes. Thus, melanoma can develop at all these sites, although cutaneous melanoma occurs most frequently (102;103).

5.6.1. Melanoma subtypes

Melanomas are morphologically divided into 4 main subgroups; superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma and lentigo maligna melanoma (102;104-106). Superficial spreading melanoma accounts for approximately 70%-80% of all melanomas. Nodular melanoma arises in about 10% of the cases, and is recognized by its rapid growth into a uniformly blue-black dome-shaped nodule (105). Acral lentiginous melanoma is not attributed to sun exposure, and arises predominantly on acral surfaces like palms, soles and under the nails (106). Lentigo maligna melanoma occurs primarily on sun-exposed skin of elderly patients, mainly located on the head and neck. Superficial spreading melanoma, acral lentiginous melanoma and lentigo maligna melanoma initially have a phase of radial growth that may last for weeks, months or years before progression to a vertical growth phase which increases the risk of metastasis. Nodular melanomas do not have this initial radial growth phase (105). This morphological classification has, however, been superseded by a system based on the histopathological parameters of excised lesions. The classification of melanoma is now

based on the vertical diameter of the lesion from the granular cell layer of the epidermis to the deepest detectable melanoma cell (tumor thickness) (107;108).

5.6.2. Epidemiology, etiology and risk factors

There are about 160 000 new cases of malignant melanoma worldwide each year, of which almost 80% are in North America, Europe, Australia and New Zealand. The disease occurs predominantly in white-skinned people (Caucasians), living in countries exposed for high intensity ultraviolet (UV) radiation (2;108). As much as 80% of malignant melanomas are caused by UV-damage to the skin during childhood and adolescence. Other risk factors, although rare, include congenital nevi, immunosuppression and excessive use of solarium (108). Melanoma patients also include those who have a sensitive skin type that freckle easily, a history of sun exposure, multiple common or dysplastic moles, a personal or family history of other types of skin cancer (squamous or basal cell carcinoma) or malignant melanoma (109). Most of the malignant melanomas occur sporadically, while around 5 % are hereditary, so called familial malignant melanoma (110). The incidence of cutaneous malignant melanoma has been rising in most European populations over the past 50 years (2), but accounts for only 4 % of dermatological cancers. However, it is responsible for 80 % of deaths from skin cancer. Only 14 percent of patients with metastatic malignant melanoma survive for five years (111).

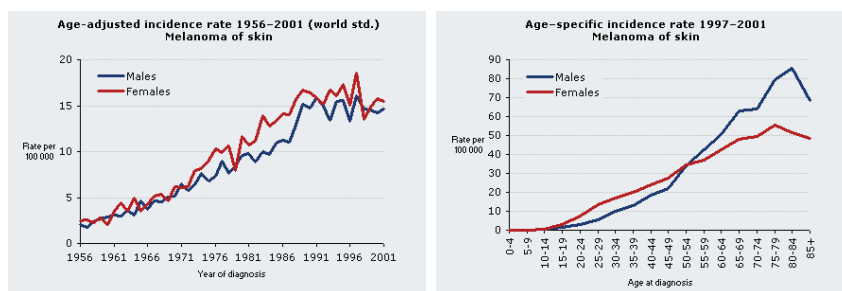


Figure 6. The age-specific incidence rate of melanoma in Norway 1997-2001. Reprinted by permission from the Norwegian Cancer Registry, ref. (112).

5.6.3. Melanoma stages and prognostic factors

The most recent changes to the American Joint Commission on Cancer (AJCC) malignant melanoma staging system was in 2002 (113). Stage I and II tumors are classified based on thickness, according to Breslow (114), with tumors less than 1 mm and between 1 and 4 mm respectively, and no spreading beyond the primary site. At stage III, the tumor involves regional lymph nodes and at stage IV, the tumor involves metastasis at a distant site (115). Tumor thickness together with ulceration, lymph node involvement and distant metastasis are regarded as the the most reliable prognostic predictors currently available in cutaneous melanoma (116;117). In addition, patient age, gender, tumor location, level of invasion, tumor vascularity, lymphovascular invasion and microsatellites have all been shown to correlate with poor prognosis (118;119). The clinical features of melanoma are asymmetry (A), a coastline border (B), multiple colours (C), a diameter greater than 6 mm (D), and as the malignant melanoma progresses, elevation (E) (108). This ABCDE detection system has been the basis for clinical diagnosis for many years. Early melanoma (stage I) is often curable with surgery, with a 5 year survival rate above 88% in men and 90% in women. When spread to regional lymph nodes (Stage III), the 5 year survival is between 50% and 60% for men and women, respectively. Most patients with distant metastases (stage IV) are incurable, and the 5 year survival decreases to 8% in men and 20 % in women (112).

5.6.4. Melanoma progression

Human skin consists of three separate layers: Epidermis, dermis and subcutis. The melanocytes are localized in the lower part of epidermis, in the basal layer which separates epidermis from dermis. This is the site at which most malignant melanomas occur. 70 % of malignant melanomas incidents originate from unblemished skin, while around 30% have their origin in benign nevi (2).

Melanomas are histologically classified according to their location and stage of progression. According to the Clark model of melanoma progression (104) there is a stepwise transformation from normal melanocytes to malignant melanoma (Figure 7). Stage I involves common acquired or congenital benign nevi, stage II involves dysplastic

nevi, with structural and architectural atypia, stage III is termed the radial growth phase (RGP) melanoma, often described as *in situ* melanoma, which grows laterally and remains largely confined to the epidermis. Transition from this stage to the vertical growth phase (VGP) in which the cells both invade the upper layer of epidermis and penetrate into the underlying dermis and subcutaneous tissue through the basement membrane, is the crucial step of the development of melanoma (stage IV). This stage is associated with acquisition of metastatic potential and poor clinical outcome (Stage V) (120).

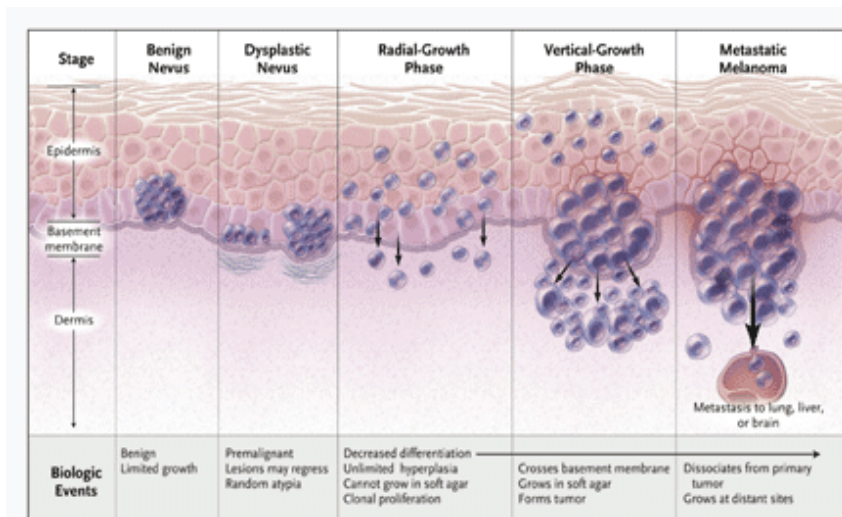


Figure 7. Simplified structure of the skin, and the classical melanoma progression model. Reprinted by permission from Massachusetts Medical Society : ref. (111), © 2006.

