ONCOGENE DEPENDENT REGULATION OF MIGRATION AND PROLIFERATION IN HUMAN TUMOR CELLS

PhD Thesis

Tamás M. Garay

Pathological Sciences Doctoral School

Semmelweis University

Supervisor: Dr. Balázs Hegedűs Ph.D

Official reviewers:

Dr. László Kőhidai MD, D.Sc

Dr. Ferhan Ayaydin Ph.D

Head of the Final Examination Committee:

Dr. Lídia Sréter MD, D.Sc

Members of the Final Examination Committee:

Dr. Balázs Sarkadi MD, D.Sc

Dr. László Herszényi MD, Ph.D

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"Our washing up is just like our language. We have dirty water and dirty dishcloths, and yet we manage to get the plates and glasses clean."

Niels Bohr

in Werner Heisenberg's The Part and the Whole

TABLE OF CONTENTS

ABBREVIATIONS

ANOVA – analysis of variance

AR – amphiregulin

ARIA – acetylcholine receptor-inducing activity

ATP – adenosine 5'-triphosphate

BMP – bone morphogenetic protein

BRAF/Braf – oncogen/protein; v-raf (viral rapidly accelerated fibrosarcoma) murine sarcoma viral oncogene homolog B1

BrdU – 5-bromo-2'-deoxiuridine

BTC – betacellulin

CDM – cell-derived matrix

CREB – cyclic AMP response element-binding protein

CPE – carboxypeptidase E

DAG – diacylglycerol

DMEM – Dulbecco's Modified Eagle's Medium

DNA – deoxyribonucleic acid

EGF / EGFR – epidermal growth factor / epidermal growth factor receptor

EMT – epithelial-to-mesenchymal transition

ECM – extracellular matrix

EPR – epiregulin

ErbB – named after similarity to avian erythroblastosis oncogene B

ERK – extracellular signal pathway regulated kinase

FCS – fetal calf serum

FDA – Food and Drug Administration

FGF / FGFR – fibroblast growth factor / fibroblast growth factor receptor

FGF2 / bFGF – basic fibroblast growth factor

FHF – FGF homologous factors

FTI – farnesyl transferase inhibitor

GAP – GTPase activating proteins

GEF – guanine nucleotide exchange factor

GF / GFR – growth factor / growth factor receptor

GGF – glial growth factor

HB-EGF – heparin-binding EGF-like growth factor

HER – human epidermal growth factor receptor

HGF – hepatocyte growth factor

HRG – heregulin

IGF – insulin-like growth factor

IGFBP – IGF binding proteins

IP3 – inositol 1,4,5-triphosphate

MAP – mitogen-activated protein kinase

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

NDF – Neu differentiation factor

NP – neuropilin

NF1 – neurofibromin 1

NOD (mice) – non-obese diabetic (mice)

NRAS/Nras – oncogen/protein; neuroblastoma v-ras (viral rat sarcoma) oncogene homolog

NRG – neuregulin

NSG (mice) – NOD scid gamma (mice)

PAGE – polyacrylamide gel electrophoresis

PCR – polymerase chain reaction

PDG / PDGFR – platelet derived growth factor / platelet derived growth factor receptor

PFA – paraformaldehyde

PKC – protein kinase C

PI3 – – phosphatidylinositide 3

PI3K – phosphatidylinositide 3-kinase

PIGF – phosphatidylinositol-glycan biosynthesis class F protein

PIP2 – phosphatidylinositol 4,5 biphosphate

PIP3 – phosphatidylinositol (3,4,5)-triphosphate

PLC-γ – phospholipase C gamma

qRT-PCR – quantitative real-time PCR

RFLP – restriction fragment length polymorphism

RNA – ribonucleic acid

SARA – Smad anchor for receptor activation

SCID – severe combined immunodeficiency

SDS –sodium dodecyl sulfate

SDS-PAGE–sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SF – scatter factor (also named HGF)

sFRP – soluble Frizzled-related proteins

SRB – sulforhodamine B

TGFα – transforming growth factor-α

TGFβ – transforming growth factor-β

TUNEL (assay)– terminal deoxynucleotidyl transferase dUTP nick end labeling (assay)

VEGF / VEGFR – vascular endothelial growth factor / vascular endothelial growth factor receptor

ZA – zoledronic acid

1. INTRODUCTION

Most of the solid tumors have a steadily growing incidence and, in the majority of cases, not primary tumor growth but distant metastases are the main cause of death. Metastasis formation is a complex process that requires a spatiotemporal regulation of cell adhesion, cell proliferation and cell migration. The available various anticancer treatment modalities often interact with different cellular targets, thus, these modalities can exert differential effects on tumor stroma, extracellular matrix (ECM) or on tumor cell death, proliferation and migration. Additionally, anticancer therapies often influence the most important regulatory mechanisms, including the various growth factor (GF) and TGF signaling pathways.

First, the connection between cell proliferation and cell division will be studied including the role of oncogenic signaling in these processes. Next, the migratory and proliferative effects of the activation of oncogenic pathways will be described. Finally, the consequence of the targeted inhibition of these pathways will be investigated.

1.1. Migration and proliferation of tumor cells

1.1.1. Invasive growth and metastasis are critical factors in tumor progression

Tumor progression is the phenomenon when tumors gain a more "malignant" and aggressive phenotype during their life time (Foulds 1957). A critical component of tumor progression is the formation of tumors distant from the primary tumor – namely metastasis – that have enormous clinical importance as they are responsible for the lethal outcome in up to 90% of cases (Sporn 1996; Hanahan and Weinberg 2000).

The study of stepwise progression of tumors, including metastasis formation is of outmost importance both at the morphological as well as at the genetic level. Nevertheless, differences and similarities between primary lesions and metastases have been in the focus of research since the end of the 19th century (Magruder 1888; Gibbes 1889). The very early findings, namely that particular tumors would give metastasis with a great probability to certain organs, led to the formulation of two major pathomechanisms known as Paget's "seed and soil"

8

hypothesis (Paget 1889) and Ewing's mechanical/anatomical theory (Ewing 1922). Paget hypothesized that metastasis formation would require both a malignant cell being able to survive in the given distant microenvironment ("seed") and a permissive microenvironment ("soil") (Paget 1889). This hypothesis could explain bone metastases of renal and prostate cancers since both tumors can readily express bone matrix proteins (Cooper et al. 2000; Weber et al. 2007). Ewing's mechanical/anatomical theory predicts that the first metastatic foci would develop in the filtering organ (Ewing 1922) which is evident in colorectal cancer and its corresponding liver metastasis as well as in lower rectal/anal cancers and their lung metastases (Mack and Marshall 2010; Kovacs et al. 2013).

Based on the growing knowledge on genetic events related to tumor progression, two major models have emerged describing the genetic similarities and differences between primary tumor and metastasis: the "linear progression" and "parallel progression" model. The "linear progression model" suggests that cancer cell dissemination occurs after extensive expansion of primary tumors having a huge number of shared genetic alterations between the primary tumor and metastasis. In contrast, the "parallel progression model" claims early tumor cell dissemination of small tumors (even smaller than the threshold for tumor detection), which is supported by cases where a large number of genetic differences exist between primary tumor and metastasis (Mack and Marshall 2010; van Zijl et al. 2011). Considering these two models, one of the most relevant questions is not whether metastasis formation is an early or late event in tumor progression, but more importantly, whether evolution of malignant cells occurs inside or outside the primary lesion (Klein 2008).

Regardless, metastasis formation can be described as a cascade or a multi-step process encompassing (I) invasion of tumor cells in the surrounding tissue, (II) intravasation – the entering of tumor cells into the lumen of blood or lymphatic vessels, (III) surviving the rigors of the circulatory system, (IV) extravasation – the arrest at distant organ sites and invasion into the parenchyma of distant tissues, and finally, (V) surviving the foreign microenvironment and forming colonies of tumor cells (Liotta 1986). Each step of the metastatic cascade is very selective, therefore, as little as 0.1% or even less of the disseminated cancer cells could successfully develop a distal metastasis (Fidler 1970; Yoshida et al. 1993; Mack and Marshall 2010).

Although there are different theories to elucidate the mechanism of tumor progression and metastasis formation, none of them can shed light on why cancer cells do proliferate and migrate. First, cells need to disentangle themselves from the well-balanced physiological

control of proliferation and apoptosis. This would enable not only excessive tumor growth but − more importantly − provide the opportunity to accumulate additional alterations that impair regulatory mechanisms such as cell-cell and cell-matrix adhesion and migration. In cancers of epithelial origin, these alterations together lead to a transition from an epithelial to mesenchymal-like phenotype, often referred to as epithelial–mesenchymal transition (EMT)(Thiery 2002; Yamaguchi et al. 2005). Alterations and loss of intercellular junctions as well as (re)gain of a migratory phenotype are the pivotal and clinically by far the most important steps in tumor progression as this provides the capacity of cells to invade locally and to form metastases (Nowell 1986; Hanahan and Weinberg 2000).

1.1.2. State-of-the-art measurement of proliferation and growth in cell cultures *in vitro*

A number of experimental methods have been developed to study migration and proliferation since these are crucial cellular processes in tumor growth and progression. All of the available *in vitro* experimental methods aim to recapitulate certain aspects of the *in vivo* situation. Although these methods do not entirely depict the *in vivo* situation, they provide a good and valuable model of it.

In-vitro-proliferation measurement of cells is often performed by quantitative evaluation of some bio-chemical activity of the living cells. Therefore the gathered data is in closer connection with cell viability than with actual proliferation. There are plenty of quick and well-reproducible assays using dyes that are converted by the metabolic capacity of the cells and the color development can be easily quantified using photometry (Vega-Avila and Pugsley 2011).

Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) or also called *Alamar Blue assay*, for example, − originally described by Pesch and Simmert in 1929 as a tool for showing disease-causative organism in milk − is based on the reduction of resazurin to resorufin and dihydro-resorufin in viable and metabolically active cells (Anoopkumar-Dukie et al. 2005). In microbiology, *crystal violet* (also known as Gentian violet, hexamethyl pararosaniline chloride or 4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) was originally used to differentiate Gram-negative and positive bacteria but since it also binds to DNA contents of cells, it is used for quantifying cell density and proliferation in monolayer

cell cultures (Saotome et al. 1989; Itagaki et al. 1991; Chiba et al. 1998). In addition, *BrdU* (5-bromo-2'-deoxiuridine) is a synthetic nucleoside analog of the DNA precursor thymidine, which is incorporated into the newly synthesized DNA in proliferating cells (Hawker 2003). In the *neutral red assay*, the uptake and accumulation of the neutral red dye in the lysosomes of uninjured cells is determined by colorimetric measurements (Borenfreund and Puerner 1985; Borenfreund et al. 1988). Among the proliferation assays, the most popular is probably the *methylthiazol-tetrazolium (MTT) assay*, which is based on the activity of succinic dehydrogenase reducing the yellow soluble MTT tetrazolium salt to a blue insoluble MTT formazan product after being taken up by the mitochondria (Mosmann 1983; Chiba et al. 1998; Wagner et al. 1999; Vega-Avila and Pugsley 2011). The *sulforhodamine B (SRB) assay*, developed by Skehan, measures the total protein content of the cells (it binds to basic amino-acid residues), and is even more sensitive and more reproducible than MTT assay (Skehan et al. 1990; Keepers et al. 1991). Nevertheless, there is a drawback of these aforementioned assays, namely that one has to assume that cells keep the same intensity in mitochondrial activity, protein accumulation or ploidy level/DNA content in response to treatment modalities and that the chemical parameters of the medium (pH, glucose concentration) are unaffected from the treatment (Jabbar et al. 1989; Vistica et al. 1991; Chiba et al. 1998; Vega-Avila and Pugsley 2011).

The *clonogenic assay* (2D colony formation assay or clonogenic cell survival assay) incorporates no such assumption but it is more time consuming than colorimetric assays (Vega-Avila and Pugsley 2011). The long duration of treatment, the metabolism of the treated cells and the stability of the active compound can result in a variation of the concentration of the substance. Additionally, many human cancer cell lines show low baseline clonogenic potential (Grenman et al. 1991). Cell proliferation and tumor growth *in vitro* can also be assessed in a more physiologically relevant three-dimensional set-up by evaluating the number and magnitude (diameter, cross section area) of 3D cell colonies, spheroids (Haji-Karim and Carlsson 1978; Kunz-Schughart et al. 1998). However, this method can only be applied in cell lines capable of sphere formation.

Considering all the available methods, one of the simplest and most unbiased ways to evaluate proliferation is to count the number of observed cell divisions. This is a more direct way to evaluate proliferation compared to the enzyme activity or total protein based assays and can be achieved by using time-lapse videomicroscopy (Teague et al. 1993; Hegedus et al.

2000; Wick et al. 2003; Hegedus et al. 2004). Therefore, this method was very often used - besides SRB assays - in most of the experiments presented in this thesis.

1.1.3. *In vitro* experimental models of migration in 2D and 3D tumor cell cultures

Structural and molecular determinants of both cellular migratory activity and tissue environment define the locomotion of cells *in vivo*. The cells locomotory activity is determined by intracellular processes including the reorganization of the cytoskeletal apparatus, the formation of pseudopodia and the allocation of energy (mainly in form of ATP) to the motion related processes such exerting force on the extracellular matrix. The tissue environment often represents a barrier for moving cells. To be able to migrate, cells need to restructure their 3D environment, for instance, by digesting the extracellular matrix (ECM) with the use of proteases.

Experimental models that can best recapitulate the *in vivo* situation are *multicellular 3D models* including heterotypic cell-cell and cell-matrix interactions. In these 3D models, cells are embedded into a biological matrix to establish platforms to investigate cell invasion, which inhere not only in cell migration but also in adhesion and proteolysis of ECM components (Hegedus et al. 2006; Kramer et al. 2013). The most complex biological matrices are cell-derived matrices (CDMs) such as the ECM of high density fibroblast cell cultures (Porter and Vanamee 1949; Bradbury et al. 2012). These CDMs are composed of fibronectin, collagen types I and II, heparin sulfate proteoglycans and hyaluronic acid in a proportion that is dependent of the particular cell culture (Kutys et al. 2013). More reproducible are the cell-line-derived basement membrane extracts such as Matrigel obtained from Engelberth-Holm-Swarm mouse sarcoma cells, which is composed of laminin, collagen IV isoforms and heparin sulfate proteoglycans (Kleinman et al. 1982; Benton et al. 2011). The major components of ECM are collagen and fibrin that are often used as single protein 3D matrix models. These fibrous gel matrices are suitable tools to study migratory events such as wound heeling or metastatic invasion (Benton et al. 2011; Bradbury et al. 2012), although they simplify the heterogeneous nature of ECM into a single component gel. Seemingly minor alterations in the protocol may cause significant changes in the properties of the gels. For instance, an increase in fiber concentration results in elevated mechanical strength and

decreased pore size of the gel or an increase in gelation temperature leads to decreased pore size and fibril diameter (McPherson et al. 1985; Miron-Mendoza et al. 2010). The experimental setups differ from each other in how cells are seeded in the matrix, which, in parallel, determines the direction of their migration and the method of evaluation. In *3D cell tracking assays*, cells are seeded in the gel in a low density and the route of the cells through the matrix is tracked via automated microscopy and state–of-the-art image analysis (Hamilton 2009; Kramer et al. 2013). It is also possible to seed the cells as a layer on the surface of the gel (vertical gel invasion assay) or as a spheroid or aggregate in the gel (spheroid gel invasion assay) and to evaluate the migration of the cells from the seeded colony (Szabo et al. 2012; Kramer et al. 2013).

Whereas *3D migration models* are experimental tools for modeling the *in vivo* cell dynamics with high fidelity, 2D models are useful instruments to provide insights of molecular machineries underlying cell adhesion and migration (Friedl and Brocker 2000; Wolf et al. 2009). Of note, some of the migration assays can be applied as 2D or 3D experimental methods, as well. Optionally, different matrices can be used in micro-fluidic chamber assays (Meyvantsson and Beebe 2008) or in the platypus assay that is a modified cell exclusion assay (Hulkower and Herber 2011; Kramer et al. 2013). In Boyden chamber or transmembrane assays, the membrane can be covered with a matrix (Boyden 1962; Albini et al. 1987; Eccles et al. 2005). In the absence of external cues, cells show more or less random migration, which is the prevalent mode of migration for immune cells and metastatic cancer cells (Huth et al. 2010). However, in Boyden chambers or in micro-fluidic chamber assays, cells exert chemotactic migration along a gradient (Toetsch et al. 2009; Prummer et al. 2013). In micro-carrier bead assays and spheroid migration assays, cells are grown to 3D colonies either on beads or in non-adherent vessels and the outward movement of cells to an adherent surface from the 3D colonies is measured. Thus, these assays show some common aspects with 2D and 3D assay, as well (Kramer et al. 2013).

In classical *2D migration assays*, cells are seeded in a particular place and are expected to migrate towards an empty area. In scratch assay, for example, the empty space is literally scratched in a confluent cell-layer (Liang et al. 2007). In cell excursion assays, cells migrate from the occupied periphery of the cell culture vessel to the central cell free area (Hulkower and Herber 2011). The advantage of this assay opposite to the similar scratch assay is that cell migration can be studied uncoupled from contributions of cell damage and permeabilization arising from the scratch (Poujade et al. 2007). Spatially seen, the opposite is going on in the

fence assay where, at seeding, a removable ring (=fence) is preventing cells to attach on the periphery of the vial (Pratt et al. 1984; Fischer et al. 1990). If seeding cells in a low confluence, individual cells will have the space and the opportunity to migrate. This migration can be tracked by videomicroscopy (Hegedus et al. 2000; Hegedus et al. 2004; Huth et al. 2010) or by coating the cell culture vial with substances (e.g. gold particles), and where the moving cells clear the substances on their way they leave a detectable route behind (colloidal particle assay) (Niinaka et al. 2001; Kramer et al. 2013). To further reduce the dimensions of cell cultures testing migration, it is worth mentioning that even 1D cell migration can be measured by seeding cells onto thin fibronectin lines that restrict the migration along this fibers (Maiuri et al. 2012). In this experimental setup, displacement of the cells could be best described as a 1D correlated random walk (Codling et al. 2008). In our investigations, random migration was measured in 2D via evaluation of videomicroscopy and invasion was observed in 3D by monitoring cells migrating outward from cell aggregates in collagen gel.