5.6.5. Molecular changes in melanoma

5.6.5.1. Familial melanoma

The transition from benign nevi to dysplastic nevi involves molecular alterations that affect cell growth, DNA repair and the susceptibility to cell death.

In 25-40% of familial melanoma cases there is a mutation in *CDKN2A*, the gene encoding for the tumor suppressor genes, *p16^{INK4A}* (INK4) and *p19^{INK4d}* (ARF) (111). Also, 2 % of the families have hereditary mutations in the *CDK4* gene affecting the INK4 binding site (121;122).

The risk of developing a melanoma in *CDKN2A* mutation carriers is high, but it varies with geographical localization as in sporadic melanoma, with increased incidence in Australia (91%) as compared to European countries (58%) (123).

5.6.5.2. Sporadic melanoma

In non-familial melanoma, 20-50% of the patients show an inactivating mutation in the tumor suppressor gene, *PTEN* (Phosphatase and tensin homologue deleted on chromosome ten), leading to over-activation of the PI3K pathway, known to play a major role in melanoma progression (111). Activation of the MAPK pathway is also very common in melanoma (124). In about 15 % of the cases, the activation is caused by mutations in N-RAS (125;126). However, it has been reported that 60-70 % of melanoma patients carry mutations in *B-RAF* (V600E) (127). As mutations in *N-RAS* and *B-RAF* occur independently of each other, almost all melanomas have some degree of MAPK activity (128). So far, *B-RAF* is the only gene known to be altered in the majority of sporadic melanomas (110), however, *B-RAF* is also shown to be mutated in most benign nevi (127-131) and it is suggested that mutated *B-RAF* may induce senescence in these pre-malignant lesions (132).

KIT is a RTK which promotes survival through both the MAPK and PI3K pathways, and amplification and activating mutations of the *KIT* gene are observed in 14% of melanomas (133).

The microphthalmia-associated transcriptional factor (MITF) is essential in melanocytic differentiation, and amplifications of the *MITF* gene have recently been demonstrated in 10% of primary melanomas and 20% of metastatic melanomas. However, its oncogenic function seems to appear mainly in cooperation with mutated B-RAF. Nevertheless, reduction of MITF activity has been shown to increase the sensitivity to

chemotherapeutic agents. Thus, MITF is a potential future therapeutic target in melanoma patients (134).

Cadherins are multifunctional transmembrane proteins that sustain cell-cell contacts, form connections with the actin cytoskeleton and influence intracellular signaling. They are divided into three subtypes: E (epithelial), P (placental) and N (Neural) (111). Progression from RGF to VGF is recognized by loss of E-cadherin and increased N-cadherin expression. By interacting with other N-cadherin expressing cells, such as dermal fibroblasts and the vascular endothelium, melanomas are able to metastasize.

The intracellular domain of E-cadherin is associated with a large protein complex that includes β -catenin, a protein playing an important role in cell adhesion as well as cell signaling. The Wnt/ β -catenin signaling pathway has been shown to be involved in the formation of many cancers, including melanoma, and several of the components in this pathway, such as APC, ICAT, LEF1 and β -catenin itself, are modified during melanoma progression. In addition to its role in cell adhesion, β -catenin signaling is also important in promoting cell proliferation, leading to transcription of proliferation inducing genes like c-Myc, MITF, cyclin D1 and MMP-7 (42;135).

Transition from RGF to VGF is associated with increased expression of the integrin α V β 3, a protein that is responsible for degradation of collagen in the basement membrane via induction of matrix metalloproteinase 2 (MMP-2). α V β 3 ligation also increases the expression of the pro-survival *Bcl-2* gene, and stimulates the motility of melanoma cells through re-organization of the cytoskeleton (111).

6. EXPERIMENTAL CONSIDERATIONS

6.1. Material

The patient material used in paper I, II and IV was selected from a malignant melanoma archive derived from patients undergoing surgery at the Norwegian Radium hospital and regional hospitals since the 1980s. Research on this material has been approved by The Regional Committee for Medical Research Ethics in Norway.

6.2. *In vitro* cell cultures

In order to study biological effects, cells derived from organ tissues are grown as *in vitro* cell cultures. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed.

The most commonly varied factor in culture systems is the growth medium, which supplies nutrients and growth factors. Animal serum added to the media is widely used to supply the cells with hormones that stimulate cell growth and attachment, as well as hormone carrying proteins, lipids and minerals (136). However, when changing batches, and sometimes the supplier of serum as well as media, problems might occasionally occur. We have not identified any differences in our cell cultures due to such changes, but exact growth conditions cannot be guaranteed. Moreover, it is estimated that about 20% of human cell lines are not the kind of cells they were generally assumed to be. The reason for this is that some cell lines, which grow faster and more vigorously than others, can cross-contaminate cultures of other cell lines and in time overgrow and displace the original cells (137).

6.3. Measurement of cell viability and apoptosis

In paper III we measured cell viability and apoptosis using simple ELISA detection systems. Viability was measured using the MTS-assay (Cell Titer Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) which is a colorimetric method for detecting the amount of living cells. The colored end product is linearly

correlated to the dehydrogenase activity in metabolically active cells. However, viable cells are not necessarily the same as proliferating cells, and other methods should be added for evaluating proliferation. Apoptosis was measured using The Cell Death ELISA Plus Kit (Roche Diagnostics, Mannheim, Germany). This assay determines the enrichment of histone-complexed DNA fragments (mono -and oligonucleosomes) in the cytoplasm of apoptotic cells. Although the ELISA results in paper III did correlate with the proliferation assay, the methods have proven to be less accurate, and other methods are needed to validate these results. Thus, in paper IV we used [³H]-thymidine incorporation to measure proliferation rates as well as measuring apoptosis-induced DNA fragmentation by the TUNEL (terminal deoxynucleotidyl transferase end labeling) assay. Moreover, viability of cells was always evaluated visually, in light microscope. Occasionally, the cells were stained with Tryphan blue, which is incorporated in dead cells, and blue cells were counted to get a more exact overview of the amount of dead cells. The results were further confirmed by detecting protein expression of proliferation and apoptotic markers by Western blotting.