1.1.4. Connection between cell proliferation and cell migration – the "go or grow" hypothesis

The connection between cell migration and cell proliferation is extensively studied since a fine adjustment and a temporal interplay between these two cellular processes are crucial for the metastatic potential of tumor cells. Based on experimental data and on the concept that cytoskeletal machinery cannot be used for proliferation and migration concurrently, the "go or grow" hypothesis - formulated for tumor cells - postulates that migration and cell division are mutually exclusive, and tumor cells defer proliferation for cell migration (Funk and Sage 1991; Giese et al. 1996; Schultz et al. 2002; Corcoran and Del Maestro 2003).

Numerous experiments on brain tumor cells support the "go or grow" hypothesis demonstrating that if proliferation is impaired migration is enhanced in cell populations or vice versa. In two dimensional cell cultures of glioma cells, TGF-β exerted a growth-inhibitory action and elicited migration and invasiveness (Merzak et al. 1994). Similarly, enhanced expression of soluble Frizzled-related proteins (sFRPs) inhibited the motility of glioma cells *in vitro* and increased their clonogenic potential *in vitro* and *in vivo* (Roth et al. 2000). Correspondingly in another study, glioma cells overexpressing carboxypeptidase E

(CPE) showed enhanced proliferation and decreased migratory activity (Horing et al. 2012). In conventional 2D cultures of astrocytoma cells, impaired migration due to cell density or vitronectin coated surface resulted in higher proliferation (Giese et al. 1996). In line with the results measured in 2D, in 3D cultures of astrocytoma cells embedded in collagen gel, the gel-invading, active migrating cells detaching from the surface of the spheroids showed less proliferative activity as cells in the inner layer of the spheroids (Tamaki et al. 1997). Similarly, no correlation was found between invasion and Ki-67 labeling in patient-derived short-time 3D cell cultures of intracranial tumor cells (Khoshyomn et al. 1999). Recently, two microRNAs have been identified in glioma cells that have a role in the regulation of proliferation and migration dichotomy. Lower glucose concentrations are linked to reduced miR-451 expression resulting in inhibition of proliferation and increase of migration (Godlewski et al. 2010). Contrariwise, reduced expression of miR-9 in the miR-9/CREB/NF1 signaling is linked to increased proliferation and decreased migration (Tan et al. 2012).

In contrast to the supporting findings, the "go or grow" hypothesis is challenged in several studies. In glioma cell spheroids, EGF treatment enhanced both migration and proliferation *in vitro* (Lund-Johansen et al. 1990). Similarly, the inhibition of EGFR resulted in the increase of both migration and proliferation in patient-derived short-time 3D glioblastoma cell cultures (Penar et al. 1997). In addition, hypericin reduced both migration and proliferation in glioma cells *in vitro* (Zhang et al. 1997). Of note, the "go or grow" hypothesis was directly tested using time-lapse videomicroscopy and Ki-67 labeling in four medulloblastoma cell lines but the hypothesis could not been confirmed (Corcoran and Del Maestro 2003).

In addition to experimental work, a number of mathematical models have been developed in order to study the cellular and molecular mechanisms being able to underline such a dichotomy (Fedotov and Iomin 2007; Wang et al. 2009; Bauer et al. 2010; Kim et al. 2011; Hatzikirou et al. 2012). Some of the theoretical approaches using stochastic and probabilistic mathematical models could recapitulate similar behavior (Fedotov and Iomin 2007; Hatzikirou et al. 2012), whereas others challenged certain aspects of the "go or grow" hypothesis (Bauer et al. 2010).

Furthermore, from the structural point of view, the actin and microtubule cytoskeletal apparatus are used to maintain changes in cell shape and mitotic cell rounding during the cytokinetic phase of cell cycle. Consequently, the normal cytoskeletal apparatus should not be available for active cell migration during cell division (Paluch et al. 2005; Stewart et al. 2011). The competition of proliferation and migration for the finite-free energy – mainly in

form of ATP – resources (Czirók et al. 1998) would also support the mutual exclusiveness of these cellular processes.

The "go or grow" hypothesis is particularly important in the development of effective anti-cancer drugs that can also target the survival-prone subpopulation of tumor cells being able to escape from the primary tumor and survive in metastatic tissue microenvironment. If tumor cells defer cell proliferation for cell migration than migrating cells should have a decreased sensitivity to treatment modalities targeting the proliferating tumor cells. Thus, anti-proliferative therapies may unintentionally select for migratory cells or even induce cell migration in surviving cell populations. Furthermore, inhibition of cell migration might induce the proliferation of disseminating cells and lead to primary or secondary tumor growth. For this reason, understanding better the connection between proliferation and migration is essential for the development of therapies inhibiting both of these cellular processes.

Since evaluation of the "go or grow" hypothesis is currently largely based on brain tumor cells, in this thesis, we extended the investigation of this hypothesis on 2D cell cultures of tumor cell lines originating from different embryonic cell layers such as cells of neuroectodermal, mesodermal and entodermal origin, using long-term time-lapse videomicroscopy.

1.2. Molecular pathways controlling migration and proliferation

1.2.1. Overview of polypeptide growth factors controlling cell migration and proliferation

A great variety of molecules organized in complex networks of signal transduction pathways are controlling cell migration and proliferation, which are involved in several non-malignant processes such as embryonic development, wound healing, immune response, angiogenesis and tissue homeostasis (Horwitz and Webb 2003; Friedl and Weigelin 2008; Hulkower and Herber 2011). Of note, malfunctions of these molecular networks result in deregulated and signal independent growth stimulation that is in turn necessary for the initiation and progression of tumors (Hanahan and Weinberg 2000; Hanahan and Weinberg 2011).

Polypeptide growth factors (GFs) are among the most important regulators of cell migration and proliferation. They differ from hormones as neither their site(s) of synthesis nor their site(s) of action is restricted to defined tissues and they operate mostly in a paracrine fashion (Carpenter and Cohen 1990). Polypeptides are unable to cross the hydrophobic cell membrane, hence, they transmit their signals via cell membrane receptors, which are often activated by ligand-induced oligomerization or polymerization (Heldin 1995; Heldin 1996). Since the activation and/or repression of GF signal transduction plays a crucial role in cell migration and proliferation, it is no wonder that malignant cells often carry molecular alterations of these factors or their receptors, or switch to an autocrine mode of action from a paracrine one (Favoni and de Cupis 2000; Hanahan and Weinberg 2011). Note worthily, as many GFs use common downstream signal transduction pathways, a malfunction in one molecule of the system may affect several molecular pathway simultaneously (Favoni and de Cupis 2000).

Despite the fact that GFs and their growth factor receptors (GFRs) are grouped into signaling families according to their biochemical structures, the nomenclature remained complex, arbitrary and in some cases even confusing (Yorio et al. 2008). The most important growth factor signaling system is probably the epidermal growth factor (EGF) family having several ligands such as epidermal growth factor (EGF), transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), epiregulin (EPR), cripto-, epigen-, neuregulin (NRG1-4; NRG-1 is also known as Neu differentiation factor (NDF)), heregulin (HRG), acetylcholine receptor-inducing activity (ARIA) and glial growth factor (GGF). These factors bind to four different EGF receptors, ErbB1 (also termed EGFR, HER1), ErbB2 (also termed HER2, p185, or neu), ErbB3 (also termed HER3 or p160) and ErbB4 (HER4), inducing cell migration, differentiation and controlling angiogenesis, wound healing, bone reabsorption, atherosclerosis and tumor growth. HER comes from human epidermal growth factor receptor and ErbB is named for its similarity to ERBB avian erythroblastosis oncogene B. Mutations in ligands and receptors of EGF family are especially important in breast, ovarian and lung cancer but malfunctions are also present in head and neck, colorectal, pancreatic, bladder, prostate and renal cancer as well as in glioma (Yarden 2001; Harris et al. 2003; Grandis and Sok 2004; Dreux et al. 2006; Dutta and Maity 2007).

Since TGFα is a member of EGF family, the signaling family mentioned next should be TGFβ, as the two classes of TGFs (TGFα and TGFβ) show no structural or genetic similarity.

There are 33 ligands in the TGFβ signaling family often grouped as TGFs, activins, and bone morphogenetic proteins (BMPs) but there are seven type I (ALK1–7) and four type II receptors (TβRII, BMPRII, ActRIIA and B) having a high level of overlap between ligands regulating cellular proliferation, survival, differentiation and migration. Failure in the function of TGFβ family proteins are found in colorectal, pancreatic, breast and lung cancer (Massague 2000; Gordon and Blobe 2008; Horbelt et al. 2012; Wiater and Vale 2012).

Another well-known GF family is the fibroblast growth factor (FGF) family. It consists of 18 ligands because four previous members, now termed FGF homologous factors (FHF1-4), have been removed from the list of originally 22 ligands as these molecules lack functional similarity to other FGF family members (Goldfarb et al. 2007). The 18 FGF ligands act on 4 FGF receptors (FGFR1-4), affecting proliferation, migration and differentiation in embryonic development and homeostatic factors tissue repair and tumor progression in the adult organism (e.g. breast, bladder, liver cancer, multiple myeloma, renal cell carcinoma as well as angiogenesis around the tumor) (Ornitz and Itoh 2001; Acevedo et al. 2009; Liang et al. 2012; Turner et al. 2012).

The insulin-like growth factor (IGF) signaling system consists of two ligands (IGF-I, IGF-II) and two receptors (IGF-I receptor, IGFIR; and IGF-II receptor, IGFIIR). In addition, seven regulator molecules, namely the six high-affinity IGF binding proteins (IGFBP1-6) and acid-labile subunit (ALS), acts together regulating cell survival, cell proliferation and invasion (Capoluongo 2011; Domene et al. 2011). It is worth mentioning that some reviews enumerate also insulin and insulin receptor (IR) to the IGF family (King and Wong 2012). Alterations in the IGF family have been described in colorectal, breast, pancreatic, lung, thyroid, head and neck, prostate, renal, ovarian, and endometrial cancer as well as in sarcomas (Pollak 2008; Gallagher and LeRoith 2011; King and Wong 2012).

Angiogenesis and endothelial cell proliferation, migration, survival and endothelium permeability in healthy as well as in cancerous tissue are controlled by the ligands and receptors of vascular endothelial growth factor (VEGF) family, which consists of seven secreted protein ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF) and VEGF-F) and five receptors (VEGFR-1, VEGFR-2, VEGFR-3 and neuropilins (NP-1 and NP-2)) (Otrock et al. 2007; Shibuya 2013).

Members of the platelet derived growth factor (PDGF) family are structurally and functionally related to the VEGF family. The PDGF ligands build homo- or heterodimers from four different polypeptide chains named PDGF-AA, -AB, -BB, -CC, and -DD.

Similarly, receptors are formed from two tyrosine kinase receptor chains combined with homo- or heterodimers (PDGFR-αα, -ββ and −αβ). PDGF family members are involved in tumors such as non-small-cell lung cancer, glioma, prostate cancer and rhabdomyosarcoma (Fredriksson et al. 2004; Andrae et al. 2008; Heldin 2012; Nazarenko et al. 2012; Ostendorf et al. 2012).

Finally, the hepatocyte growth factor (HGF) family with one known ligand (HGF also named as scatter factor (SF)) and its receptor encoded by the MET gene is of particular importance because malfunction of this signaling pathway contributes to tumor formation in several cancers (e.g. lung, esophageal, gastric, breast, prostate head and neck and papillary renal cancer) and promotes aggressive cellular behavior that is linked to metastasis formation (Toschi and Janne 2008; Cecchi et al. 2012; Gherardi et al. 2012).

This short enumeration of the most important growth factor families should emphasize the complexity of GF signaling and their importance in tumor diseases (Favoni and de Cupis 2000). In the present work, the role of EGF, FGF2 and activin ligands were investigated in relation to malignant cell migration and proliferation.

1.2.2. The EGF and FGF2 activated receptor tyrosine kinase pathways

EGF and FGF2 are single-chain polypeptides consisting of 53 and 155 amino acid residues, respectively (Favoni and de Cupis 2000). EGF was one of the first GFs discovered (Cohen 1986; Favoni and de Cupis 2000). The role of EGF signaling is well established in many types of cancer (Dutta and Maity 2007). The first FGF cloned was FGF2, also known as basic fibroblast growth factor (bFGF), which is the prototypical FGF ligand with considerable literature about its role in several carcinogenic processes (Kurokawa et al. 1988; Turner et al. 2012)

EGF exerts its function on EGFR (also termed ErbB1) (Harris et al. 2003; Dreux et al. 2006), whereas FGF2 ligands activate all four types of FGFRs (Ornitz et al. 1996; Zhang et al. 2006; Cotton et al. 2008; Heinzle et al. 2011). Upon activation, the downstream elements of EGF and FGF2 signaling are extensively overlapping. Some of the most important downstream elements of EGF/FGF2 signaling are shown in *Figure 1*. Both EGF and FGF2 signaling acts through the activation of Ras, subsequently Raf and extracellular signal pathway regulated kinase (ERK)/mitogen-activated protein kinase (MAP) kinase cascade. In addition,

EGF/FGF2 signals lead to the activation of phospholipase C gamma (PLC-γ) that initiates the hydrolysis of phosphatidylinositol 4,5 biphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG), which in turn activates protein kinase C (PKC). Furthermore, both EGF and FGF2 signaling activates, either directly or through Ras, PI3 kinase (PI3K), which generates PIP3 by phosphorylating PIP2 and leads to the activation of AKT. The phosphatase PTEN is responsible for the dephosphorylating of PIP3 to PIP2 and, hence, for the deactivation of AKT. (For review see Maruta and Burgess (1994), Dutta and Maity (2007), Ghosh and Chin (2009), Liang et al. (2012).)
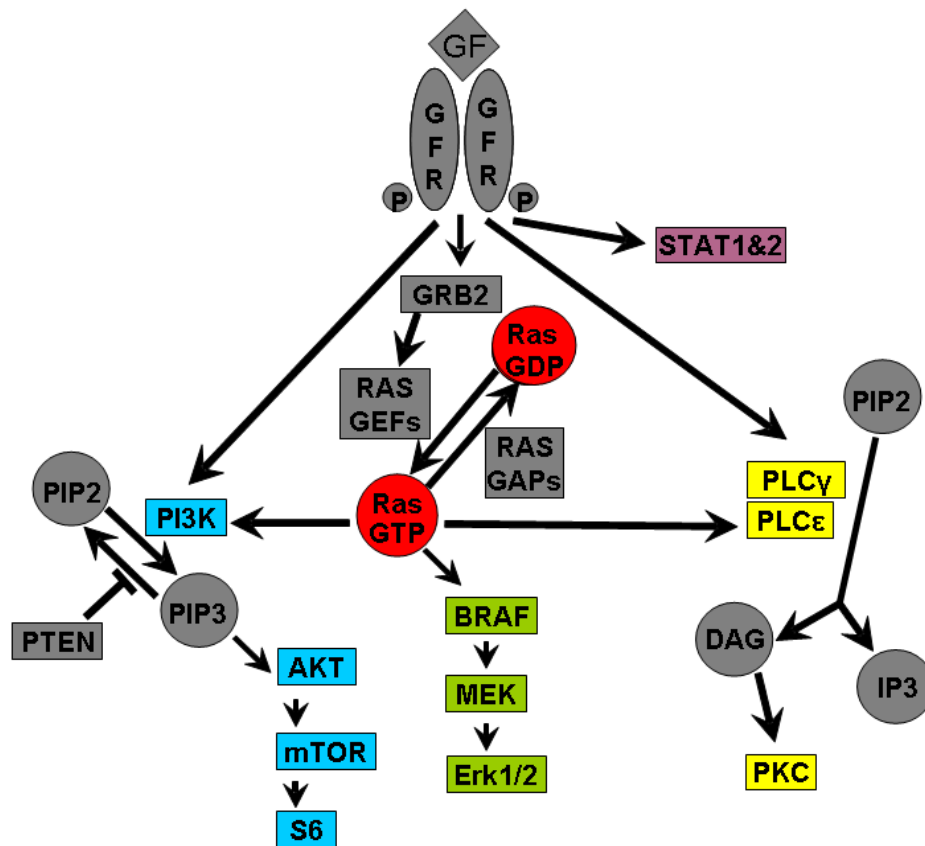
Figure 1. The EGFR/FGFR signaling pathway. Upon activation, GFRs form dimmers and activate the downstream effector, which induces activation of the RAF/MEK/ERK (green), the PI3K/(PTEN)/AKT/mTOR (blue) and the PLC/PKC (yellow) pathways as well as alters transcription by the activation of STAT1 and STAT3 (purple). It is important to note that a constitutively activated mutant Ras can activate all three major signal transduction pathways and oncogenic BRAF activates the RAF/MEK/ERK signaling. Modified after (Timar et al. 2010)

Although the role of EGF and FGF2 signaling in non-malignant tissue could provide useful information to cancer research, the majority of published works elucidate EGF / FGF2 signaling in tumors. Under normal conditions, EGF signaling is involved in epidermal proliferation, gastric acid secretion, urothelial regeneration, corneal wound healing, periodontal repair, regulation of apoptosis and even in placental development (Carpenter and Cohen 1990; Danielsen and Maihle 2002; Daher et al. 2003; Marzioni et al. 2005; Dereka et al. 2006; Yu et al. 2010). FGF2 exerts its function in regulating processes of hematopoiesis and regulation of growth and function of endothelial and smooth muscle cells (Allouche and Bikfalvi 1995; Nugent and Iozzo 2000).

Impaired EGF and FGF2 signaling is involved in a great variety of malignancies such as breast, ovarian, lung, head and neck, colorectal, pancreatic, bladder, prostate cancer, renal cell carcinoma, multiple myeloma, glioma as well as tumor angiogenesis (Yarden 2001; Grandis and Sok 2004; Dutta and Maity 2007; Acevedo et al. 2009; Liang et al. 2012). Accordingly, the targeting of EGFR has become an efficient therapeutic option for certain malignancies. The anti-EGFR1 monoclonal antibody cetuximab (Erbitux©) is approved by the Food and Drug Administration (FDA) as targeted therapy in colorectal cancer and investigated as a promising treatment modality in head and neck cancer (Denaro et al. 2013) and non-small cell lung cancer (Pirker 2013). Small molecule tyrosine kinase inhibitors (TKI) erlotinib (Tarceva©) and gefitinib (Iressa©) are approved by the FDA for the treatment of non-small cell lung cancer and considered as potential therapeutics in colorectal cancer (Gravalos et al. 2007) and breast cancer (Normanno et al. 2006; Khajah et al. 2012). Nevertheless, EGF and FGF2 signaling is particularly important in malignant melanoma because signal transduction of the receptors is affected by oncogenic driver mutations in BRAF or NRAS, which are present in about 40 to 70% and in 10 to 30% of melanoma cases, respectively (Demunter et al. 2001; Davies et al. 2002; Kumar et al. 2003; Maldonado et al. 2003; Houben et al. 2004; Tsao et al. 2004; Curtin et al. 2005).

Several studies demonstrated that EGF signaling is indeed affected in melanocytic malignancies. In a great variety of benign and neoplastic melanocytic lesions increased EGFR expression was demonstrated by immunohistochemistry (Ellis et al. 1992). Interestingly, expression of EGFR was found to be positively and the expression of EGF negatively correlated with a more malignant phenotype in melanocytic tumors (Lazar-Molnar et al. 2000). Furthermore more intense EGFR expression was detected in melanoma metastases than in dysplastic nevi (Elder et al. 1989) and the amplification of EGFR gene is also

considered to correlate with tumor progression (Rakosy et al. 2007; Feinmesser et al. 2010; Boone et al. 2011). However, there is a varying degree of expression of EGFR in melanoma cells and some cell lines lack expression (Gordon-Thomson et al. 2001). *In vitro* studies have shown that EGF signaling can stimulate proliferation and migration of melanoma cells (Lazar-Molnar et al. 2000). Furthermore, EGF was shown to facilitate melanoma lymph node metastases by affecting lymphangiogenesis (Bracher et al. 2013). Of note, recent studies claim EGF signaling to be responsible for resistance against BRAF inhibitors (Girotti and Marais 2013; Girotti et al. 2013).

Although normal and malignant melanocytes express predominantly FGFR1 (Becker et al. 1992), there is an increase in overall expression of growth factor receptors and the transcription of FGFR4 was detected only in malignant melanoma cells (Easty et al. 1993; Yayon et al. 1997). Furthermore, the expression of FGFR4 is thought to be a potential prognostic marker for melanoma (Streit et al. 2006). The importance of FGFR1 is underlined with experiments, in which melanoma cells expressing truncated FGFR1 and lacking the intracellular kinase domain showed dramatically reduced cell proliferation and survival *in vitro* as well as decreased tumorigenic potential *in vivo* (Yayon et al. 1997). In addition, FGF2 is not expressed in normal melanocytes but it is in melanoma cells (Halaban et al. 1988). Furthermore, FGF2 signaling is involved in processes leading to melanocytic tumors and melanoma and several FGFR2 loss-of-function mutations have been identified in melanoma (Gartside et al. 2009). It has been reported that forced expression of FGF2 in melanocytes resulted in autonomous and increased growth *in vitro* but not in increased tumor forming capacity *in vivo* (Dotto et al. 1989; Nesbit et al. 1999). In contrast, inhibition of FGF2 signaling by either specific neutralizing antibodies or by antisense oligonucleotides resulted in decreased migration and proliferation *in vitro* and prolonged survival time and suppression of tumor growth in animal models (Wang and Becker 1997; Ozen et al. 2004; Chalkiadaki et al. 2009; Li et al. 2010; Aguzzi et al. 2011; Metzner et al. 2011; Yu et al. 2012).

The facts that both EGF and FGF2 act on extensive overlapping downstream signaling networks and that the most common oncogenic mutations in malignant melanoma are activating mutations of their downstream effectors led us to investigate the activation and inhibition of EGF and FGF2 signaling on melanoma cells with different NRAS and BRAF mutational status.

1.2.3. Activin-activated receptor protein serine/threonine kinase signal transduction

Activin is a member of TGFβ signaling family and - in contrast to EGF and FGF2 - it is a homo- or heterodimeric protein. Altogether five subunits have been described (activin subunits βA, βB, βC , βD and βE), nevertheless, activin βD has been identified only in *Xenopus laevis* (Oda et al. 1995). Activin ligands have been named according to their building monomers. Thus, for example activin A contains two βA monomers and activin BE consists of one βB and one βE subunit.

Activin binding receptors are composed of two dimeric proteins, namely type I and type II receptors, being the former the most common. Type I activin receptor is known as Alk4 (also known as ActRIB) but Alk7 (ActRIC) and Alk2 (ActRIA) can also mediate activin signaling, whereas type II activin receptor is a dimmer of ActRIIA or ActRIIB peptides (Tsuchida et al. 2008; Antsiferova and Werner 2012). A schematic view of the activin signaling pathway is shown in *Figure 2*. Canonical activin signaling starts with the binding of activin to a dimeric type II activin receptor, which leads to the recruitment, phosphorylation and activation of type I activin receptor. The activated type I activin receptor phosphorylate SMAD2[1] and SMAD3 molecules, which then become liberated from SARA (SMAD anchor for receptor activation) proteins. Subsequently, SMAD2/3 interacts with SMAD4 (the so called common mediator SMAD4 – Co-SMAD4) and translocates to the nucleus, where the complex directly regulates gene expression (Schmierer and Hill 2007; Antsiferova and Werner 2012). SMAD6 and SMAD7 are cytosolic inhibitors of the canonical SMAD signalization. Besides the canonical SMAD pathway, activin interacts, in a cell type-dependent manner, with other intracellular signals such as the pituitary transcription factor Pit-1, RAS or Erk1/2 (Cocolakis et al. 2001; Bao et al. 2005; Tsuchida et al. 2009; Grusch et al. 2010).

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[1] The acronym SMAD is a portmanteau of the *Drosophila* protein mothers against decapentaplegic (MAD) and the *Caenorhabditis elegans* protein SMA (responsible for small body size)
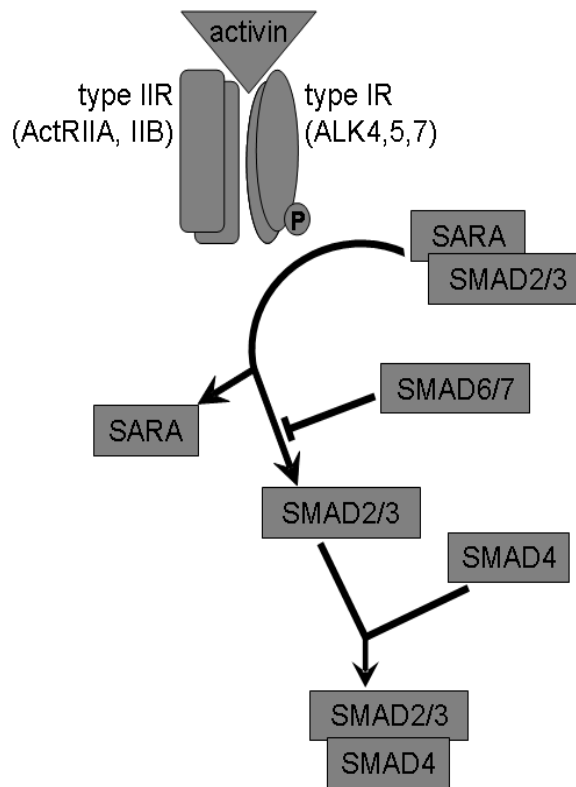
Figure 2. The canonic activin signaling pathway. Binding of activin to type II activin receptor induces recruitment and phosphorylation of type I activin receptor, which leads to the liberation of SMAD2 and SMAD3 from SARA. Liberated SMAD2/3 is able to form a complex with SMAD4, which may act as a transcription factor in the nucleus. SMAD6 and SMAD7 are inhibitors of the canonic activin pathway. Modified after Risbridger et al. (2001) and Tsuchida et al. (2009).

In non-malignant tissues, activin is involved in pancreatic development and homeostasis (Wiater and Vale 2012), inflammation (Hedger et al. 2011; Fearon et al. 2012), wound healing processes (Antsiferova and Werner 2012), reproduction (de Kretser et al. 2002), stem cell biology and regulation of apoptosis, cell proliferation (Beattie et al. 2005) as well as regulation of the hypothalamus-pituitary-gonadal axis (Tsuchida et al. 2008).

In tumors, unlike EGF and FGF2, activin signaling can be associated with both inhibition and promotion of cell proliferation and tumor progression. For example, in hepatocellular carcinoma (Chen et al. 2000; Deli et al. 2008), breast cancer (Burdette et al. 2005; Katik et al. 2009) and prostate cancer (Wang et al. 1996; Risbridger et al. 2001), activin signaling takes part in inhibition of cell proliferation and tumor progression. In line with this observation,

these tumors often overexpress activin antagonizing proteins (Grusch et al. 2006; Razanajaona et al. 2007).

In contrast, activin can promote cell proliferation in endometrial carcinoma (Tanaka et al. 2004; Ferreira et al. 2008), oral squamous cell carcinoma (Chang et al. 2010), testicular cancer (Devouassoux-Shisheboran et al. 2003) gastric cancer (Takeno et al. 2008; Wang et al. 2012) and in a great variety of thoracic tumors like esophageal squamous cell carcinoma (Yoshinaga et al. 2008; Puhringer-Oppermann et al. 2010), esophageal adenocarcinoma (Seder et al. 2009) and lung adenocarcinoma (Seder et al. 2009). In line with this, overexpression of activin is found in these thoracic tumors and is often related to poor prognosis, enhanced metastasis and, thus, to shorter disease-free survival time (Yoshinaga et al. 2003; Seder et al. 2009; Chang et al. 2010).

Exogenous antagonists against the activin type I (SB-431542 and SB-505124) and type II (activin-M108A) receptors have been developed (Harrison et al. 2005). SB-431542 acts on all three activin type I receptors, namely on ALK4, ALK5 and ALK7 (Inman et al. 2002; Laping et al. 2002). Treatment with SB-431542 showed antitumor effect on clear cell renal cell carcinoma cells (Bostrom et al. 2013). Similarly SB-431542 treatment inhibited proliferation of human osteosarcoma cells (Matsuyama et al. 2003) and decreased proliferation and migration of glioma cells (Hjelmeland et al. 2004) *in vitro*.

Since mesothelioma lacks targeted therapy (Jackman 2009) and, in some other thoracic tumors, the inhibition of activin leads to a decrease in cell proliferation and tumor invasion (Seder et al. 2009; Seder et al. 2009; Chang et al. 2010) our aim was to investigate the potential effect of inhibiting activin signaling in human mesothelioma cells.

1.3. Targeted inhibition of GF pathways controlling migration and proliferation

1.3.1. Molecularly targeted inhibition of cancer treatment in clinical practice

Molecularly targeted inhibition of a signaling system can be exerted on many levels, such as ligand, binding site of the receptor, intracellular kinase domain of the receptor and downstream elements. Inhibition on ligand level is often used when the aim is to inhibit the production of hormones, as in the case of aromatase inhibitors and estrogen hormones (e.g.

exemestane (Aromasin[®])) (Decensi et al. 2012). The extracellular, active site of receptors can be blocked by antibodies, for example cetuximab (Erbitux[®]) that binds and inhibits EGFR1 (Bou-Assaly and Mukherji 2010). To inhibit signaling at the site of the intracellular kinase domain of receptors small-molecule inhibitors are used since they can cross the cell membrane (e.g. erlotinib (Tarceva[®]), tyrosine kinase inhibitor of EGFR1) (Siegel-Lakhai et al. 2005). Of note, many downstream elements of receptor signaling pathways are kinases that can also be blocked by small-molecule inhibitors (e.g. the mTOR inhibitor temsirolimus (Torisel[®])) (Klumpen et al. 2010). A summary of the FDA-approved (Food and Drug Administration) targeted therapies inhibiting GF signaling pathways in cancer patients is listed in **Table 1.** (http://www.cancer.gov/cancertopics/factsheet/Therapy/targeted).

Table 1. The list of targeted therapies acting on GF pathways approved by the Food and Drug Administration (FDA) for cancer treatment.

Generic name	Trade name	Approved for	Molecule type	Target molecule
Bevacizumab	Avastin	glioblastoma; non-small cell lung cancer; metastatic colorectal cancer; metastatic kidney cancer	monoclonal antibody	VEGF
Bosutinib	Bosulif	CML	small-molecule	BCR/ABL, SRC
Cabozantinib	Cometriq	metastatic medullary thyroid cancer	small-molecule	VEGFR2, FLT3, KIT, MET, RET, TEK
Cetuximab	Erbitux	squamous cell carcinoma of the head and neck; colorectal cancer	monoclonal antibody	EGFR1
Crizotinib	Xalkori	metastatic non-small cell lung cancer	small-molecule	EML4-ALK
Dasatinib	Sprycel	CML; acute lymphoblastic leukemia	small-molecule	wide range of tyrosine kinases
Erlotinib	Tarceva	metastatic non-small cell lung cancer; pancreatic cancer	small-molecule	EGFR1

Everolimus	Afinito	advanced kidney cancer; advanced breast cancer; pancreatic neuroendocrine tumors; subependymal giant cell astrocytoma;	small-molecule	mTOR
Gefitinib	Iressa	non-small cell lung cancer	small-molecule	EGFR1
Imatinib mesylate	Gleevec/ Glivec	gastrointestinal stromal tumor; dermatofibrosarcoma protuberans; acute lymphoblastic leukemia (philadelphia cromosome +); myelodysplastic/myeloproliferative disorders; systemic mastocytosis	small-molecule	BCR/ABL, KIT, PDGFRβ
Lapatinib	Tykerb	metastatic breast cancer	small-molecule	EGFR1, EGFR2 (HER2)
Nilotinib	Tasigna	CML	small-molecule	BCR/ABL
Panitumumab	Vectibix	metastatic colon cancer	monoclonal antibody	EGFR1
Pazopanib	Votrient	advanced renal cell carcinoma; soft tissue sarcoma	small-molecule	VEGFR1, VEGFR2, VEGFR3, PDGFRα, PDGFRβ, KIT
Pertuzumab	Perjeta	in combination with trastuzumab and docetaxel in metastatic breast cancer that expresses HER-2	monoclonal antibody	EGFR2 (HER2)
Regorafenib	Stivarga	metastatic colorectal cancer	small-molecule	VEGFR1, VEGFR2, VEGFR3, RAF, RET, PDGFRβ, KIT

Sorafenib	Nexavar	advanced renal cell carcinoma; hepatocellular carcinoma	small-molecule	VEGFR2, VEGFR3, RAF, PDGFRβ, KIT, FLT3
Sunitinib	Sutent	metastatic renal cell carcinoma; gastrointestinal stromal tumor that is not responding to imatinib; pancreatic neuroendocrine tumors	small-molecule	VEGFR1, VEGFR2, VEGFR3, PDGFRα, PDGFRβ, KIT, FLT3, CSF-1R
Temsirolimus	Torisel	renal cell carcinoma	small-molecule	mTOR
Trastuzumab	Herceptin	breast cancer; gastric or gastro-esophageal junction adenocarcinoma	monoclonal antibody	EGFR2 (HER2)
Vandetanib	Caprelsa	metastatic medullary thyroid cancer	small-molecule	EGFR1, VEGFR2, RET
Vemurafenib	Zelboraf	metastatic melanoma with BRAF V600E mutation	small-molecule	BRAF (with V600E mutation)
Ziv-aflibercept	Zaltrap	metastatic colorectal cancer	recombinant fusion protein	VEGF-A, VEGF-B, PlGF

New targeted therapies are emerging day after day, nevertheless, mesothelioma completely lacks for any approved targeted therapy and only one such therapy is available for the subgroup of melanoma patients with mutant BRAF. Unfortunately, despite the promising first results, BRAF-mutant melanoma often shows intrinsic or acquired resistance against vemurafenib treatment (Bollag et al. 2010; Flaherty et al. 2010; Paraiso et al. 2010; Ribas and Flaherty 2011).

3.3.2 Novel molecular targets in development

The development of new targeted therapies involves not only the invention of novel treatment modalities against new or well-known target molecules, but also the identification of new indications for compounds and targets already in use. Finding new indications is not always obvious because, the same treatment can have opposite effect on cancer cells. As mentioned above, activin treatment inhibited cell proliferation in breast cancer and hepatocellular carcinoma but promoted proliferation of gastric cancer and squamous cell carcinoma (Deli et al. 2008; Takeno et al. 2008; Katik et al. 2009; Chang et al. 2010). Furthermore, combined treatments can lead to a more efficient usage of known targeted therapies and even to successful treatment of resistant cases.

For example, the FDA-approved bisphosphonates and amino-bisphosphonates, such as zoledronic acid (Zometa®) is a palliative treatment in cancer bone metastases but a new indication of zoledronic acid (ZA) could be the treatment of tumors with RAS mutations such as malignant melanoma. In line with this, a large number of *in vitro* and *in vivo* experimental results suggest that ZA and other bisphosphonates may have, beside the inhibiting effect on osteoclasts, a specific antitumor activity like inhibition of proliferation and/or apoptosis induction in myeloma (Derenne et al. 1999; Iguchi et al. 2003; Guenther et al. 2010), osteosarcoma (Sonnemann et al. 2001; Kubista et al. 2006), prostate (Lee et al. 2001; Corey et al. 2003) or breast cancer (Senaratne et al. 2000; Jagdev et al. 2001). Even in preclinical studies running on cancer types without preferential spreading to bone as pancreatic cancer (Tassone et al. 2003) and neural crest derived neuroblastoma (Peng et al. 2007), the cells have shown sensitivity to ZA treatment. Moreover, the antitumor effect exerted by ZA is especially interesting in melanoma treatment because ZA inhibits farnesyl-diphosphate synthase and as a result, the lack of the substrate of geranylgeranyl transferase and farnesyl transferase (two enzymes being responsible for prenylation) impairs the posttranslational modification of Ras (Amin et al. 1992; van Beek et al. 1999). The earlier *in vitro* studies in melanoma cells have shown proliferation inhibiting and apoptosis inducing effect of ZA (Forsea et al. 2004). Furthermore, ZA treatment could contribute to the regression of pulmonary and bone metastases of a melanoma patient (Laggner et al. 2009). Nevertheless, the effect of ZA on melanoma cells *in vivo* and the dependence of biological response on the BRAF or NRAS oncogenic mutation status have not yet been studied.