6.4. Transfection experiments

RNA interference (RNAi) represents a form of post-transcriptional gene silencing (PTGS). Artificially synthesized single interference RNA (siRNA) is incorporated into a RNA-induced silencing complex (RISC) which probes the target mRNA for complementary sequences and then mediates mRNA cleavage followed by gene silencing. Antisense oligonucleotides are also used in this manner. Antisense-RNA bind to mRNA strands and prevent protein translation while antisense DNA can be used to target a specific, complementary RNA sequence. Transfer of siRNA or antisense oligonucleotides into cells has now become a routine tool for investigating gene function and biological pathways.

In paper III we used antisense oligonucleotides targeting PKC- α . The set of oligonucleotides as well as procedure were chosen based on the findings of Dennis *et al.*, who first reported decreased malignancy in nude mice following PKC- α downregulation in human melanoma cells (138). Simultaneously, the same oligonucleotides were used in a clinical trial at the Norwegian Radium Hospital. In

paper IV we used transient transfection of FAPB7 specific siRNA (Stealth RNAi, Invitrogen, San Diego, CA) in order to study the effect of down-regulation of FAPB7 in the early stage melanoma cell line WM35 and the metastasis-derived cell line WM239. The method was optimized to achieve the greatest amount of target-specific knock-down of expression, as well as a low rate of cell death in control cells. We transfected the cells with three different sets of oligonucleotides at three different concentrations (200 nM, 100 nM and 50 nM). The best results were obtained with 50 nM siFABP7 oligonucleotide set #1 or Stealth RNAi negative control duplex (Medium GC Duplex) in combination with 50 nM LipofectamineTMRNAiMAX transfection reagent (all reagents from Invitrogen). The negative siRNA control affected cell viability as the amount of living cells decreased compared to untreated cells. Hence, the effect of siFABP7 had to be related to the negative siRNA control and not to untreated cells.

6.5. Microarray

In paper IV we used gene expression profiling (Affymetrix) to identify differentially expressed genes in WM35 cells grown as multicellular aggregates (spheroids) after treatment with the PKC activator, PMA, and/or the MEK1 inhibitor, PD98059. cDNA microarray expression analysis was performed using commercial slides (Affymetrix U133 Plus 2.0 arrays, Affymetrix, Santa Clara, CA), containing 47 000 probes of high specificity. Three hybridizations were performed, from three biological experiments, using untreated monolayer WM35 cells as a reference. The analysis was done commercially, and resulted in a variety of up and down-regulated genes. To avoid false positive results a 2.5 fold change in ratio was defined as a cut off, which is quite strict, and it should be taken into consideration that other interesting genes might have been excluded. The microarray results needed to be confirmed, and both real- time RT-PCR and Western blotting were used to validate selected results.

6.6. Immunohistochemistry

In paper I, II and IV we performed immunohistochemistry on paraffin embedded tissue using commercially available polyclonal antibodies against pJNK, p38 and FABP7. pERK1/2 was detected with a monoclonal antibody. Cross-reactivity might occur due to sequence homology between different proteins. To validate the specificity of our

antibodies, western blot was performed to confirm the size of the protein. The immunohistochemical methods were optimized by qualified persons. All series included positive control samples. Negative controls included substitution of the primary antibody with mouse myeloma protein of the same subclass and concentration as the mono/polyclonal antibodies used. All controls gave satisfactory results. In paper I we used the biotin-streptavidin-peroxidase (ABC) method for detection of activated ERK1/2. This method is still in widespread use, but the presence of endogenous biotin in tissues can lead to increased background staining. Formalin fixation and paraffin embedding decreases the endogenous biotin level, but the biotin might recover when the tissue sections are treated with heat prior to hybridization. To avoid these limitations, methods that do not rely on biotin have been developed. The EnVision+ system, used in paper II and IV, is based on a polymer backbone which is attached to multiple enzymes as well as secondary anti-mouse or anti-rabbit antibodies. Therefore, the method can be used to detect any tissue bound protein of mouse or rabbit origin. Besides being both easier and more time saving, EnVision + has also been shown to be equally, if not more sensitive than biotin based methods. The substrate used also varied between the experiments. In paper I and paper IV, diaminobenzidine tetrahydrochloride (DAB) was used, and since the resulting staining is brown, it can be difficult to separate stained proteins from melanin within the cells. Therefore we also tried 3-amino-9-ethylcarbazole (AEC), which gives a red staining. However, the scoring was done by an experienced pathologist, and should be reliable regardless of the substrate used. Since only 1 of 10 000 cells in a primary tumor is able to metastasize, it is important to identify tumor subpopulations that have markers of increased aggressiveness. We therefore chose the 5 % cut off to distinguish between negative and positive cells.

7. BRIEF SUMMARY OF PAPERS

Paper I: Expression of activated extracellular signal-regulated kinases 1/2 in malignant melanomas: Relationship with clinical outcome.

The protein expression of activated extracellular signal-regulated kinases 1 and 2 (ERK1/2) in a panel of benign nevi, primary and metastatic melanomas was examined by immunohistochemistry and correlated to clinicopathological parameters and cell cycle markers. We found a heterogeneous expression of activated ERK1/2 in primary and metastatic lesions, with a decrease in the expression level in the metastases compared to the primary melanomas. No immunoreactivity was detected in benign nevi. When analyzing the melanoma subgroups separately, we found that activated ERK1/2 expression varied significantly with the thickness of superficial spreading melanomas, with lower expression in thinner lesions (Mann-Whitney $P=0.016$). We also observed significant correlation between activated ERK1/2 and cyclin D1 ($P=0.031$) in nodular as well as between activated ERK1/2 and cyclin D3 ($P=0.030$) in superficial spreading primary melanomas. The protein level of p27^{Kip1} correlated with nuclear ERK1/2 activation ($P=0.048$) in superficial spreading melanomas. Furthermore, a strong correlation between activated ERK1/2 and membrane bound β -catenin ($P=0.004$) in nodular melanomas was revealed. Activation of ERK1/2 did not have any impact on relapse-free or overall survival. In conclusion, these results suggest that activation of ERK1/2 may be involved in cell cycle regulation in superficial spreading melanomas. In the nodular subtype ERK1/2 activation might lead to destabilization of membrane bound β -catenin.

Paper II: Activation of c-Jun N-Terminal Kinase (JNK) is associated with cell proliferation and shorter relapse-free period in superficial spreading malignant melanoma

The panel of benign nevi, primary and metastatic melanomas were immunohistochemically analyzed for expression of the activated MAPK family members, p38/MAPK (p-p38) and JNK (p-JNK). The findings were correlated with known prognostic variables and cell cycle markers. There was a heterogeneous

expression of both p-JNK and p-p38, with increased expression in the metastases for both proteins. Surprisingly, while only 7 % of the benign nevi showed positive immunostaining for p-p38, 73.5% of the benign nevi showed strong activation of JNK. When separating the melanoma subgroups, we found that high level of cytoplasmic p-JNK was associated with thicker tumors ($P=0.017$) and shorter disease-free survival ($P=0.003$) in patients with superficial spreading melanomas. Moreover, cytoplasmic p-JNK correlated with cyclin A ($P=0.017$) and p21 ($P=0.021$) in the same patient group. In patients with nodular melanomas, nuclear p-p38 was associated with Ki-67 expression ($P=0.012$), but neither cytoplasmic nor nuclear localized p-p38 was associated with disease outcome. All three MAPK signaling pathways were positively correlated to each other in superficial spreading melanoma, and p38 activation was also positively associated with p-Akt ($P=0.047$) in this melanoma subgroup. In contrast, except for a positive correlation between nuclear p-p38 and membranous p-TrkA ($P=0.02$), no correlation between the activation status of the different signaling pathways was observed in nodular melanomas. In conclusion, our results suggest that activated JNK in benign nevi may have a role in restricting uncontrolled cell proliferation or survival. However, during tumor progression the functional role of JNK activation is switched, and activated JNK is associated with cell proliferation and shorter relapse-free period. Altogether, these results suggest that the JNK activation status may be a marker for clinical outcome for patients with at least the superficial spreading subgroup of malignant melanoma. The activation status of p38, on the other hand, seems to play a less important role in development and progression of malignant melanomas.

Paper III: Phorbol ester phorbol-12-myristate-13-acetate promotes anchorage-independent growth and survival of melanomas through MEK-independent activation of ERK1/2

In this study we examined the effect of the PKC activating tumor promoting phorbol ester, PMA, on proliferation and survival of melanoma cells grown as multicellular aggregates in suspension (spheroids). In contrast to what was found in monolayer cells, PMA increased cell proliferation in spheroids. Interestingly, we also observed that PMA protected melanoma cells against suspension induced apoptosis (“anoikis”). To further elucidate whether PKC was of importance for the ability of melanoma cells to proliferate and survive in suspension, anoikis-resistant melanoma cell lines were

transfected with antisense oligonucleotides directed against the PKC- α isoenzyme or treated with the general PKC inhibitor Gö6976. We found that PKC down-regulation was associated with a strong induction in suspension induced cell death. Of particular interest, we provided evidence of PMA-mediated activation of ERK1/2 independently of its upstream activator, MEK1/2. In conclusion, our results suggest that PMA increases anchorage-independent growth at least partly through PKC activation and MEK-independent activation of ERK1/2.

Paper IV: The fatty acid protein 7 (FABP7) is involved in proliferation and invasion of melanoma cells

We used gene expression profiling (AffymetrixTM) in order to identify genes that are involved in PMA induced survival of melanoma spheroids (Paper III). Among the most significantly differentially expressed genes was the *fatty acid-binding protein 7 (FABP7)*. We found that FABP7 mRNA and protein levels were down-regulated following treatment of melanoma cell lines with PMA and/or the MEK1 inhibitor PD98059. Moreover, down-regulation of FABP7 using siRNA decreased cell proliferation and invasion, but did not affect apoptosis. The protein expression of FABP7 were immunohistochemically analyzed in a panel of benign nevi, primary and metastatic melanomas and correlated to clinicopathological parameters and cell cycle markers. FABP7 was heterogeneously expressed, with the highest expression levels in nevi compared to primary and metastatic lesions. In superficial spreading melanomas we observed positive correlation between cytoplasmic FABP7 and tumor thickness (P = 0.021) as well as a trend for correlation with Ki-67 scores (P = 0.070). A trend for negative association between cytoplasmic FABP7 expression and relapse-free survival (P = 0.069) was also observed in this patient group. Taken together, these results suggest that FABP7 can be regulated by both PKC and the MAPK/ERK1/2 pathway in melanoma cells, but is not involved in anoikis. Furthermore, FABP7 is involved in cell proliferation and invasion *in vitro*, and may be associated with melanoma progression.

8. RESULTS AND DISCUSSION

8.1. Expression and subcellular localization of MAPK

In paper I and paper II we reported the immunohistochemical staining of activated ERK1/2, JNK and p38 in a panel of primary and metastatic human malignant melanomas as well as benign nevi. In agreement with other studies (60;139-146), we observed a heterogeneous cytoplasmic and/or nuclear staining pattern for p-Erk1/2 (54%), p-JNK (35%) and p-p38 (25%) in the primary malignant melanomas. Activated ERK1/2, p38 and JNK was found in cytoplasm alone in 21 %, 13 % and 6 % of the tumors, respectively, whereas exclusively nuclear staining was found in 3 % for p-ERK1/2, 7 % for p-p38 and 21 for p-JNK.

Interestingly, a lower number of tumors expressed activated ERK1/2, p38 and JNK in the metastases compared to the primaries, as only 33 %, 9 % and 25 % showed positive immunostaining in the cytoplasm and/or nucleus, respectively. The reason for this decrease needs to be elucidated, but there has been increasing evidence that the MAPK phosphatases (MKPs/DUSP-proteins), which negatively regulate MAPKs, can be abnormally regulated in certain types of cancer (147). Thus, it may be speculated that over-expression of MKPs can contribute to dephosphorylation and inactivation of MAPKs in the metastases.

While several studies have revealed that B-RAF mutations are very frequent in both malignant melanoma and benign nevi (127-131), our results showed no immunoreactivity of p-ERK1/2 in benign nevi (paper I). This observation is supported by other studies (148-150), strongly suggesting that B-RAF mutation is not sufficient to activate the RAS-RAF-MEK-MAPK pathway in melanoma tissue. The lack of correlation between B-RAF mutations and activation of ERK1/2 could be explained by additional inhibitory mechanisms that suppress the expression or activity of B-RAF or its downstream targets (148). Melanocytes, like other normal cells, have defense mechanisms against oncogenic activation, and involved in this defense are the MKPs, resulting in dephosphorylation and inactivation of MAPK kinases (147). It has previously been shown that MKPs are not present in atypical nevi, further suggesting

that loss of MKP activity may be a part of early melanomagenesis (151). To the best of our knowledge, there have been no studies on MKP expression in benign nevi, but it is reasonable to suggest that MKPs have a protective role by inactivating MAP kinases in these lesions.

Another inhibitory molecule that could explain the lack of ERK1/2 activity in benign nevi is the RAF kinase inhibitor protein (RKIP), which prevents MEK activation by RAF-1. Recently, it was shown that the expression of RKIP decreased in primary melanoma tissue as compared to benign nevi (152). Finally, most benign nevi do not progress to melanoma but are growth arrested, establishing a senescent state which needs to be evaded before further melanoma progression (153). It has been questioned whether this senescence is induced by B-RAF. For example, it has been shown that sustained B-RAF expression in human melanocytes induces cell cycle arrest which is accompanied with hallmarks of senescence, like up-regulation of p16^{INK4} and senescence associated acidic β -galactosidase (SA- β -GAL) (154). In a recent study it was revealed that oncogenic B-RAF induced senescence and apoptosis through an autocrine/paracrine pathway which involved insulin growth factor binding protein 7 (IGFBP7) (155).