Gefitinib and *erlotinib* are two well-known inhibitors of EGFR1 and are in clinical use for the treatment of non-small cell lung and pancreatic cancer. Both inhibitors are also promising therapeutics in colorectal cancer (Gravalos et al. 2007). Similarly, gefitinib and erlotinib showed inhibitory effect on the proliferation and migration of breast cancer cells (Normanno et al. 2006; Khajah et al. 2012). In addition, gefitinib inhibited proliferation of malignant melanoma cells harboring wild type BRAF and NRAS (Djerf et al. 2009) but failed to show significant clinical efficacy as a single-agent therapy for unselected patients with metastatic melanoma (Patel et al. 2011). As a single therapy, erlotinib failed to reduce proliferation of melanoma cells but in combination with bevacizumab, a VEGF-A binding antibody, the decrease in proliferation was significant *in vitro* (Schicher et al. 2009). Similarly, monotherapy in *in vivo* xenografts of melanoma with unknown oncogenic mutations showed modest inhibition of tumor growth but, in combination with bevacizumab, tumor growth was significantly inhibited (Schicher et al. 2009). An additional EGFR inhibitor, *pelitinib* (EKB-569), binding irreversibly to EGFR, inhibited the proliferation of hepatocellular carcinoma cells *in vitro* (Kim and Lim 2011). In another *in vitro* study, pelitinib inhibited proliferation of gefitinib- and erlotinib resistant non-small cell lung cancer cell lines (Kwak et al. 2005). In a phase I study, clinical benefit was seen with temsirolimus administered in combination with pelitinib (Bryce et al. 2012). A further compound inhibiting EGF signaling is the pan-EGFR tyrosine kinase inhibitor *CI-1033* (also called *canertinib* or PD183805), which effectively inhibited the growth of esophageal cancer cells in a dose-dependent manner both *in vitro* and *in vivo* (Ako et al. 2007). Furthermore, CI-1033 was shown to be effective in inhibiting proliferation *in vivo* and tumor growth *in vitro* in malignant melanoma harboring wild type BRAF and NRAS (Djerf Severinsson et al. 2011). It can be seen from the above mentioned examples that EGFR inhibitors are effective in different kinds of solid tumors, though their systematical testing on melanoma cells with known oncogenic mutations have not performed yet.

Targeted therapies against FGF signaling have not been approved yet, nevertheless, numerous molecules inhibiting FGF signaling are available today. One of them is the small molecule kinase inhibitor *BIBF-1120* (also known as *nintedanib* or intedanib or vargatef) inhibiting also VEGF and PDGF receptors. BIBF-1120 inhibits the proliferation of a large panel of tumor cells including kidney, pharyngeal, ovary, lung, colon, pancreatic cancer and glioma cells *in vitro* and antitumor effect *in vivo* (Hilberg et al. 2008; Torok et al. 2012; Katoh and Nakagama 2013). Furthermore, BIBF-1120 is considered to be a suitable treatment for

idiopathic pulmonary fibrosis (Antoniu 2012). Besides FGFRs, the tyrosine kinase inhibitor *ponatinib* (also named AP24534) has an affinity to VEGFR and ABL as well. *In vitro* treatment with ponatinib resulted in decreased proliferation of breast, lung, gastric, endometrial, bladder, colon cancer cells and reduced growth of tumor xenografts and prolonged survival of host mice *in vivo* (O'Hare et al. 2009; Gozgit et al. 2012; Katoh and Nakagama 2013). Due to the affinity to ABL, ponatinib has recently been approved for the treatment of chronic myeloid leukemia and Philadelphia chromosome positive acute lymphoblastic leukemia (http://clinicaltrials.gov; NCT01592136). Another substance, *BGJ-398*, is a novel and highly selective inhibitor for FGFRs, which effectively reduces proliferation of bladder cancer cells *in vitro* and the amount of circulating tumor cells and lymph node as well as distant metastases *in vivo* (Guagnano et al. 2011; Cheng et al. 2013). Recently, a phase II clinical study has started, where BGJ-398 is going to be tested in combination with the RAF inhibitor LGX818 on BRAF-mutant advanced melanoma (http://clinicaltrials.gov; NCT01820364). A further FGFR selective inhibitor is *AZD-4547*, which reduced the proliferation of breast cancer, multiple myeloma, acute myeloid leukemia and myeloproliferative syndrome-derived cells *in vivo* and demonstrated antitumor effect on colon cancer xenografts *in vivo* (Gavine et al. 2012; Katoh and Nakagama 2013). Thus, similarly to targeting EGFRs, anti-FGFR therapies are effective in different kinds of solid tumors. In addition, EGF and FGF signaling are potential emerging targets for tumor therapy, since these are central pathways and since these pathways are especially affected by the most common oncogenic mutations in malignant melanoma.

The small molecule inhibitor *SB-431542* antagonizes activin signaling by binding to the type I activin receptors ALK4, ALK5 and ALK7 (Inman et al. 2002). It inhibits proliferation of osteosarcoma and proliferation as well as motility of glioma cells *in vitro* (Matsuyama et al. 2003; Hjelmeland et al. 2004; Harrison et al. 2005). Furthermore, SB-431542 augmented immune reactivity against cancer cells *in vitro* and *in vivo* (Tanaka et al. 2010).

Taken together, the investigation of the inhibition of GF signaling is still one of the promising leading edges in the development of anti-cancer therapies.

2. OBJECTIVES

In this thesis, we aimed to investigate the interplay between proliferation and migration in 2D and 3D cultures of human tumor cells. Further aim was to explore how this interplay would be regulated upon stimulation and inhibition of GF receptor pathways and would depend on oncogenic mutations. Thus, our questions were as follows.

1. *Do human tumor cells defer proliferation to cell migration in adherent cultures as it had been postulated in the "go or grow" hypothesis?* Accordingly, we measured proliferation, migration and length of cytokinesis in 35 lung cancer, melanoma and mesothelioma cell lines by videomicroscopy and performed correlation analysis between these cellular processes at both single cell and population level.

2. *Does invasion of the ECM from multicellular spheroids require concurrent cell proliferation?* We raised this question because a number of mathematical models of 3D matrix invasion of tumor cells incorporate the assumption that proliferation is a prerequisite to invasive behavior, however, there was no experimental evidence available for this. In order to evaluate this hypothesis we characterized the invasion pattern of proliferating and proliferation-inhibited cells from multicellular spheroids into collagen type I gel.

3. *Do BRAF and NRAS oncogenic mutations determine the migratory and proliferative response of melanoma cells to activation and inhibition of EGFR and FGFR?* First, BRAF and NRAS oncogenic mutations as well as EGFR and FGFR expression of melanoma cells were determined. Then, migration and proliferation as well as the activation of downstream signaling were explored under baseline conditions and after treatment with EGF and/or FGF or with the inhibitors of these receptors.

4. *Does prenylation inhibition interfere with migration, proliferation and the activity of Ras signaling pathway in human melanoma cells in vitro and in vivo?* BRAF and NRAS mutation dependent effect of ZA treatment on cell migration, proliferation and apoptosis induction was determined in melanoma cells *in vitro*. Furthermore, the effect of ZA on

primary tumor growth and metastasis formation was assessed using animal models of melanoma cells with different mutational status.

5.   *Does activin signaling support or interfere with migration and proliferation of human mesothelioma cells?* Accordingly, we assessed the effect of activin and activin-receptor inhibitor SB431542 treatment on cell proliferation, cytokinesis and migration in human mesothelioma cells via videomicroscopy.

3. METHODS

3.1. Cell cultures

The complete list of cell lines used in this thesis together with their tumor of origin, histological subtype and references are presented in ***Table 2***.

Table 2. Complete list of cell lines used in this thesis.

Cell line / alias	Tumor type	Hystological subtype	Reference
A2058; CRL-11147	melanoma	derived from metastatic site: lymph node	American Type Culture Collection (ATCC)
A375; CRL-1619	melanoma	malignant melanoma (primary site)	ATCC
CRL5820; NCI-H28	mesothelioma	derived from pleural effusion	ATCC
CRL5915; NCI-H2052	mesothelioma	derived from pleural effusion	ATCC
EKVX	lung	non-small-cell lung carcinoma; adenocarcinoma	(Hubbard et al. 1988)
GBM1; formerly HB	brain	glioblastoma multiforme	(Hegedus et al. 2006)
H146; HTB-173	lung	small cell lung cancer; carcinoma; derived from metastatic site: bone marrow	ATCC
H1650; CRL-5883	lung	adenocarcinoma; bronchoalveolar carcinoma; derived from pleural effusion	ATCC

H1975; CRL-5908	lung	adenocarcinoma; non-small cell lung cancer	ATCC
HCC-15; ACC 496	lung	squamous cell carcinoma	Deutsche Sammlung von Microorganismen and Zellkulturen GmbH (DSMZ)
HCC3	liver	hepatocellular carcinoma	(Sagmeister et al. 2008)
HL-HE	lung	small cell lung cancer; derived from metastatic site: brain	Institute of Cancer Research, Vienna, Austria
HT168	melanoma	A2058 subline with low liver metastatic capacity in immunosuppressed mice	(Ladanyi et al. 1990)
HT168-M1	melanoma	A2058 subline with high liver metastatic capacity in immunosuppressed mice	(Ladanyi et al. 1990)
HT199	melanoma		(Ladanyi et al. 1995)
HTB-182; NCI-H520	lung	squamous cell carcinoma	ATCC
I-2	mesothelioma		University of Milano, Italy
I-9	mesothelioma		University of Milano, Italy
LC42	lung	adenocarcinoma	Institute for Cancer Research, Oslo, Norway
LCLC103H; ACC 384	lung	large cell lung carcinoma; derived from pleural effusion	DSMZ
M24met	melanoma	M24met is isolated from a nude mice xenograft of M24 (a lymphnode metastasis)	(Mueller et al. 1991)
M38K	mesothelioma	mixed histological type	University of Helsinki, Helsinki, Finland
MEWO; HTB-65	melanoma	derived from metastatic site: lymph node	ATCC

P31cis	mesothelioma	human pulmonary mesothelioma cell line; cisplatin resistant	University of Umea, Sweden
P31wt	mesothelioma	human pulmonary mesothelioma cell line; wild type	University of Umea, Sweden
SELS	lung	adenocarcinoma; derived from lymph node metastasis	(Endresen et al. 1985)
SPC111	mesothelioma	from pleural effusion with mixed histology (male)	University of Zurich, Switzerland
SPC212	mesothelioma	from tumor with mixed histology (female)	University of Zurich, Switzerland
SW900; HTB-59	lung	squamous cell carcinoma	ATCC
U87; HTB-14	glioblastoma	glioblastoma; astrocytoma	ATCC
VM-1 FTSLA	melanoma	derived from metastatic site: lymph node	Institute of Cancer Research, Vienna, Austria
VM-15 MJZJ	melanoma	derived from metastatic site: lymph node	Institute of Cancer Research, Vienna, Austria
VM-21 RHTP	melanoma	nodular melanoma	Institute of Cancer Research, Vienna, Austria
VM-24 SHJT	melanoma	derived from metastatic site: lymph node	Institute of Cancer Research, Vienna, Austria
VM-47 HOST	melanoma	derived from metastatic site: brain	Institute of Cancer Research, Vienna, Austria
VMC23	mesothelioma	epithelioid mesothelioma	Institute of Cancer Research, Vienna, Austria
VMC33	mesothelioma	epithelioid mesothelioma	Institute of Cancer Research, Vienna, Austria
WM35	melanoma	superficial spreading melanoma	Wistar Collection; Wistar Institute, Philadelphia, USA

WM983A	melanoma	unclassified radial growth phase melanoma (primary site from WM983B)	Wistar Collection; Wistar Institute, Philadelphia, USA
WM983B	melanoma	derived from metastatic site: lymph node	Wistar Collection; Wistar Institute, Philadelphia, USA

Cell cultures were maintained, unless otherwise stated, at 37°C in a humidified 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 4500mg/dm^3 glucose, piruvate and L-glutamine (Lonza, Switzerland) and supplemented with 10% fetal calf serum (FCS; Lonza, Switzerland) and 1% penicillin-streptomycin-amphoterycin (Switzerland). For conventional 2D cultures, cells were grown in tissue culture flasks. For examining the EGFR and FGFR activation, cells were kept in medium supplemented with 5% FCS.

3.2. Collagen invasion assay

For 3D cell aggregates, a modified version of our previous protocol (Hegedus et al. 2006) was applied. Briefly, GBM1 and U87 cells were trypsinized (0.1% trypsin, Sigma) at 80% confluent conventional cell culture and cell suspensions were centrifuged at 2000×g for 3 min. Pellets were drawn into pipette tips of 200 μl and incubated for 30 minutes at 37°C. The resulting cylindrical aggregates were immediately embedded into collagen gel.

Collagen gel was prepared from rat-tail collagen type I (Sigma-Aldrich) by dissolving the powder in 1M acetic acid. Neutral pH of the solution was adjusted by adding 7.5% $NaHCO_3$. The final concentration of 1 mg/ml was achieved by diluting the gel with regular culture medium or with medium complemented with the inhibitor compound. For the assay, 200 μl ice-cold collagen solution was added to each well of 96-well plates. Before embedding the cell aggregates, the plate had been kept at room temperature for 10 minutes and when seeding the cells, the plate was transferred to a 37°C incubator to achieve complete gelation. After 30 minutes of incubation, the gel was covered with 100 μl of complete growth medium and the samples were kept at 37°C in a humidified 5% CO_2 atmosphere. Medium was changed every other day. To follow the invasion, images were taken daily using a phase-contrast Nikon

microscope and a Nikon Coolpix 4500 digital camera. The experiments were quantified by measuring the distance between the tips of the radially migrating cells from the outer border of the sphere after 24 hours.. Experiments were repeated independently thrice.

3.3. Analysis of oncogenic mutation in melanoma cells

For mutation analysis, adherent and 80% confluent cells were detached (0.1% trypsin, Sigma-Aldrich, St. Louis, MO) and centrifuged. From the cell pellet genomic DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to manufactures' instructions, except for the deparaffinization steps that were skipped.

BRAF codon 600 mutations were analyzed by a two-step polymerase chain reaction (PCR) and restriction digestion as described previously (Nagasaka et al. 2004). The first primer sequences were as follows: forward (Mt-F) 5'-TAAAAATAGGTGATTTTGGTCTAGCTGC-3' and reverse (Wt-R) 5'-CCAAAAATTTAATCAGTGGAAAAATA-3'. The products obtained were then used in a second-stage PCR with the primer pair Mt-F and Mt-R (5'-AAAAATTTAAGCAGTGGAAAAATAGC-3') under the same conditions as the first-stage PCR. In the last step PCR products were digested with BtsI (New England Biolabs, Beverly, MA). All products were visualized on 3% agarose gels stained with ethidium bromide.

The base pair substitutions in NRAS and BRAF were determined by sequencing. The isolated DNA was amplified by PCR using the primers of hBRAFex15F: 5′-GGAAAGCATCTCACCTCATCC-3′ and hBRAFex15R: 5′-TGGTTTCAAAATATTCGTTTTAAGG-3′ for BRAF and hNRASex2F: 5′-CACCCCCAGGATTCTTACAG-3′ and hNRASex2R: 5′-TCGCCTGTCCTCATGTATTG-3′ for NRAS. After the PCR reaction, samples were purified with Applied Biosystems BigDye® XTerminator™ Purification Kit and mutations were verified through sequencing on ABI 3130 genetic Analyser System (Life Technologies, Carlsbad, CA) with BigDye® Terminator v1.1 Kit (Applied Biosystems, Foster City, CA). The sequencing analysis was carried out by Dr. Erzsébet Rásó and Tamás Barbai.

3.4. Investigation of expressed GFRs - qRT-PCR for EGFR, FGF1-4 receptor expression

Total ribonucleic acid (RNA) was isolated using TRIzol (Invitorgen, Carlsbad, CA) according to manufactures' instructions. In the next step messenger RNA (mRNA) levels of EGFR and FGFRs were determined by quantitative real-time PCRs (qRT-PCRs). TaqMan qRT-PCR Master Mix containing the appropriate TaqMan probe (11 µl) and cDNA (1 µl) were mixed in a MicroAmp Optical 96-well Reaction Plate (Applied Biosystems, Foster City, CA). The following TaqMan probes were used: EGFR - Hs01076078 m1, FGFR1 - Hs00915135 m1, FGFR2 - Hs01552926 m1, FGFR3 - Hs00179829 m1, FGFR4 - Hs00608751 g1, and GAPDH - Hs99999905 m1. PCR and fluorescence measurement after each cycle was performed and in an ABI Prism 7000 SDS Thermocycler (Applied Biosystems, Foster City, CA). As a reference GAPDH, a housekeeping gene, was used for normalization and additionally the size of PCR products were verified by agarose gel electrophoresis. Each sample-preparation and measurement was performed twice. These measurements were carried out by Karin Schelch at the Institute of Cancer Research of the Medical University of Vienna, Vienna, Austria.

3.5. Videomicroscopy

Videomicroscopy measurements were performed as described previously (Hegedus et al. 2000; Hegedus et al. 2004). Briefly, cells were seeded in the inner 8 wells of 24-well plates (Corning Incorporated, Corning, NY). To have the cells attached, they were kept overnight in DMEM medium supplemented with FCS. Upon start of the measurement, culture medium was changed to CO_2-independent medium (Gibco-BRL Life Technologies, Carlsbad, CA) supplemented with FCS and 4 mM glutamine. Outer wells of the plate were filled with medium to reduce evaporation from the inner wells. Cells were kept in a custom designed incubator built around an inverted phase-contrast microscope (World Precision Instruments, Sarasota, FL) at 37°C and room ambient atmosphere. During the recording, images of 3 neighboring microscopic fields in each well were taken in every 5 min for at least 48 hours if no treatment was scheduled for the cells. If cells were to be treated, recording started 1 day before – to obtain baseline data – and ended at least 2 days after the treatment.

3.6. Cell migration analysis

Migration data were retrieved from the captured phase contrast images analyzing them individually with a cell-tracking program that enables manual marking of individual cells and to recording their positions into data files. By connecting the marks of one cell's position on each consecutive picture during the whole recording the path – the trajectory – of a given cell can be obtained (*Figure 3.*). Cell motility was quantified as the net displacement of tracked cells during the relevant 24 hours of the recorded time period. To characterize a cell line population, single cell displacements were determined in two independent time-lapse recordings, each containing three non-overlapping microscopic fields. The obtained displacement magnitudes were pooled and averaged.
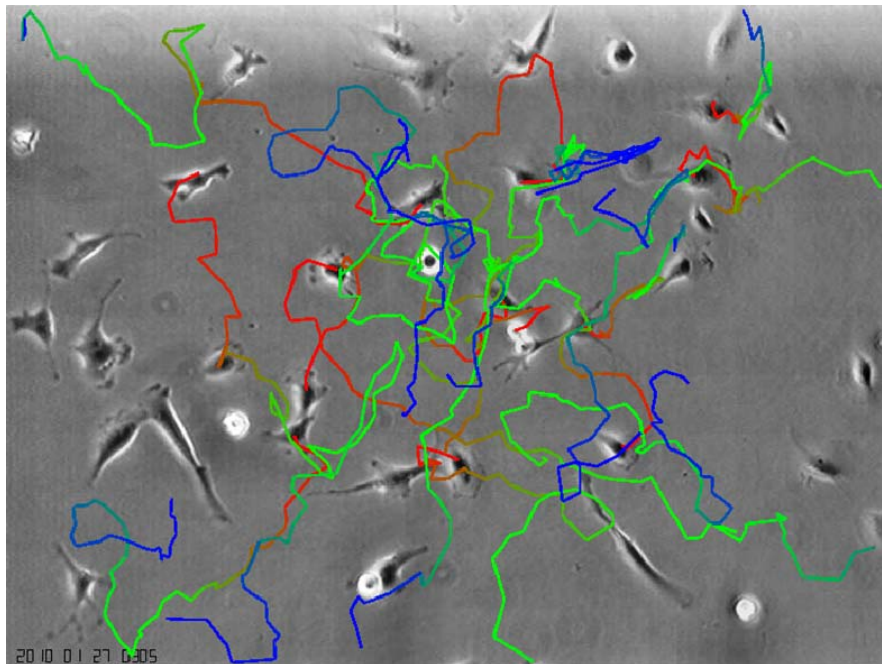
Figure 3. Trajectories of cells. By marking the position of a given cell on each consecutive picture and connecting these points during the whole recording the path of a given cell i.e. its trajectory can be drown. The color of the depicted trajectories refers to the time elapsed in the order of red-green-blue.

3.7. Videomicroscopy based proliferation and cytokinesis analysis

Cell proliferation was expressed as the "expected value of daily divisions per cell", calculated as <dN/N>, where dN is the increment in cell numbers within one microscopic field during a 24 h-long time period, N is the initial number of cells within the field, and <..> denotes averaging over at least 4 microscopic fields obtained from at least two independent recordings. Thus, expected value of cell division shows how many divisions can be expected while observing a single cell for 24 hours.

Average duration of one cell division – cytokinesis length – was also assessed using videomicroscopy recordings (*Figure 4.*). To characterize the population of a given cell line, cytokinesis-lengths were evaluated and averaged for at least 30 cells, obtained from at least three independent microscopic fields.
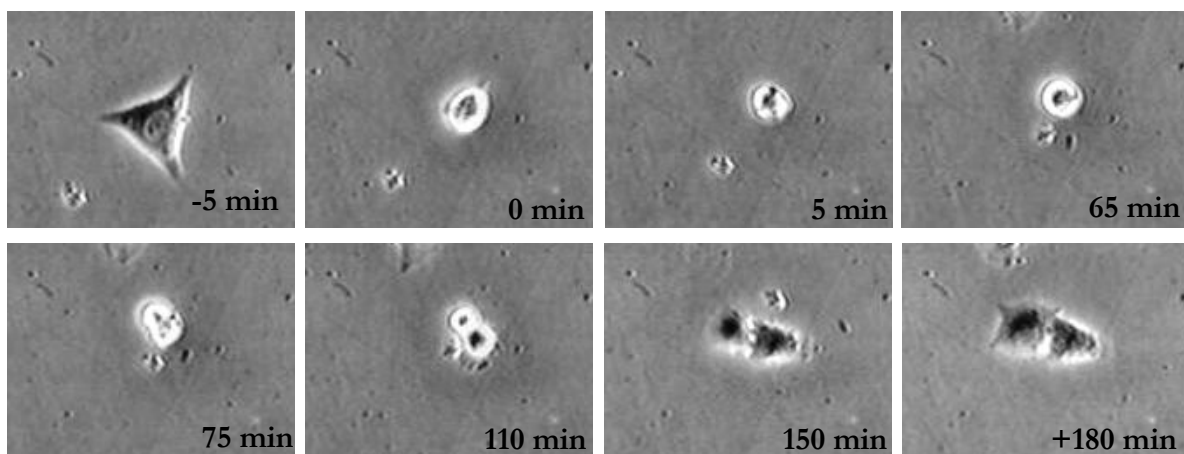
Figure 4. Example for a 150-minutes-long cytokinesis on the videomicroscpic recording.

3.8. Single cell based correlation analysis

Time-lapse recordings and cell tracking data also allow us to probe for correlations between cell motility and cell proliferation at the individual cell level. Therefore, measures of individual cell motility were linked to further parameters. (i) We included information whether the traced cell underwent cell division, and if so, what was the duration of cytokinesis. (ii) To characterize local cell density, we specified the number of cells that were

either in physical contact with, or closer than 10 micron from the traced cell. If there were no cells closer than a single cell diameter, we categorized the tracked cell as solitary. We employed scatter plots to detect correlations between two continuous variables (like cell motility versus cytokinesis-length). To assay the relevance of discrete parameters such as the presence of adjacent cells (cells in contact vs. solitary cells) or whether the traced cell underwent cell division during the 48 hours of the measurement (dividing vs. non-dividing cells), we directly compared distribution functions of the continuous variable (like cell speed), each for a distinct value of the discrete parameter.

Some of the correlations between our cell phenotype measures can be traced back to the fact that certain cell lines have intrinsically different cell motility or cell division rates than others, even if obtained from similar tumor types. To minimize this effect on the single-cell data, both continuous variables (speed, cytokinesis-length) were normalized as $x'=(x-X)/X$, where x and X denotes the value characterizing the individual cell and the population, respectively, and x' is the introduced new measure. Thus x' characterizes the value of a certain measure relative to the population average.

3.9. SRB proliferation assay

SRB assay was performed to analyze cell proliferation, based on the measurement of cellular protein content. Prior to measurement cells were plated in the inner 60 wells of a 96-well plate and incubated for 24 h to adhere. After 24 or 72 hours of treatment, cell monolayers were fixed with 10% trichloroacetic acid and stained for 15 min with SRB. Excess dye was removed by repeated washing with 1% (vol/vol) acetic acid, and then the protein-bound dye was dissolved in 10 mM Tris and optical density was determined at 570 nm using a microplate reader (EL800, BioTec Instruments, Winooski, VT). Proliferation data were averaged of independent experiments and effect of treatment was expressed as control to treated ratio.

3.10. TUNEL assay

For apoptosis detection cells were seeded on 24 well plates and left to adhere overnight. After treatment cells were fixed with 4% buffered PFA and labeling of terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) was performed according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland). Quantification was done by direct counting of the TUNEL-positive cells on at least five 20× microscopic fields.

3.11. Immunoblot measurements

Immunoblot analysis was performed to quantitate the activating phosphorylation of Erk1/2, S6, FAK and Src proteins at Thr202/Tyr204, Ser240/244, Tyr576/577 and Tyr416, respectively, in human melanoma cells. Cells were plated in six-well dishes and upon attachment and treatment cells were collected on ice in RIPA Buffer (Thermo Scientific, Waltham, MA) supplemented with 1% Halt Protease Inhibitor Single-Use Cocktail (Thermo Scientific). Total protein concentrations were measured using Pierce BCA Protein Assay kit (Thermo Scientific). Equal amounts of denatured protein were loaded on sodium dodecyl sulfate-polyacrylamide gel, separated by electrophoresis (SDS-PAGE; 12%) and transferred to nitrocellulose membrane (Whatman, Maidstone, UK). Incubation with anti-p-Erk1/2/Erk1/2 (Cell Signaling Technology, Danvers, MA; Cat no.: #9101, #9102, respectively), anti-p-S6/S6 (Cell Signaling; Cat no.: #2215, #2217, respectively), anti-p-FAK/FAK (Cell Signaling; Cat no.: #3281, #3285 respectively) anti-p-Src/Src (Cell Signaling Technology; Cat no.: #2101, #2123, respectively) and as loading control anti-tubulin (Cell Signaling Technology; polyclonal, rabbit) was performed overnight at 4°C in a dilution of 1:2000. HRP-labeled anti-rabbit secondary antibody was applied in a dilution of 1:2000 for 30 min at room temperature. Visualization was achieved using the Amersham ECL Advance Western Blotting Detection kit (GE HealthCare, Little Chalfont, UK). Densitometry measurements were carried out using ImageJ software (National Institutes of Health, Bethesda, MD). Activation of signaling was quantified as the ratio of phosphorylated and total protein.

3.12. In vivo experiments

All animal-model protocols were carried out in accordance with the Guidelines for Animal Experiments and were approved for the Department of Experimental Pharmacology in the National Institute of Oncology, Budapest, Hungary (permission number: 22.1/722/3/2010).

For subcutaneous xenograft models, human melanoma cells (10^6 HT168-M1 and MEWO, 10^5 M24met) were subcutaneously injected into male NSG (NOD scid gamma) mice at a weight of 30-33g having 10 animals per group. Since the HT168-M1 xenografts tend to outgrew rapidly, mice transplanted with HT168-M1 were treated and sacrificed at earlier time points. After randomization, animals were treated intraperitoneally on a weekly basis for three weeks. The treatment with ZA started when tumors were measurable, consequently at day 10 for mice injected with HT168-M1 or after two weeks for animals injected with M24met or MEWO. Control animals received 100 µl of 0.9% NaCl. The subcutaneous tumors were measured with a caliper and tumorvolumes were calculated using the formula for volume of a prolate ellipsoid (length × width^2 x π/6) and expressed in cm^3. After the last measurement of tumor size animals were sacrificed by cervical dislocation.

In the spleen to liver colonization assay, melanoma cells ($5×10^2$ HT168-M1, 10^5 M24met or 10^6 MEWO) were injected into the spleen of male NSG mice under Nembutal anesthesia, having 10 animals per group. ZA (50 or 500 µg/kg) or saline as a control was administered intraperitoneally starting from day 7 for HT168-M1 and from day 10 for M24met and MEWO cells and continued for 3 weeks. Then, animals were sacrificed by cervical dislocation, spleen and liver were removed and weighed. Animal experiments were carried out at the Department of Experimental Pharmacology in the National Institute of Oncology, Budapest, Hungary.

3.13. Statistical methods

To determine statistical differences between groups, ANOVA test with the post hoc tests Tukey-test or Dunnett's multiple comparison test was performed for datasets with normal distribution. Otherwise, non-parametric Kruskal-Wallis and post hoc Dunn's multiple comparison test was used. To determine differences between pairs T-tests were computed.

To determine the correlation Kolmogorov-Smirnov test was applied first, and in case no differences were detected to Gaussian distribution Pearson-correlation was calculated subsequently.

Statistical significance was established at $p<0.05$. All statistical analyses were computed in GraphPad Prism 5 (GraphPad Software Inc, San Diego, CA).

4. RESULTS

### *4.1. Migration/proliferation dichotomy in 2D cell cultures*

Since migration, proliferation and the interplay of these cellular processes, postulated by the "go or grow" hypothesis, are crucial in terms of tumor progression and investigated currently in central nervous system tumors we evaluated this hypothesis on tumor cell lines with neuroectodermal, mesodermal and entodermal origin. Videomicroscopy recording and assessment of migration, proliferation and cytokinesis-length were performed on both individual cells and on population levels in 2D cell cultures of thirty-five (12 mesothelioma, 13 melanoma and 10 lung) cancer cell lines. Migrated distance, average expected number of cell divisions within 24hs and average duration of cytokinesis are shown on *Figure 5 A-C*.

Migrated distances were highest in mesothelioma ranging from 45 to 300 microns. In melanoma and lung cancer cell lines the migration distance ranged from 35 to 210 microns and from 10 to 150 microns, respectively. Significantly higher averaged 24-hour migration distance was found in mesothelioma cells (160 microns; $p = 0.0014$) when compared to melanoma and lung cancer cells that migrated 80 and 50 microns, respectively (*Figure 5 A*).

Average expected number of cell divisions within 24hs showed a range of one order of magnitude from 1.8 to 0.18. The greatest variety was observed in case of lung cancer cells, where the most proliferative cell line was characterized with 1.8 and the less proliferative with 0.19 expected division in 24hs. The highest averaged proliferation was found in mesothelioma cells (the mean expected value of cell divisions in a 24-hour time interval was 0.80) followed by proliferation of melanoma and lung cancer cells with average rates of 0.68 and 0.65 respectively. There was no significant difference between the average proliferation rates of the examined cancer subtypes (*Figure 5 B*).

Average duration of cytokinesis was determined on the basis of videomicroscopic recordings. The duration of cytokinesis was the shortest, thus the cell division was the fastest, in melanoma cell lines (ranging from 50 to 108 minutes). The duration of one cell division was quite similar in lung cancer and mesothelioma cells ranging from 78 to 186 and from 58 to 172 minutes, respectively. The average duration of one division showed no significant differences between the three tumor subtypes, although lung cancer cells tended to spend

more time (103 minutes) on one division than melanoma or mesothelioma cells (88 and 85 minutes, respectively) (***Figure 5 C***).
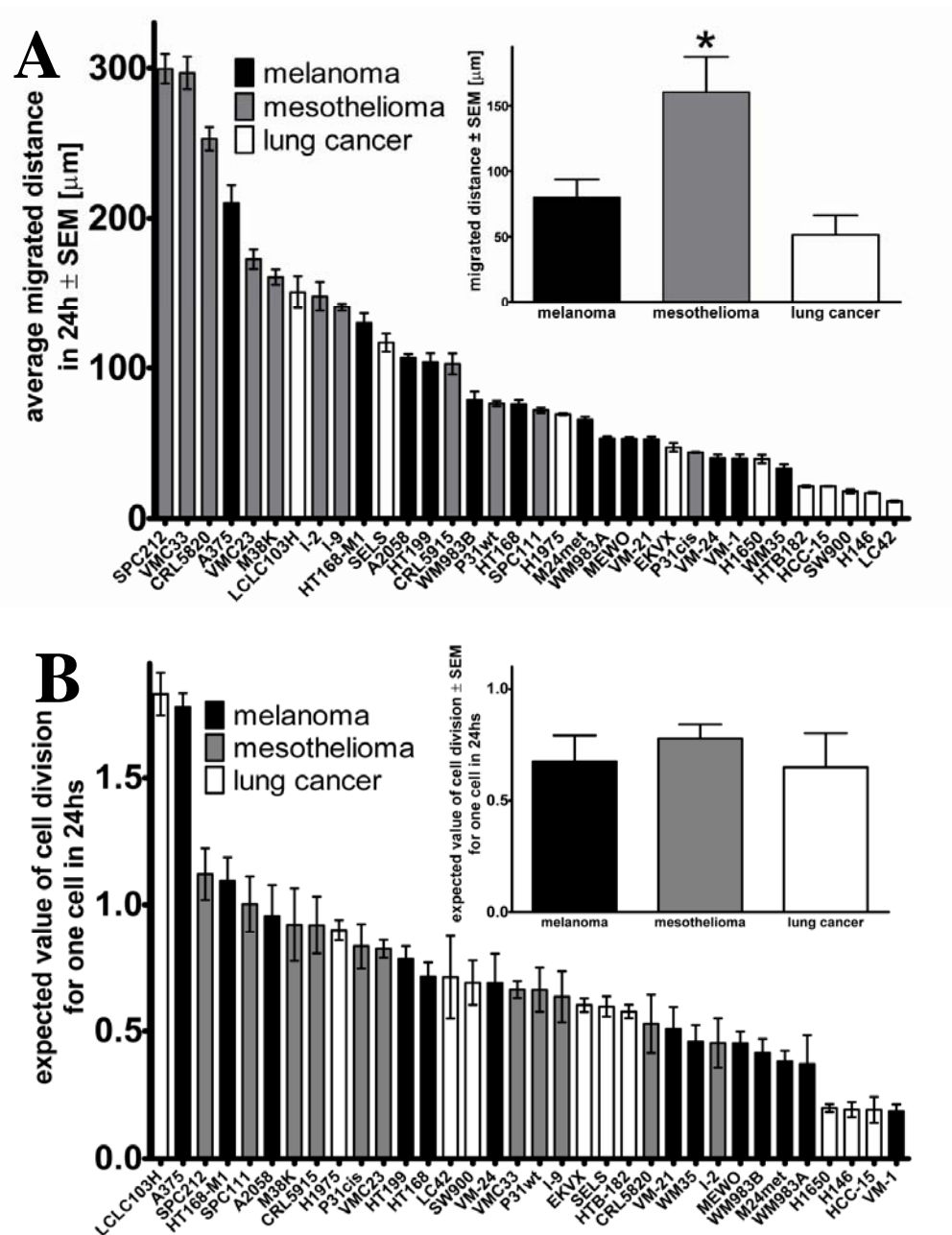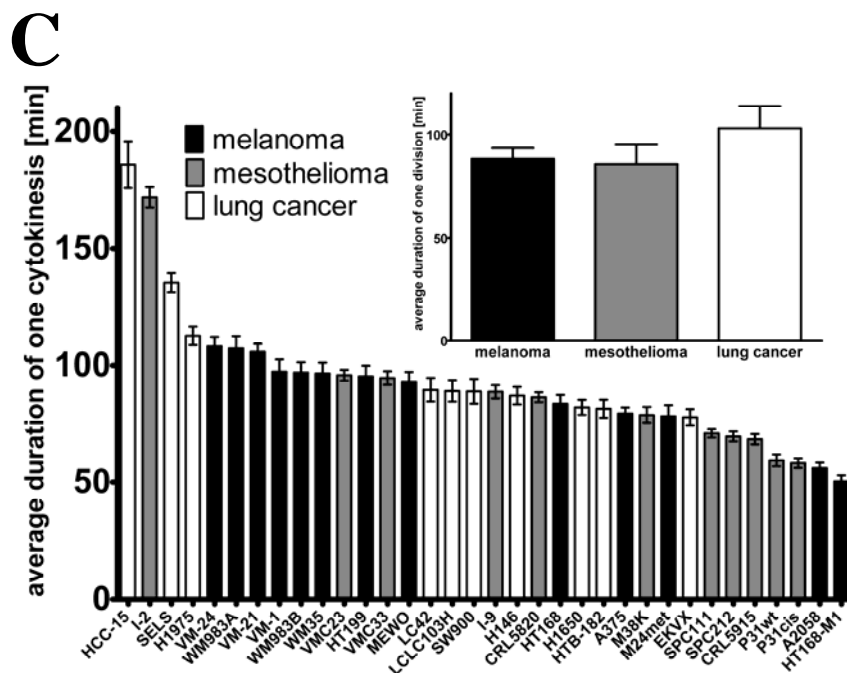
Figure 5. Migrated distance, proliferation and cytokinesis length for different cancer cell lines. (**A**) Migrated distance in 24h for the examined cell lines evaluated on the basis of videomicroscopy recordings. *Average migrated distance in 24h was significantly higher in mesothelioma cells compared to melanoma and lung cancer cells. (**B**) Proliferation of the tumor cell lines. Cell proliferation was quantified by counting cell divisions for 48 hours and normalized for initial cell number (i. e. the inverse of doubling time). (**C**) Average duration of cytokinesis determined by videomicroscopy. The inserts shows the measured parameter averaged for each cancer type. Colors black, grey and white indicate melanoma, mesothelioma and lung cancer cells, respectively. Data shown is the average of at least 4 independent measurements. Asterisk designates significant differences (p<0.05).