It has also been suggested that Akt3 in the PI3K pathway cooperates with mutated B-RAF in promoting melanoma development by directly phosphorylating and thereby inactivating the B-RAF protein, decreasing ERK1/2 activity to a level that enhances rather than inhibits melanocytic cell proliferation (156).

Only 7 % of the nevi showed positive immunostaining for p-p38 (paper II). Similarly to ERK1/2 activation, the biological effect of p38 depends on the strength and duration of its activity. Following stimulus, p38 is transiently activated within a very short time (157). Supporting our suggestion that the MKPs are active in benign nevi, it is reasonable to hypothesize that p38 is instantly dephosphorylated in early stage melanoma. In contrast, a total of 74 % of the nevi were positive for p-JNK, a finding which is contradicted by Kunz *et al.* (158) who showed no p-JNK immunoreactivity in

benign nevi. This discrepancy, however, could be explained by the small number of nevi tested by Kunz *et al.* compared to our study (8 vs.27). Moreover, it is possible that they have used a higher cutoff, which will define a larger number as negative. Further supporting our findings is the fact that benign nevi are located on the skin surface where they are thoroughly exposed to UV-radiation. UV exposure may result in JNK activation followed by induction of c-jun, activation of the AP-1 transcription factor complex and apoptosis (159;160). As discussed by Shen *et al.* (161), sustained activation of JNK has been shown to suppress ERK1/2 activation in transformed liver cell lines, a finding which may partly explain the high expression of p-JNK and the low expression of p-ERK1/2 in benign nevi. Interestingly, JNK activation in the benign nevi was solely localized to the nucleus, which suggests that JNK is involved in transcriptional regulation. Our results, altogether, suggest that neither ERK1/2 activation nor p38 activation play key-roles in the early stages of melanoma development, and that the profound JNK activation has a protective role restricting uncontrolled growth or survival of the nevus cells.

8.2. MAPK in a clinical setting

In paper I and II we examined whether activation of the different MAPK pathways has any impact on clinical outcome for melanoma patients. As described in the chapter above, there was a decline in ERK1/2 activation as well as in JNK and p38 activation in the metastases compared to the primary melanomas. These results are, however, supported by studies showing an inverse correlation between ERK1/2 activation and progression of prostate cancer (162;163). Similarly, deactivation of JNK and p38 concomitant with increased tumor aggressiveness has been previously described in rat prostate and human colon cancer (164;165) and JNK activation has been associated with early-stage non-small cell lung cancer (145). Deactivation of p38 has also been associated with progression of hepatocellular carcinomas (164). Hence, it has been speculated that deactivation of p38 and JNK in advanced tumors provides cells with an anti-apoptotic mechanism and growth advantage (52).

The ERK1/2 pathway and the PI3K pathway are both activated in different types of cancer (53;166), and cross-talk between these two pathways has been reported (167-169). It has been shown that inactivation of ERK1/2 by the PI3K pathway accompanies

disease progression, and that phospho-Akt is able to inactivate RAF and thereby its downstream targets (17;162;163;170). This is, however, contradicted by our group's recent findings, showing activated Akt in both benign nevi and in primary and metastatic melanoma tissue, as well as a positive association between p-Akt and p-ERK1/2 in superficial spreading melanomas (171). The positive correlation between these two pathways may be explained by their simultaneous parallel activation instead of the influence on each other (171).

Although our panel of melanomas has not been examined for over-expression or mutations in the RAS genes, it is of interest to note that N-RAS mutations have been associated with better clinical outcome for melanoma patients (172-174) which may partly explain the observed decline in the activation of ERK1/2, JNK and p38 in metastases.

Our results showed that activated ERK1/2 expression varied significantly with tumor thickness for patients with superficial spreading melanoma, with less expression in thinner tumors. No association to relapse-free or overall-survival was, however, revealed (Paper I). These results are supported by a study by Branca *et al.* who reported no association between phosphorylated ERK1/2 and clinical outcome in cervical cancer (175), but are in contrast to studies on pancreatic carcinoma and liver cancer, in which poor prognosis was associated with ERK1/2 activation (60;141;143;176;177). Similarly, in a study by Svensson *et al.* (178), ERK1/2 activation correlated with smaller tumors and a less aggressive tumor type, as well as with better prognosis in untreated breast cancer patients. *In vitro* studies, however, suggest that sustained activation of ERK1/2 may lead to transformation in some but not all cell types (59). Together, these results suggest that the importance of the ERK1/2 signaling pathway in tumor progression may be tumor specific.

In paper II we showed that high levels of cytoplasmic p-JNK was associated with thicker tumors and shorter relapse-free survival in patients with superficial spreading melanoma, while there was no correlation between p38 activation and clinical parameters. Earlier studies have demonstrated that activation of JNK and p38 is associated with poor prognosis for patients with breast cancer (140;179), while one study also showed correlation between JNK phosphorylation and prolonged recurrence-

free survival (180). Moreover, in a study by Aguirre-Ghiso *et al.* (181), they report that in breast, prostate, melanoma and fibrosarcoma cell lines, the ratio between ERK1/2 and p38 signaling was significant for cell behaviour and prognosis. A high ERK1/2/p38 ratio enhanced cell proliferation and growth while an inversion of this ratio caused cell cycle arrest and dormancy *in vivo*. In contrast to the other cell lines tested in the study by Aguirre-Ghiso *et al.*, the melanoma cell line used (M24met) seemed to escape this negative feedback mechanism by p38, and remained tumorigenic. However, we cannot conclude that the ratio between ERK1/2 and p38 signaling is of no importance in our melanoma panel, based on previous results from one, single cell line.

About half of the p38 substrates identified so far are transcription factors, pointing at p38 in having a role in gene regulation at a transcriptional level (182). However, with only a small proportion of melanoma specimens showing positive p38 immunostaining, and with an even smaller percentage showing nuclear expression we suggest that gene regulation is not a major task for p38 in malignant melanoma. Moreover, Aguirre-Ghiso *et al.* suggested that the p38 pathway may be altered or dysfunctional in melanomas (181). Also, p38 is stimulated by a multiplicity of external factors, mediating a variety of p38 signaling pathways, which is dependent on cell type (182).

Like p38, JNK has many nuclear substrates with both tumor suppressive as well as oncogenic effects. It has been emerging evidence that phosphorylation of non-enzymatic scaffold or adaptor proteins by JNK and other MAP kinases may alter the interactions with their binding partners and thereby mediate changes in the signaling pathways (56). With this in mind, it is possible for cytoplasmic JNK to alter its signal transduction pathway and thereby induce cell transformation.

Taken together, our results suggest different roles of the MAPKs in disease progression, and that more than one signaling pathway is involved in development and progression of a malignant phenotype.