The statistical correlations between the probability of cell division, the average migrated distance and the duration of cytokinesis has been established in all three tumor types (***Figure 6 A-I***). Interestingly, a strong positive correlation (p < 0.0001, r = 0.92, R^2 = 0.86; ***Figure 6 A***) was found between cell proliferation and cell migration in melanoma cells and in lung cancer cells (p = 0.015, r = 0.73, R^2 = 0.54; ***Figure 6 C***), as well; whereas, no correlation was observed in the examined mesothelioma cell lines (***Figure 4 B***).

Correlation between average duration of cytokinesis and cell migration was calculated for each tumor type. Interestingly, significant negative correlation was found between duration of cytokinesis and cell migration in melanoma cell lines (p = 0.0372, r = -0.5814, R^2 = 0.3380;

Figure 6 D). There was no significant correlation between cytokinesis and migration in mesothelioma and lung cancer cells (*Figure 6 E and F*). Correlation between cell proliferation and duration of cytokinesis was significant in mesothelioma cells (p = 0.0448, r = -0.61, R^2 = 0.38; *Figure 6 H*) but failed to show significance in melanoma and lung cancer cells (*Figure 6 G and I*).
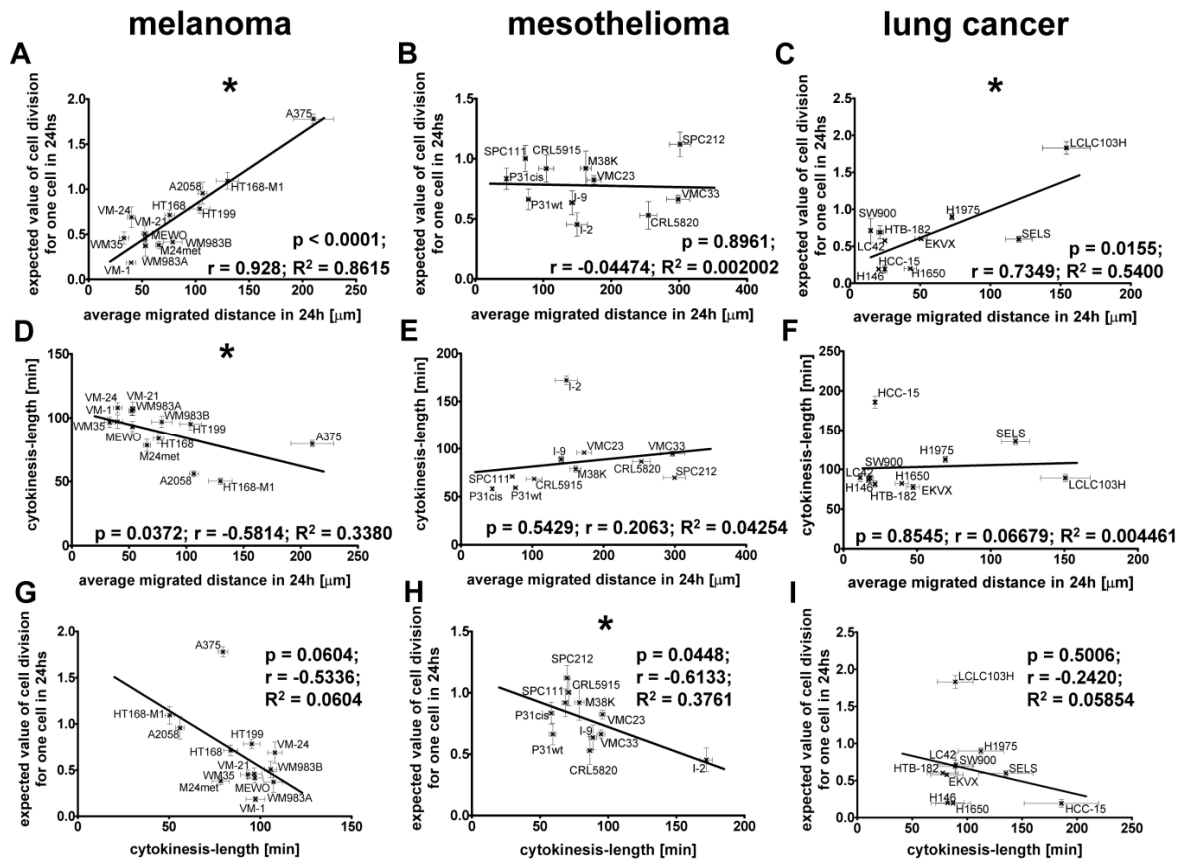

Figure 6. Correlations between mean cell proliferation, migration and duration of cytokinesis. (*A-C*) Correlation of cell proliferation and migration. *Significant positive correlation was found in melanoma and lung cancer cells. (*D-F*) Correlation of cytokinesis-length and cell migration. *There was a significant negative correlation in melanoma. (*G-I*) Correlation of cell proliferation and cytokinesis-length. A strong tendency and a significant negative correlation was characteristic of melanoma and *mesothelioma, respectively. Parameters of the calculated Pearson correlation p, r and R^2 are shown in the diagrams. Asterisks indicate statistical significant correlation.

In order to investigate whether the observed correlations at the level of cell population could also be detected at the level of single cells, we analyzed individual cells from previous

measurements of the three cell lines with low, medium and high migratory activity and established statistical correlations between migration distance and duration of cytokinesis of individual cells (***Figure 7 A-C***). However, no significant correlation was found in either of the cell lines studied (***Figure 7 A***). The velocity distribution of the dividing individual cells (cells that divided during the 48 hours of the videomicroscopy measurements) showed an increased migratory activity in melanoma and lung cancer but not in mesothelioma (***Figure 7 D-F***). In order to investigate whether local cell density would influence migratory activity, we plotted the velocity distribution of solitary and "in-contact" cells; nevertheless, no statistical differences were found in either of the three tumor types (***Figure 7 G-I***). Therefore, our findings at population-level displayed on ***Figure 6*** are also valid at individual cell level.
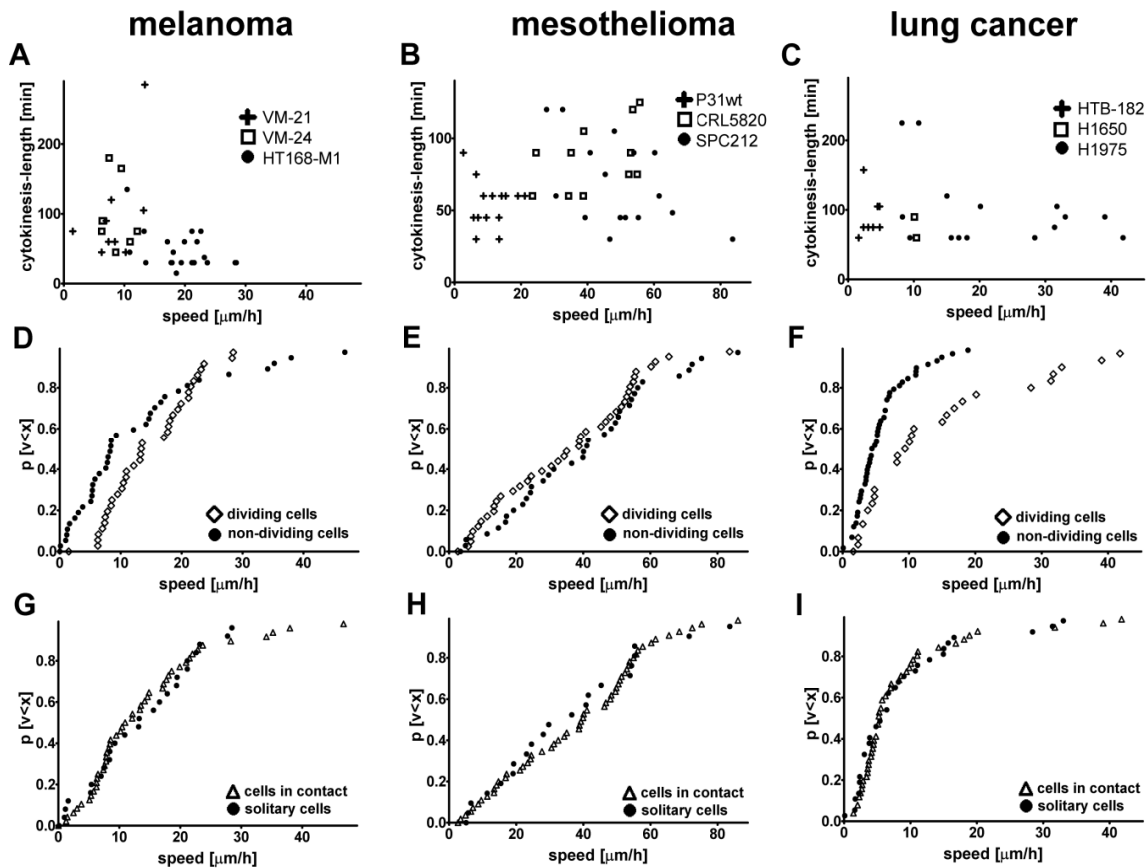
Figure 7. Correlation between migration and duration of cytokinesis, cell division and local cell density observed at the level of single cells. Individual cells from previous measurements of three cell lines with low, medium and high migratory activity from each tumor type had been analyzed. (***A-C***) We found no significant correlation between cytokinesis-length and cell migration speed. (***D-F***) Dividing melanoma and lung cancer cells displayed a higher migration speed than non-dividing cells while there was no difference in mesothelioma cultures. (***G-I***) The solitary cells and cells in contact demonstrated no difference in migratory activity. (The parameter p[v<x] stands for the probability that a randomly chosen cell shows a smaller speed than indicated at the x axis.)

Given the existing differences at the population level, we also determined if single cell motility parameters are correlated with local cell density, the presence of cell divisions or the duration of cytokinesis. Thus, we calculated the relative motility index for each cell as the difference of the values characterizing the particular cell and the population (cell line) average, and normalized the difference to the population average. In other words, we distinguished between faster and slower cells within a given population. Similarly, we related

the duration of each cytokinesis event to the average characterizing the corresponding cell line. Scatter plots and distribution functions of the normalized quantities revealed no correlations, except: non-dividing melanoma cells that exhibited a broader distribution of cell velocities than their dividing counterparts (*Figure 8*). As it can be seen from the results, the various cell lines exhibited distinct motile and proliferative characteristics. Beyond these differences, the speed and proliferative characteristics of individual cells appeared as random, free from constraining effects of the "go or grow" regulation.
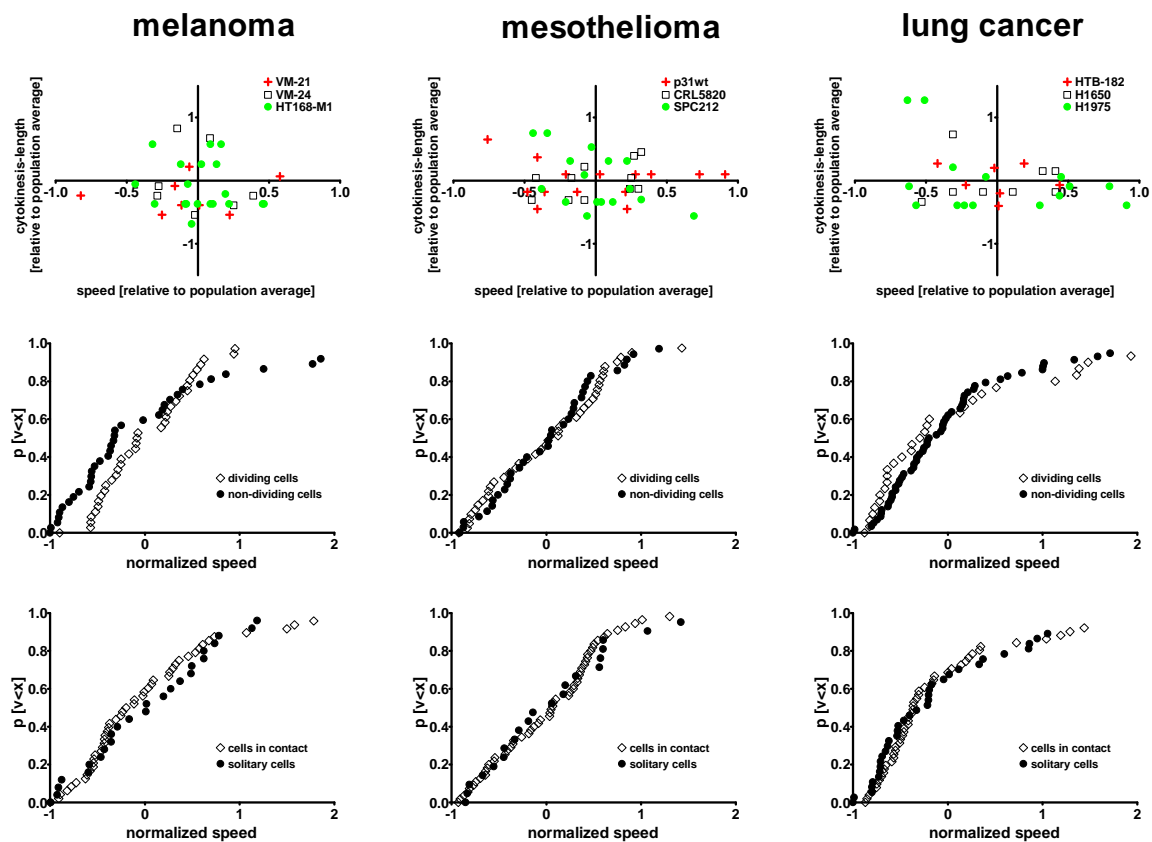
Figure 8. The influence of various potential factors on the motility of individual cells. Measures characterizing each cell are calculated by normalization to the population average of the corresponding cell line. The only statistically significant difference was found in melanoma cells, where non-dividing cells exhibited broader distribution of cell velocities than their dividing counterparts. While individual cells exhibited distinct motile and proliferative characteristics, these appeared to be random with no obvious interdependence. (The parameter p[v<x] stands for the probability that a randomly chosen cell shows a smaller speed than indicated at the x axis.)

Since the FAK/Src signaling is an important regulatory pathway of 2D migration, activation of FAK and Src kinases were explored by examining total and phosphorylated amount of proteins via immunoblot assay. Further, ranging cells according their migratory potential and dividing them into two groups at their median, average activation was calculated for the six slowest and six fastest migrating melanoma cells (***Figure 9***). Interestingly, activation of FAK tended (p=0.0796) to be higher in fast migrating melanoma cells. In contrast, activation of Src was essentially equal in fast and slow migrating cells.
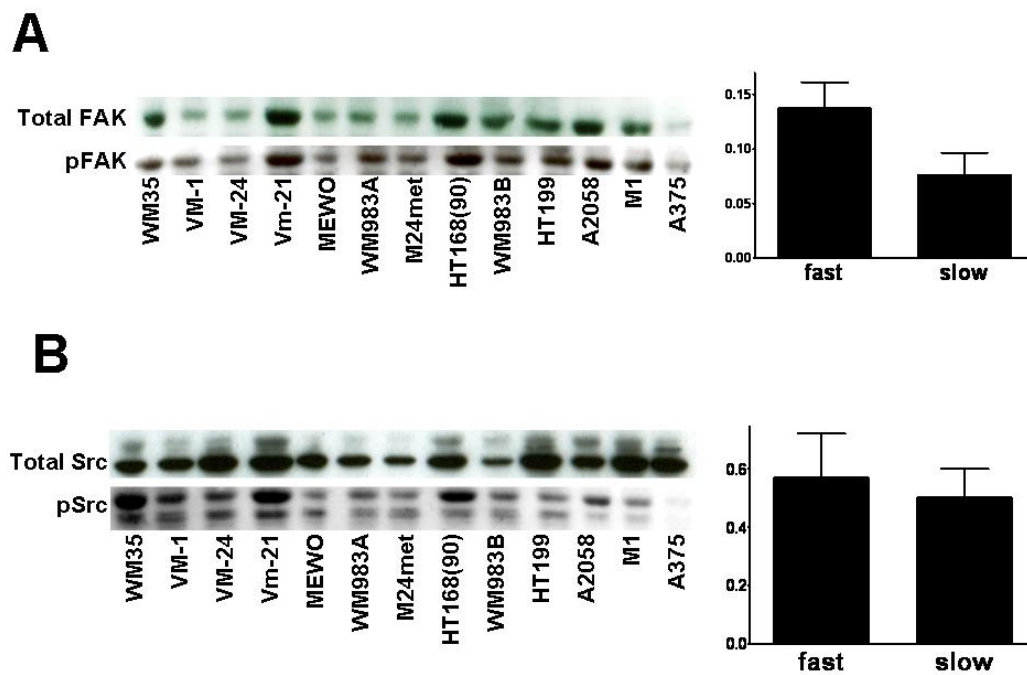
Figure 9. Representative immunoblots and quantification of the phosphorylated FAK (***A***) and Src (***B***) kinases in human melanoma cells. FAK activation proved to be considerably (p=0.0796) higher in fast migrating melanoma cells. Intensity is expressed in relation to total FAK and Src and is averaged in the six slowest and six fastest migrating melanoma cell lines (average±SEM).