8.3. Relationship between the expression of MAPK and cell cycle markers

Inappropriate regulation of the cell cycle has been demonstrated to play a major role in the tumorigenesis of human cancers. In paper I and II we explored the relationship between MAPKs and cell cycle markers in melanoma tissue. Several studies, including work from our own group, have shown that cyclin D1 is up-regulated in primary and metastatic melanoma as compared to benign nevi, suggesting that cyclin D1 is associated with proliferation during melanomagenesis (183-186). Knowing that activated ERK1/2 could be seen as a proliferative marker in melanoma, we expected an association between cyclin D1 and p-ERK1/2. We observed significant correlation between activated ERK1/2 and cyclin D1 in nodular melanomas, but surprisingly, only cyclin D3 and not cyclin D1 was associated with activated ERK1/2 in superficial spreading melanomas. So far, little is known about the interplay between cyclin D3 and cell signaling in human cancer. We have previously reported an association between the protein level of cyclin D3, cell proliferation and disease progression in superficial spreading melanoma, and suggested that cyclin D3 is the most frequently expressed D-type cyclin (183). Supporting these findings, other studies have suggested the D-type cyclins to be differentially expressed in cancer, thereby playing different roles (187;188). Thus, it may be speculated that in superficial spreading melanomas, ERK1/2 activation may induce cyclin D3 expression, thereby leading to increased cell proliferation and tumor thickness. The lack of association between cyclin D1 and ERK1/2 activation in this melanoma subgroup is also supported by others, showing that in a melanoma cell line incubated with a MAPK inhibitor, expression of cyclin D1 remained unchanged (124). Several studies have provided evidence that the induction of cyclin D1 and thereby progression through G1 requires only moderate ERK activity, but the activity has to be sustained for several hours (189). It may therefore be speculated, that the duration of MAPK activation is not optimal to induce cyclin D1.

Also in support of ERK1/2 regulating cell proliferation in melanoma, we observed a trend for correlation between activated ERK1/2 and cyclin A as well as between activated ERK1/2 and Ki-67 in superficial spreading melanomas. We have previously suggested that both cyclin A and Ki-67 may be prognostic markers for patients with superficial spreading melanoma (190). In paper II, we found significant positive

association between cytoplasmic p-JNK and cyclin A and p21^{WAF1/CIP1} in superficial spreading melanomas as well as between activated p38 in the nucleus and Ki-67 in nodular melanomas. In agreement with our results, Margheri *et al.* (191) observed a decline in JNK phosphorylation concomitantly with inhibition of cyclin D3 and cyclin A in response to blocking the urokinase receptor. Moreover, Nuntharatanapong *et al.* (192) and Kim *et al.* (193) reported positive correlation between the cdk-inhibitor p21^{CIP1/WAF1} and cytoplasmic p-JNK. In contrast to p-JNK, p38 activation was not associated with markers of cell cycle progression or disease outcome in superficial spreading melanomas (Paper II). In nodular melanomas, on the other hand, the presence of activated p38 in the nucleus was associated with Ki-67 expression. However, we have previously shown that the level of Ki-67 has little impact on disease progression of this subtype (190). Together these findings suggest that p38 does not play a major role in development or progression of melanocytic tumors and that the observed activation is rather an effect of other signaling pathways. As we also report a positive association between activation of ERK1/2, JNK and p38, it is reasonable to suggest a positive cross-talk mechanism between the three different MAPKs. In addition, we have to take into consideration that over-expression of MKK kinases is followed by activation of p38 as well as JNK, which might explain why these two pathways are often co-expressed (182).

8.4. ERK1/2 is associated with β -catenin

β -catenin signaling has been shown to be involved in several types of cancer, including melanoma, and in addition to its role in cell adhesion, β -catenin also promotes cell proliferation through activation of transcription factors (135). We have previously shown inverse association between cytoplasmic β -catenin and cyclin D1 in superficial spreading melanoma and hypothesized that β -catenin had a protective role in early melanoma development (194). This spurred us to further investigate whether activation of the MAPK pathways had any impact on the expression of β -catenin.

In paper I we observed inverse correlation between ERK1/2 activation and membrane-bound β -catenin ($P=0.004$) in nodular melanomas. We suggested that destabilization of β -catenin following direct or indirect phosphorylation by active ERK1/2 was leading to decreased homeotypic interactions and increased invasive potential in this melanoma subtype. Interestingly, in a recent study using a mouse melanoma model, Delmas *et al.*

(195) reported that β -catenin induced immortalization of melanocytes by suppressing p16^{INK4} expression and that β -catenin promoted melanoma progression in cooperation with N-RAS. It must be considered that when our melanoma panel was examined for β -catenin expression, only 8 % of the primary lesions were positive, and since the level of nuclear staining always was in agreement with the cytoplasmic staining, only cytoplasmic and membrane-bound expression was included in the statistical analysis (194). A hallmark for the activation of wnt/ β -catenin signaling in melanoma is the presence of β -catenin in the nucleus, and about 30 % of melanomas express nuclear β -catenin, suggesting an important role for this signaling pathway in melanoma (135). It would therefore be interesting to examine whether ERK1/2 activation is associated with the wnt/ β -catenin signaling pathway. However, we will need to expand our melanoma panel to elucidate this.

We did not find any association between activated JNK or activated p38 and the expression of β -catenin, but a potential correlation cannot be excluded since nuclear expression of β -catenin was not a part of this study.

8.5 PKC and its influence on MAPK signaling

Several studies have documented the importance of PKC activation in regulating cell growth, differentiation and survival (196). It has been anticipated that increased activation of PKC- α is associated with melanoma progression (84;197-199). In paper III we explored the effect of phorbol-12-myristate-13-acetate (PMA), a potent activator of classical and novel PKC isoforms, on growth and survival of human melanoma cells grown as multi-cellular aggregates (spheroids). We found that PMA, in contrast to monolayer cultures, increased cell proliferation and protected cells from suspension-induced apoptosis (anoikis). To investigate the impact of PKC in this process, we knocked out PKC- α using antisense oligonucleotides. Anoikis was strongly induced, a finding also supported by Dennis *et al.* (138), who reported that transfection of human melanoma cell lines with antisense oligonucleotides against PKC- α suppressed metastasis formation in nude mice. We observed, furthermore, that PMA induced anchorage-independent growth and reversed the down-regulation of ERK1/2 caused by MEK inhibition, suggesting a MEK-independent activation of ERK1/2. To further

ensure that the reversible effect of PMA on ERK1/2 activation was mediated by PKC, melanoma cells were grown in the presence of both PD98059 and a potent inhibitor of all classical PKCs, Gö6976. ERK1/2 can be activated by a variety of signaling events. Thus, Gö6976 alone had no effect on ERK1/2 phosphorylation. However, in combination with PD98059, Gö6976 at least partly prevented PMA-mediated reactivation of ERK1/2. The mechanism for this alternative ERK1/2 activation remains to be elucidated. However, a possible explanation might be drawn by linking our results from paper II and III together. In paper II, we demonstrated that the JNK and ERK1/2 pathways are positively correlated to each other. According to Buchner *et al.*, (200), the phorbol ester TPA (with similar functionality as PMA) induces JNK activation via the classical PKC isoforms, and we may speculate that the MEK-independent activation of ERK1/2 upon PMA treatment progresses via the JNK pathway. Moreover, the protein tyrosine kinase Src plays a role in cell proliferation, differentiation, adhesion and migration, and change in Src activity is involved in growth, progression and metastasis in several human cancers, including melanoma (201;202). Recent research has shown that increased Src activity protects lung adenocarcinoma and colon cancer cells from anoikis (203;204). PKC is a known activator of Src, and we wanted to investigate whether Src is our missing link in MEK-independent ERK1/2 activation and anoikis resistance in melanomas. However, preliminary experiments do not support this hypothesis at present.

It can also be questioned whether the inhibitors used to downregulate ERK1/2 activation show the appropriate selectivity. PD98059 inhibits MEK1 only, and even though ERK1/2 is significantly downregulated using this inhibitor, it is still a possibility that the reversible effect of PMA progresses through MEK2. However, using U0126, a more selective inhibitor of both MEK1 and MEK2, we obtained the same results.

Following PMA treatment we also observed increased phosphorylation and deregulation of the pro-apoptotic proteins Bad and Bim, which was related to increased activation of ERK1/2 and anoikis resistance (Paper III). In support of this, a recent study reported that B-RAF mutations induced phosphorylation of Bad and Bim, leading to increased anchorage-independent growth of melanoma cells. Moreover, depletion of both Bad and Bim further increased the protection of B-RAF knock-down cells from anoikis,

suggesting that the Bad and Bim proteins play a major role in B-RAF regulated apoptosis in melanoma cells (38).

Upon activation, PKC will fulfill its effect within a very short time before the protein is degraded in the cytoplasm (205-208). In this regard, our group observed increased activation of ERK1/2 even after 5 minutes of PMA treatment (data not shown). It is uncertain whether the effect of PMA is due to activation or down-regulation of PKC. For instance, Lu *et al.* (209) showed that 3Y1 rat fibroblasts are anoikis resistant when treated with TPA. When Bryostatin 1, previously shown to prevent tumor promotion by TPA, was added to TPA treated and untreated cells, they observed increased anoikis and that down-regulation of PKC- δ , but not the other isoforms expressed in this cell type, was prevented. Hence, they concluded that down-regulation of PKC- δ was responsible for the tumor-promoting effect of TPA in rat fibroblasts. Our results in paper III, however, strongly suggest that PKC activation is responsible for the observed effects of PMA.

8.6. FABP7 in melanoma

In paper III we reported that the PKC activator PMA increased cell proliferation and promoted anchorage-independent survival of melanoma cells. Furthermore, this protective action seemed to be at least partly mediated through PKC and MEK-independent activation of MEK. In an attempt to identify other genes involved in survival and apoptosis of melanoma cells, high throughput gene expression profiling was used to identify differentially expressed genes in untreated cells cultured as monolayer and spheroids, as well as in spheroids treated with PMA and/or the MEK inhibitor PD98059 (paper IV). Among the genes showing highest differentiation was fatty acid binding protein 7 (FABP7), a gene that is normally expressed in radial glial cells in the brain. However, two recent studies have reported FABP7 expression in melanoma (100;101) urging us to pursue this finding. Melanocytes originate from progenitor neural crest cells, which also have the potential to differentiate into glial cells, and melanoma and glioma cells have many tumor-associated markers in common

as well as similar biological properties (210). Hence, the observed expression of a brain specific protein in melanoma could be explained by the common origin of melanocytes and glial cells. We observed that FABP7 was down-regulated following both PKC activation and inhibition of MEK1, but knowing that the two treatments have an opposite effect on anchorage-independent survival (paper III), this observation argues against FABP7 involvement in promotion of anchorage-independent survival in melanoma cells. We further observed that PMA treatment down-regulated FABP7 even in the presence of activated ERK1/2, and suggest that this down-regulation is likely to be PMA/PKC-mediated but MAPK-independent. We therefore hypothesize that FABP7 can be regulated independently by both signaling pathways in melanoma cells.

As discussed in paper IV, it has been shown that activation of the MAPK pathway can induce increased activity of peroxisome proliferator-activated receptors α/γ (PPAR α/γ). Similarly, PKC can both positively and negatively regulate PPAR α -dependent transcription (211-213). Binding of PPAR γ to its response element, PPRE, has been shown to up-regulate FABP1 and FABP4 (88;214;215). It is reasonable, therefore, to assume that FABP7 might also be regulated through this mechanism.

To further clarify the role of FABP7 in melanomas we used siRNA to down-regulate its expression in the primary WM35 and metastatic WM239 melanoma cell lines (paper IV). This down-regulation significantly inhibited proliferation in both cell lines without affecting the degree of apoptosis. Moreover, we showed that down-regulation of FABP7 negatively influenced the invasive potential of melanoma cells. Our results are supported by Goto et al. who demonstrated increased proliferation and unaffected apoptosis, as well as reduced invasiveness, in 2 of 6 melanoma cell lines following FABP7 down-regulation (101). Together these results suggest a role for FABP7 in regulation of proliferation and invasion in melanoma.

Several members of the FABP family have been reported to be differentially expressed in cancer. Loss of expression of FABP4 has been reported in bladder cancer and FABP1 and FABP2 have been shown to be over-expressed in prostate and breast cancers (94-97). We also found, in accordance with others (101), that FABP7 was expressed in both primary and metastatic melanoma cell lines. Moreover, in clinical samples, FABP7 protein expression was increased in nevi compared to primary and metastatic melanoma,

while there was no difference in FABP7 expression between the latter two. This is further supported by another microarray study where they reported that FABP7 is down-regulated in malignant melanoma compared to nevi (100). The higher expression of FABP7 in nevi compared to malignant melanomas seems contradictory to the *in vitro* data in the present study, as well as to the association with clinical parameters of disease progression. We are unable to explain this discrepancy at present. However, the majority of benign nevi are terminal lesions that do not progress to melanoma and it is possible that the molecular events regulating these processes differ.

Analysis of the clinical data showed that thicker superficial spreading melanomas expressed higher levels of FABP7 (paper IV). We also observed a trend towards association between high levels of FABP7 and reduced disease-free survival, as well as a positive trend between FABP7 and the proliferation marker Ki-67 in these patients. This suggests that FABP7 may contribute to disease progression, possibly by increasing the cell proliferation and invasive potential of melanoma cells.

In our melanoma cohort we observed heterogeneous expression of the FABP7 protein. Such variation in sub-cellular localization of FABP7 has also been reported in developing radial glia cells, glioma cell lines (89;216) and glioblastoma multiforme (GBM) specimens (217). Since FABP proteins are considered to be co-activators in PPAR-mediated gene transcription control, this could in part explain FABP7 translocation to the nucleus (88). However, nuclear FABP7 expression did not correlate with the examined markers or with clinical parameters, which indicates that FABP7 in this compartment is of little importance in melanoma progression.