4.2. Proliferation and migration in 3D cell cultures

Since interplay between proliferation and migration plays an important role in 3D cellular models, and furthermore proliferation is thought to be a prerequisite of the 3D invasion of tumor cells in the extracellular matrix, the independence of proliferation and migration was studied in glioblastoma cells. Furthermore this evaluation could support a novel mathematical model that describes the invasion of tumor cells into the surrounding matrix. Accordingly cell invasion from an aggregate into a surrounding ECM was studied in the presence/absence of a cell division inhibitor Q50 (research compound; Avidin Ltd, Hungary). Nevertheless, the invasion patterns and migrated distances in the first 24 hours after treatment were essentially the same in division inhibited and control cells (***Figure 10***).
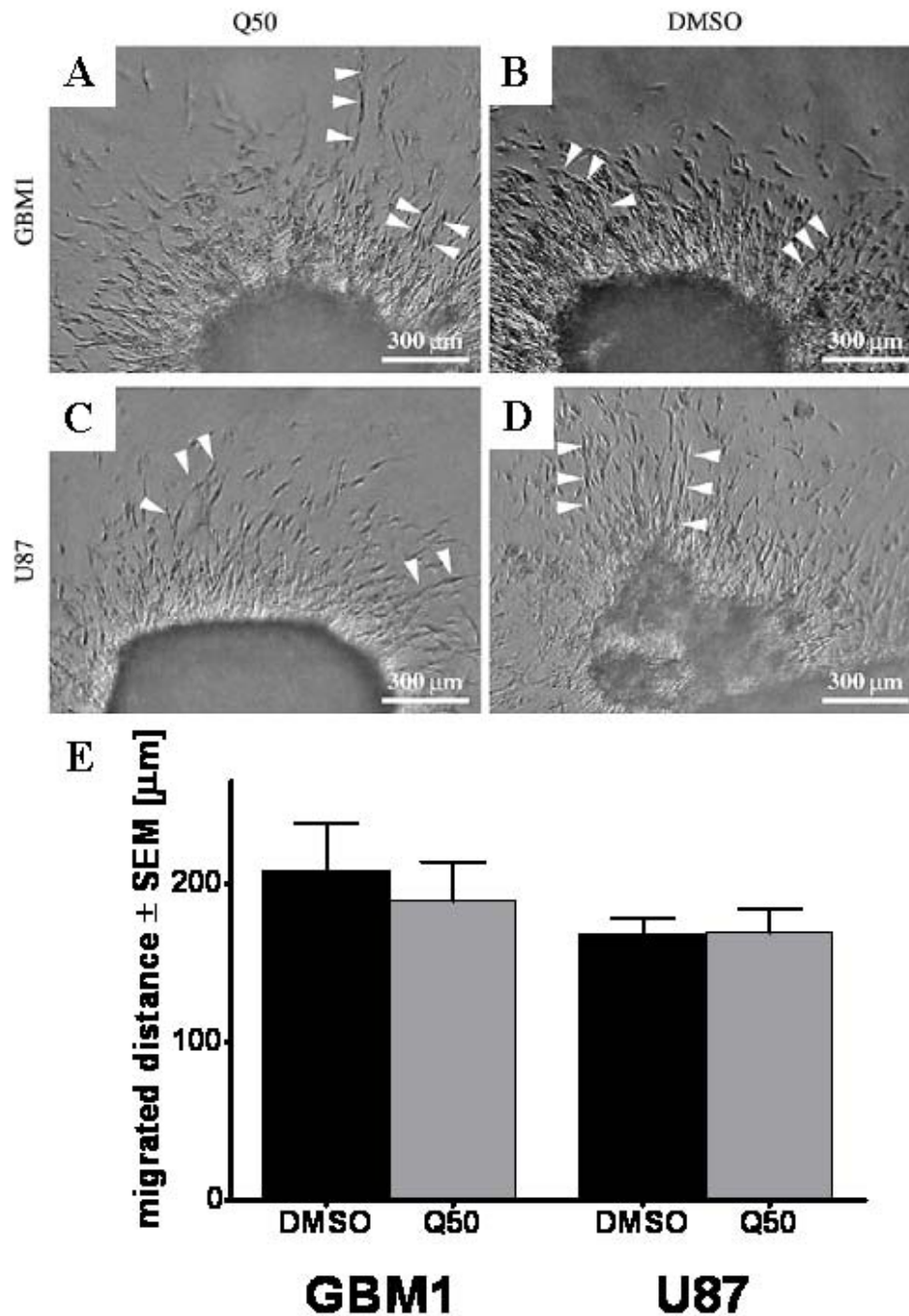
Figure 10. Representative pictures of gel invasion of glioblastoma cells. Invasion of GBM1 (*A* and *B*) and U87 (*C* and *D*) cells into collagen gels after one day in culture and quantification of the migration (*E*). The initial invasion patterns are independent of cell division: cells treated with Q50 (*A* and *C*) show a similar invasion pattern as the control cultures (*B* and *D*). In each experiment cells invade the matrix radially outward from the aggregate with essentially the same velocity (*E*) and often form radially oriented chains (arrowheads). Data is shown as average ± SEM of at least four independent measurements.

4.3. Major oncogenic mutations in melanomas

In order to evaluate whether the most important oncogenic mutations would influence migration and cell division in a distinct manner in melanoma, mutational status of the investigated cell lines were determined or confirmed if already known. Base pair substitutions in BRAF and NRAS were determined by sequencing (***Table 3***). The mutational status was confirmed in BRAF-mutant A375 and A2058, in NRAS-mutant M24met and in double wild type MEWO cells. Furthermore, BRAF mutation was determined in HT168-M1 and HT199 cells, NRAS mutation was found in VM-15 cells and VM-47 was proved to have wild type of NRAS and BRAF.

Table 3. Oncogenic BRAF and NRAS mutation in the examined human melanoma cell lines detected by direct sequencing method.

	BRAF	NRAS
A375	c.1799T>A: p.Val600Glu (V600E)	wild-type
A2058	c.1799T>A: p.Val600Glu (V600E)	wild-type
HT168-M1	c.1799T>A: p.Val600Glu (V600E)	wild-type
HT199	c.1799T>A: p.Val600Glu (V600E)	wild-type
VM-15	wild-type	c.181C>A: p.Gln61Lys (Q61K)
M24met	wild-type	c.182A>G: p.Gln61Arg (Q61R)
MEWO	wild-type	wild-type
VM-47	wild-type	wild-type

4.4. Ligand dependent activation of EGFR and FGFR in melanoma

Prior to testing the activation of EGFR and FGFR receptors on melanoma cells with known mutational status, expression of EGFR and FGFR relative to GAPDH was investigated by qPCR. High expression of EGFR, FGFR1 and FGFR4 was confirmed in each of the investigated cell lines (***Figure 11***). Interestingly, FGFR2 and FGFR3 were not expressed in

the two NRAS-mutant cell lines. In average, the lowest expression of growth factor receptors was found in the double wild-type cells.
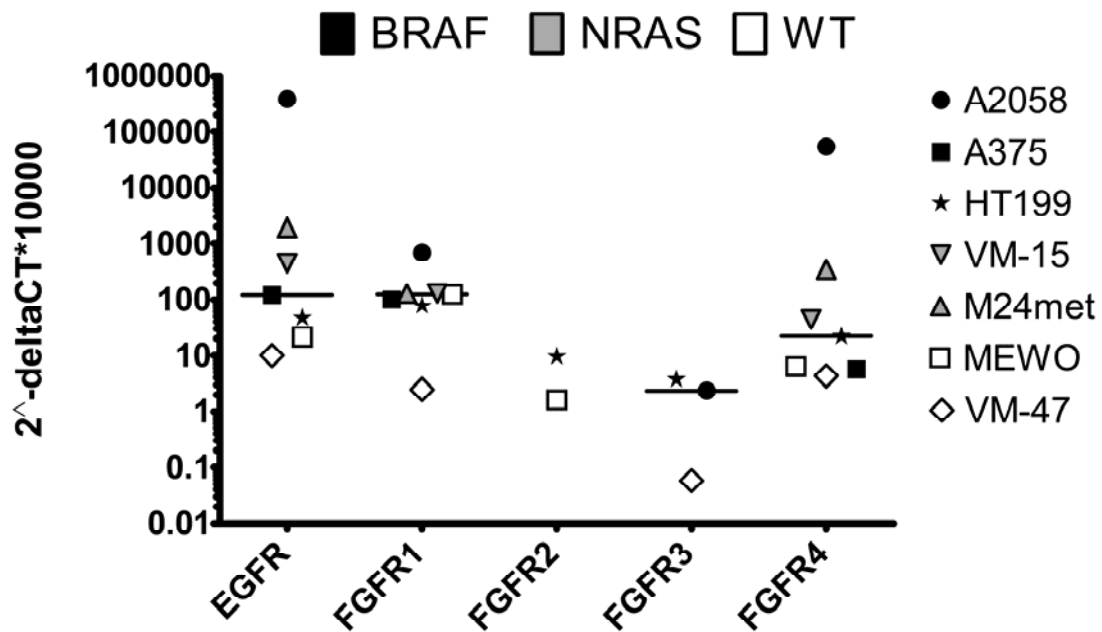

Figure 11. Relative expression of EGFR and FGFRsrelative to GAPDH in melanoma cell lines with different mutational status determined usingRT-qPCR. EGFR, FGFR1 and FGFR4 were expressed in each investigated cell line. Interestingly, the double wild-type cells showed the lowest expression of GFRs, in average. Black: BRAF, and grey: NRAS mutation and clear markes: wild type.

Ligand activation of EGFR and FGFR was investigated by treating cells with 50 ng/ml EGF, FGF2 or both. Changes in morphology after 24 h treatment with EGF and/or FGF2 were most striking in double wild type cells. Furthermore, a similarly elongated form was taken by NRAS-mutant cells following FGF2 treatment (*Figure 12*).
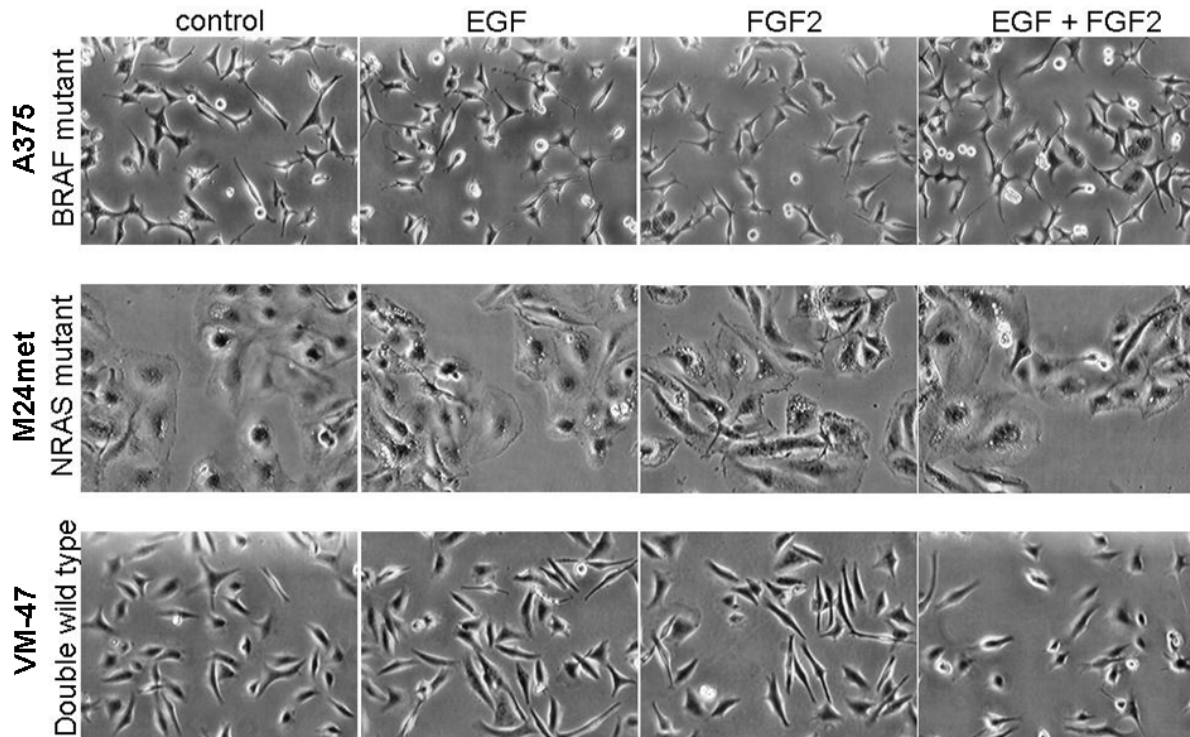
Figure 12. Morphological changes 24 h following EGF and/or FGF2 treatment in melanoma cells. The most striking effect was seen in double wild type cells where the majority of cells obtained an elongated morphology upon treatment. A modest alteration was found in NRAS-mutant cells following the addition of FGF2.

Evaluating the videomicroscopy recordings, proliferation and migration of untreated cells with different oncogenic mutations was compared first (***Figure 13***). Importantly, both BRAF and NRAS activating mutations resulted in elevated levels of migration and proliferation compared with double wild type cells. Of note, the difference between the average migrated distance of BRAF-mutant and double wild type cells was statistically significant ($p < 0.05$ by Kruskal-Wallis test post hoc Dunn's multiple comparison test).
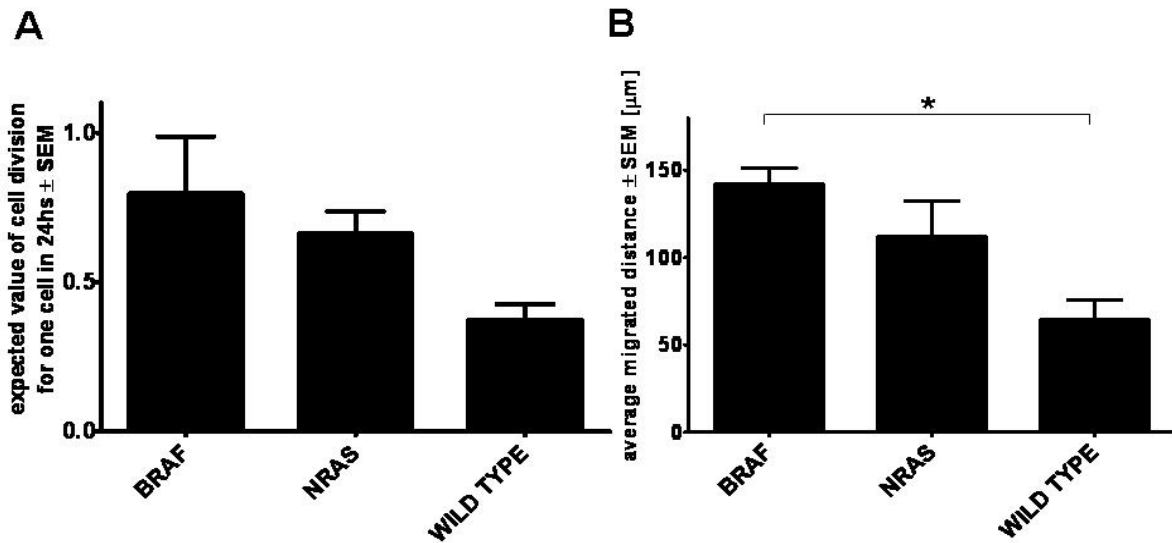
Figure 13. Proliferation (A) and migration (B) of untreated melanoma cells averaged according to mutation status, BRAF-, NRAS-mutant or double wild type. Of note, both proliferation and migration was the highest in BRAF-mutant cells and the difference in migration of BRAF-mutant cells was significantly higher compared with that in double wild type cells ($p < 0.05$ by Kruskal-Wallis test post hoc Dunn's multiple comparison test). Data is shown as average ± SEM of at least four independent measurements.

Mutational dependent effect of EGF and/or FGF2 treatment (50 ng/ml each) on proliferation and cell viability of melanoma cells was tested via videomicroscopy and SRB-assay, which generated essentially comparable results (***Figure 14***). The only significant difference in proliferation compared with control was observed in the double wild-type cell line VM-47 upon FGF2 treatment ($p < 0.05$ by Kruskal-Wallis test and post hoc Dunn's multiple comparison test). However, the proliferation promoting effect of FGF2 was not seen in the combination treatment. In addition, there was a modest increase of cell proliferation in double wild-type cells, but not in cells with BRAF or NRAS oncogenic mutations.
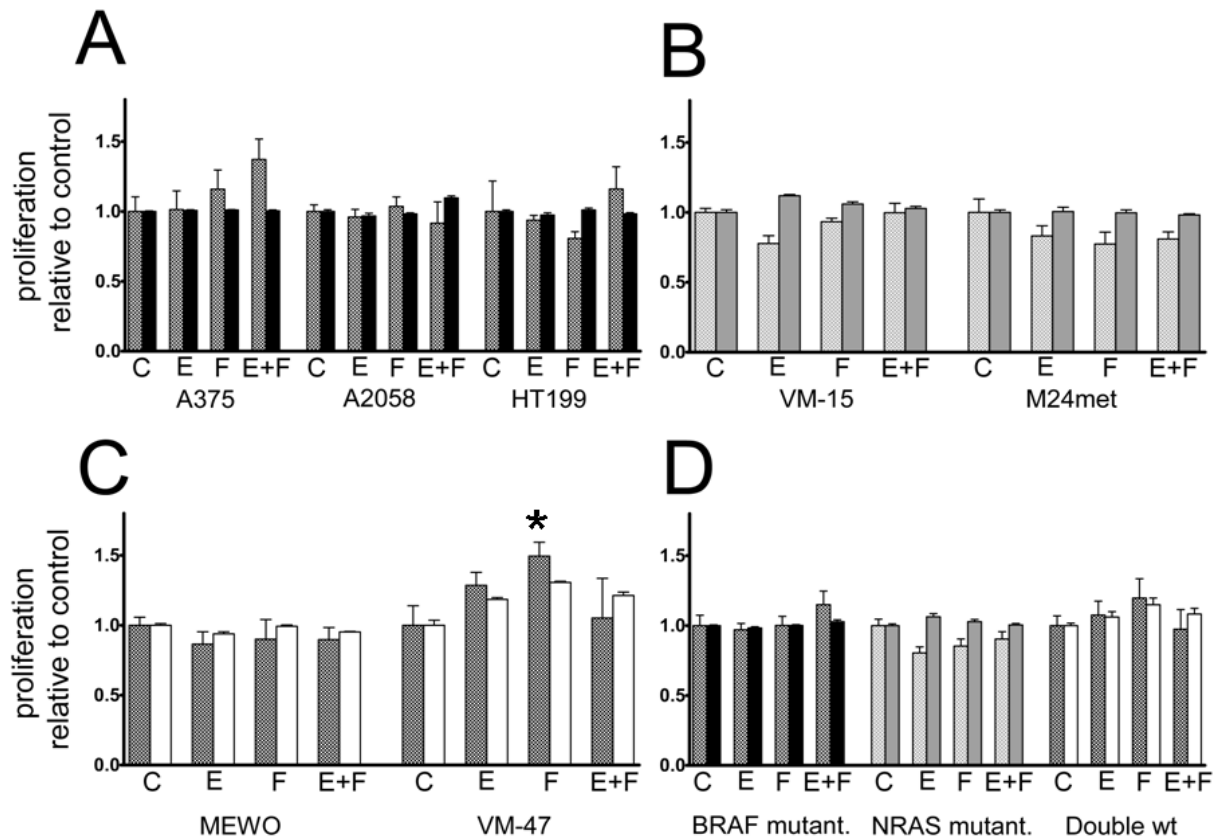
Figure 14. Effect of EGF and/or FGF2 treatment on proliferation of melanoma cells measured using videomicroscopy and SRB assay. Cell lines are plotted on separate graphs according to their mutation status (***A***) BRAF, (***B***) NRAS and (***C***) wilde type. (***D***) Average proliferation in cell lines having the same mutation status. The pair of columns for each treatment on each graph represents proliferation detected using SRB (left) and videomicroscopy (right) Data is shown as average ± SEM of independent measurements, which was 6 in case of SRB assays and 4 in case of videomicroscopy measurements. Asterisk indicates significant elevation ($p < 0.05$ by Kruskal-Wallis test and post hoc Dunn's multiple comparison test) in proliferation compared with control and detected using SRB assay. C = control; E = EGF; F = FGF2; E+F = EGF and FGF2 treatment.