9. CONCLUSIONS

In this thesis we have tried to reveal some of the signaling mechanisms underlying melanoma development and progression. We show that the MAPK signaling pathways, represented by ERK1/2 and JNK, are both involved in this process. We suggest that activation of ERK1/2 is associated with cell cycle regulation in superficial spreading melanomas and also with β -catenin destabilization and decrease in homotypic interactions in nodular melanomas (Paper I). Moreover, while activated JNK plays a role in restricting uncontrolled cell proliferation in benign nevi, its functional role is switched during tumorigenesis, and JNK activation is associated with cell proliferation and shorter relapse-free survival in patients with superficial spreading melanoma (Paper II). Activation of p38 does not seem to play an important role in melanoma progression.

PMA induces anchorage-independent survival through MEK1-independent ERK1/2 activation (Paper III). In our search after genes involved in anoikis resistance, microarray studies revealed that FABP7 is differentially expressed in melanoma cells treated with PKC activating and/or MEK1/2 inhibiting agents (Paper IV). We conclude that FABP7 seems to be regulated both by PKC and ERK1/2 through independent mechanisms. Moreover, FABP7 is not involved in anoikis resistance, but still we show that FABP7 is involved in regulation of cell proliferation and invasion. Finally, FABP7 is associated with poor prognosis in patients with superficial spreading melanoma.

Alltogether, our results point to the ERK1/2 and JNK signaling pathways, as well as PKC and FABP7, as potential targets for melanoma therapy.

10. FUTURE ASPECTS

From our studies we know that PMA mediates MEK1-independent ERK1/2 activation and survival. To ensure that the observed effect of PMA is actually independent of MEK and is not due to some unspecific effects of the inhibitors, we will use siRNA oligonucleotides directly targeted against MEK1/2 and then perform the same experiments as with the cells treated with pharmaceutical MEK inhibitors.

The pathway from PKC to MEK-independent activation of ERK1/2 remains to be elucidated. However, our microarray study has provided us with a great amount of data which needs to be further studied. Among several interesting genes that were differentially expressed was *MKP3*, also called *DUSP6*, which showed a 4 fold down-regulation in spheroids after treatment with the MEK1 inhibitor PD98059. PMA had no impact on the *MKP3* expression levels, a finding that excludes the PKC pathway as a regulator of MKPs. Yet, being important regulators of MAPK activity, it will be of interest to investigate the roles of MKPs in melanoma cell lines and tissue.

NUR77 is a nuclear orphan receptor that is able to activate transcription independently of an exogenous ligand, and controls both apoptosis and survival in cancer cells, depending on its subcellular localization (218). *NUR77* has been shown to be regulated by RSK, a down-stream substrate of ERK1/2 (219). Moreover, export of *NUR77* from the nucleus, where it functions as an oncogenic survival factor, to the cytoplasm, where it interacts with Bcl-2 and trigger apoptosis, has been shown to be regulated by JNK (220). Our microarray data showed that PD98059-treated spheroids have a 2.6 fold decrease in the *NUR77* expression compared to the spheroid control, supporting MAPK regulation of *NUR77*. Interestingly, PMA treated spheroids showed a 3.4 fold increase in *NUR77* expression compared to the control spheroids, an increase which was sustained also when the spheroids were treated with PD98059 and PMA in combination. It will be of interest to reveal a potential association between *NUR77* and both PMA/PKC activation and the JNK pathway in melanoma cells.

As mentioned in chapter 9.5, recent research has shown that increased Src activity protects lung adenocarcinoma and colon cancer cells from suspension-induced cell death

(anoikis). We have initiated studies in order to elucidate the role of Src in melanoma, hoping for an association with PMA mediated, anchorage-independent survival of melanoma cells. However, our results so far show no tendency in that direction. Nevertheless, our results are not less interesting. During our preliminary investigation we inhibited Src activity using a specific inhibitor, PP2. We found that PP2, similarly to PMA, promoted cell survival, increased levels of the cyclins A and D1 and enhanced phosphorylation and thereby inactivation of the pro-apoptotic proteins Bim and Bad. These results were supported by proliferation assay (MTS) and flow cytometric analysis showing increased proliferation and survival, respectively, for PP2-treated spheroids. Several studies show that Src can regulate ERK1/2 activation. To study whether Src activation influenced any of the MAPK pathways in our cells or vice versa, spheroids were treated with PP2 as well as inhibitors of MEK, p38 and JNK. Inhibition of Src promoted sustained phosphorylation of ERK1/2 and JNK, but decreased phosphorylation of p38 under anchorage-deprived conditions. However, inhibition of both the ERK1/2, p38 and JNK pathways induced a decrease in Src phosphorylation. So far, our results suggest that inactivation of Src promotes better cell survival in WM35 melanoma cells and that Src might be regulated by the MAPKs rather than vice versa. Our further aim is to transfect WM35 cells with siRNA directly targeting Src to investigate whether the better cell survival is due to PP2 mediated specific inhibition of Src or not. Moreover, we wish to characterize the upstream and downstream activators of Src in an attempt to elucidate the role of Src in melanoma cell signalling.

JNK has also been connected to anchorage-independent growth. In this regard, it has been reported that mouse embryo fibroblasts lacking the *JNK1/2* genes showed reduced anoikis resistance (221). Moreover, transfecting lung cancer cells with antisense oligonucleotides against JNK inhibited EGF-stimulated cell proliferation and anchorage-independent growth (222). To follow the possible link between JNK and PKC-induced, MEK-independent ERK1/2 activation (paper II and III), it would be interesting to study whether JNK plays a role in anoikis resistance in melanoma cells and to further examine the regulation of JNK activity in these cells.

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ERRATA

1. Acknowledgements, page 5, final part; Change from September 2007 to September 2008

5.3. Apoptosis. Page 14, second line from below; change from signalling to signaling.

5.4.1.1. RAFs. Page 18, second line from above; change from Raf to RAF

5.6.2. Epidemiology, etiology and risk factors. Page 26, 12th line from above; change from familiar to familial.

5.6.3. Melanoma stages and prognostic factors. Page 27, 12th line from above; change from 6mm to 6 mm.

5.6.5.2. Sporadic melanoma. Page 29, 8th line from above; change from B-RAF occurs to B-RAF occur.

6.1. Material. Page 31, first line; change from paper III to Paper II.

6.3. Measurement of cell viability and apoptosis. Page 31, first line; Change from paper II to paper III.

Page 32, line 6; Change from paper II to paper III.

6.4. Transfection experiments. Page 32, second section, first line; Change from paper II to paper III.

6.6. Immunohistochemistry. Page 33, first line; Change from paper III to paper II.

Page 34, 12th line from above, change from paper III to paper II.

8.2. MAPK in a clinical setting. Page 41, third section, 4th line; Change from Branca et al. to Branca *et al.*

8.6. FABP7 in melanoma. Page 48, First section, last line; Change from signalling to signaling.