Videomicroscopy recordings were also used to define the mutation-dependent effect of EGF and/or FGF2 treatment on migration of melanoma cells. The migratory effect was more profound compared to the effect seen in proliferation (***Figure 15***). Migration in both double wild type cell lines increased upon treatment with GFs but EGF caused more elevated migratory activity than FGF2 in both cell lines. Importantly, the combined treatment resulted in more increased migration than a single treatment with EGF or FGF2. Although

significantly elevated migration was observed in the NRAS-mutant cell line M24met after FGF2 treatment, this increase was considerably smaller than those observed in double wild type cells. When taking the average migration distance in cell lines having the same mutation status, BRAF-mutant cells failed to show altered migration after treatment with GFs and NRAS-mutant cells showed only a modest increase in migration in response to FGF2 or combined growth factor treatment.
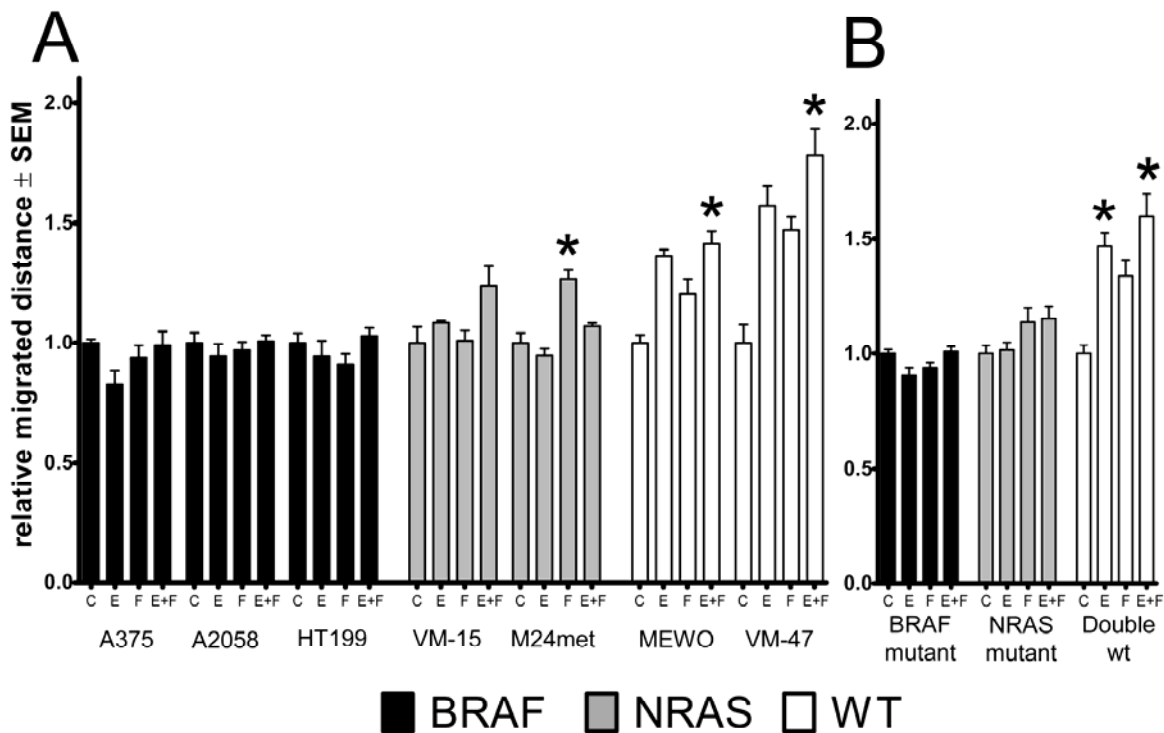

Figure 15. Effect of EGF and/or FGF2 treatment on migration of melanoma cells measured by videomicroscopy and grouped by cells (***A***) and averaged for mutations (***B***). Convincing effect on migration was only seen in double wild type cells. On average NRAS mutant cells responded to FGF2 or EGF and FGF2 combined treatment with a modest increase in migration. Colors black, grey and white indicate BRAF, NRAS mutation and wild type. Data shown is average ± SEM and results of 3 independent measurements. Asterisks indicate significance of $p < 0.05$ by Kruskal-Wallis and Dunn's multiple comparison test. (C = control; E = EGF; F = FGF2; EF = EGF and FGF2 treatment)

Phosphorylation of the two downstream effectors Erk1/2 and S6 were explored using immunoblot measurement to assess the activation of growth factor receptor pathway (***Figure 16*** and ***17***). Under baseline conditions, BRAF oncogenic mutations resulted in a 2.4 and 1.4

times higher phosphorylation of Erk1/2 and S6, respectively, as compared to wild type cells. In NRAS mutant cells, the phosphorylation of Erk1/2 and S6 was 3.2 and 1.9 times higher compared to that in double wild type cells (*Figure 16*).
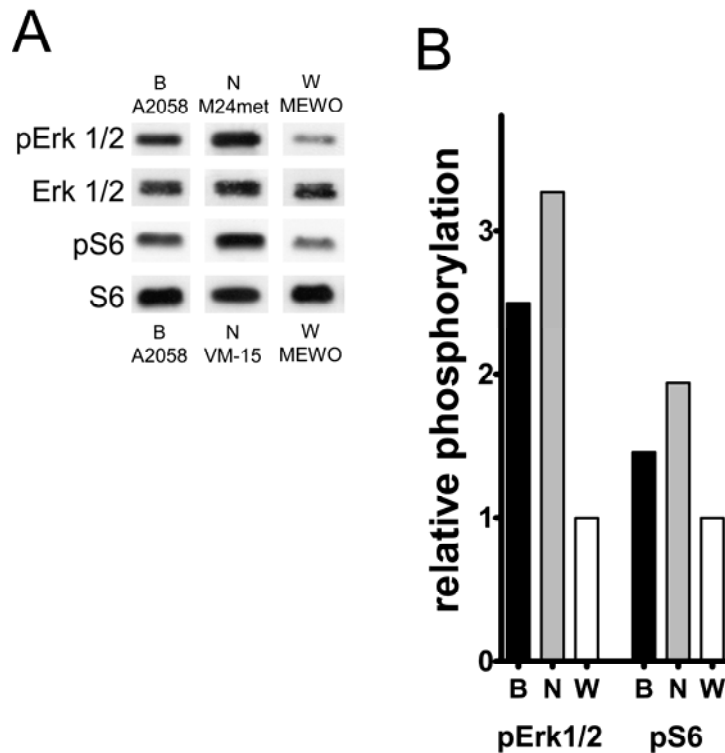

Figure 16. Representative immunoblots (*A*) and quantification (*B*) of the phosphorylation of GF receptor pathway effectors Erk 1/2 and S6 in untreated cells relative to double wild cells. Both Erk 1/2 and S6 showed a higher level in baseline activation in BRAF and NRAS mutant cells compared to wild type cells. Data shown is an average result of 3 independent measurements and averaged for the types of oncogenic mutations. Colors black, grey and white indicate BRAF, NRAS mutation and wild type. (B = BRAF mutant; N = NRAS mutant; W = double wild type)

Treatment with GFs elevated the level of phosphorylation of Erk1/2 and S6 in double wild type cells in a much higher proportion as compared to either BRAF or NRAS mutant cells (*Figure 17*). In double wild type cells a 1.6 to 2.9 fold increase in phosphorylation of GF effectors was measured. Whereas, the highest increase found in the mutant cells was only a 2.04-fold increase in the phosphorylation of Erk1/2 measured in NRAS mutant cells after combined treatment. Generally, the alteration of Erk1/2 and S6 phosphorylation measured in

cells harboring oncogenic mutations was rather modest when compared to the double wild type response.
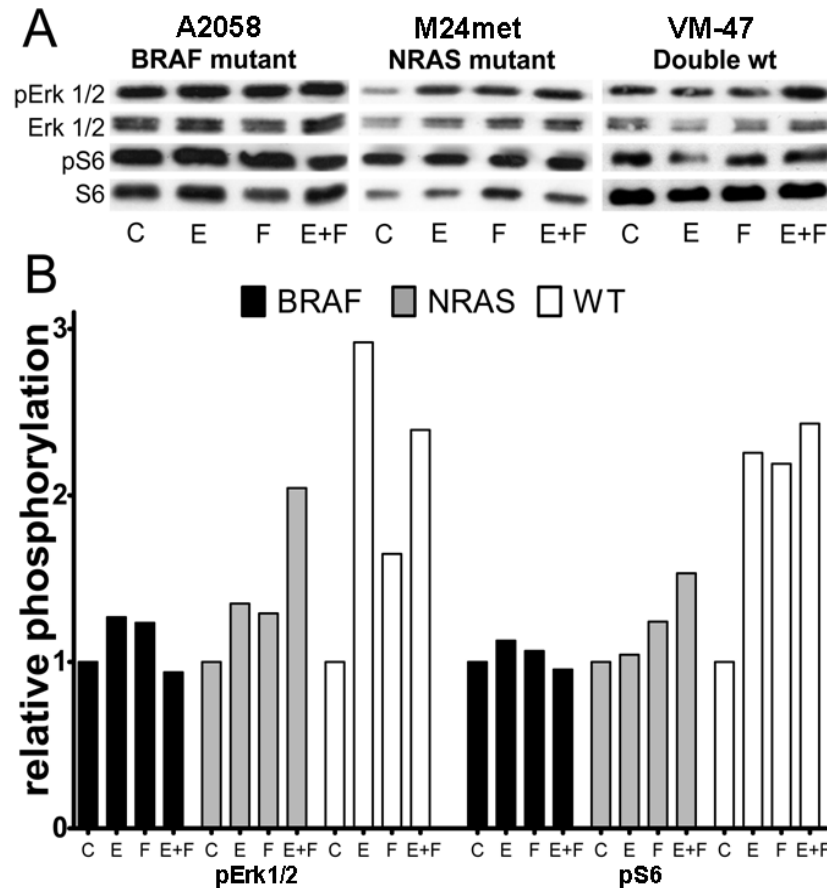

Figure 17. Representative pictures of the immunoblots (***A***) and quantification (***B***) of the phosphorylation of GF receptor pathway effectors Erk 1/2 and S6 after EGF and/or FGF2 treatment relative to untreated control. High increase was found in the activation of the examined receptor targets after the combined treatment in NRAS mutant cells. However, generally higher elevation of phosphorylation was found in the double wild type cells upon treatment. Colors black, grey and white indicate BRAF, NRAS mutation and wild type for these genes. Data shown is an average result of at least 3 independent measurements and averaged for the types of oncogenic mutations. (B = BRAF mutant; N = NRAS mutant; W = double wild type; C = control; E = EGF; F = FGF; EF = EGF and FGF treatment)

4.5. Inhibition of EGFR and FGFR in melanoma

Since activation of the EGF/FGF signal transduction was found to be dependent on the oncogenic mutation in melanoma cells, the pharmacological inhibition of EGFR and FGFR was also compared in the melanoma cells with different oncogenic mutations.

The oncogenic mutation dependent inhibition of EGF and FGF signaling was performed by treating the cells for 72 h with EGFR (gefitinib, erlotinib CI-1033 and pelitinib; *Figure 18 A*) and FGFR (ponatinib, BGJ-389, BIBF-1120 and AZD-4745; *Figure 18 B*) inhibitors and viability was measured via SRB assay. Independent of their mutational status, cell lines were largely insensitive to gefitinib and erlotinib treatment. Although CI-1033 and pelitinib treatment were somewhat effective, there was no difference between the sensitivity of cells having different mutations. Similarly, the effect of FGFR inhibition on cell viability was also independent of cells being BRAF- or NRAS-mutant or wild type for these genes.
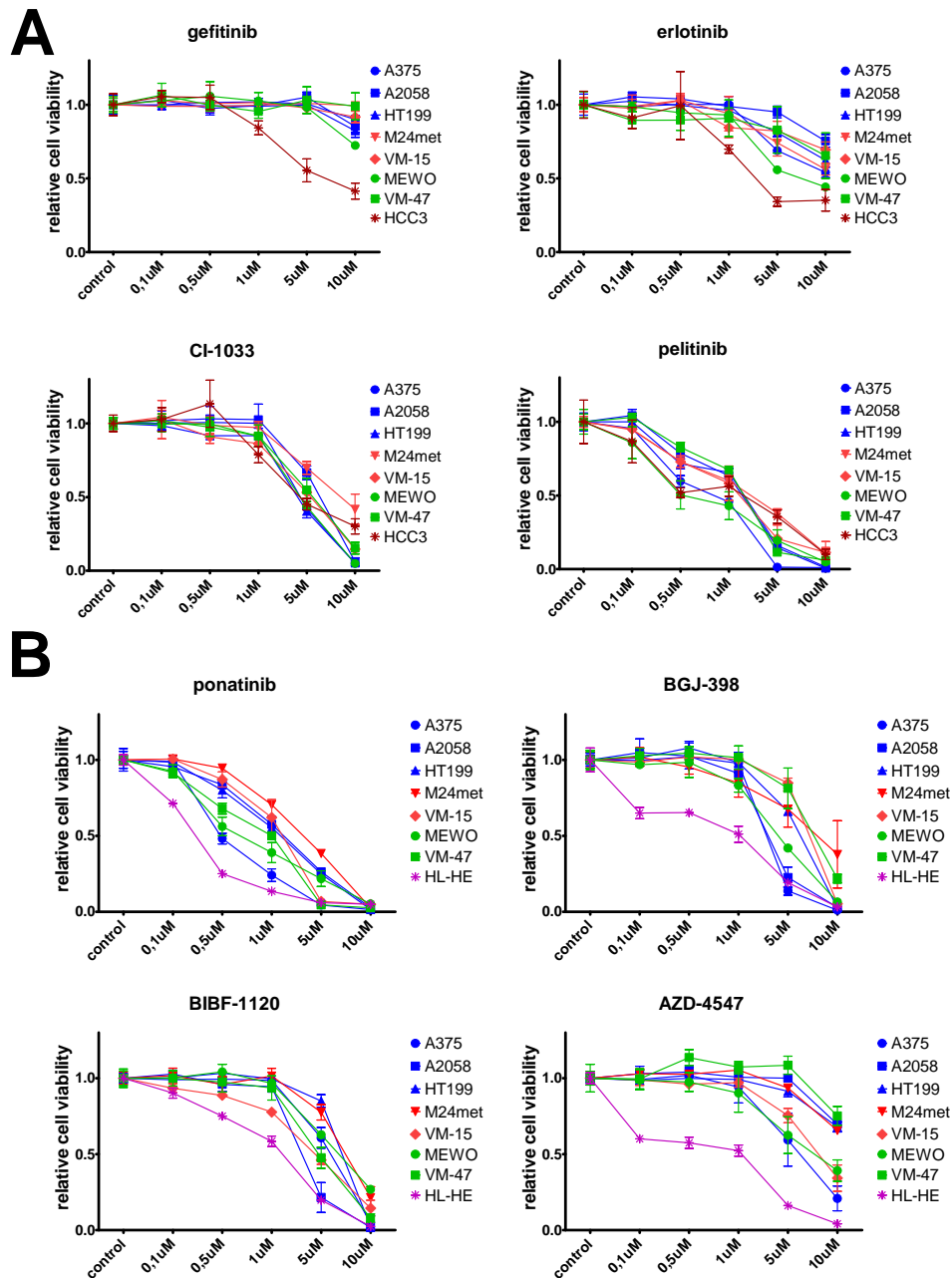
Figure 18. Proliferation inhibition of EGFR (**A**) and FGFR (**B**) inhibitor treatment on melanoma cells. There was no mutation dependent difference in the sensitivity of cell lines in any of the eight different inhibitors. Of note, currently approved EGFR inhibitors had no effect on melanoma cells when compared to a sensitive hepatocellular carcinoma line (HCC3, brown line). However, novel inhibitors have shown increased efficacy. Also melanoma cells demonstrated lower sensitivity to FGFR inhibition when compared to a sensitive small cell lung cancer line (HL-HE, magenta line). Colors blue, red and green indicate BRAF, NRAS mutation and wild type for these genes, respectively. Data shown is average ±SEM of at least 10 repeats in 2 independent measurements.

4.6. Oncogenic mutation-dependent prenylation inhibition response in melanoma

Posttranslational modification − including prenylation among others − of Ras is one of the major regulators of its activity and oncogenic RAS mutations play a major role in malignant melanoma. Thus, the effect of prenylation inhibition was examined by zoledronic acid (ZA; 25μM) treated human melanoma cell lines carrying either mutant BRAF or NRAS or none of them. After the 24-hour-treatment, the BRAF-mutant cells displayed a profound change in morphology (***Figure 19***). These cells obtained a rather elongated form, whereas only modest or no change was found in NRAS-mutant or in double wild-type cells.
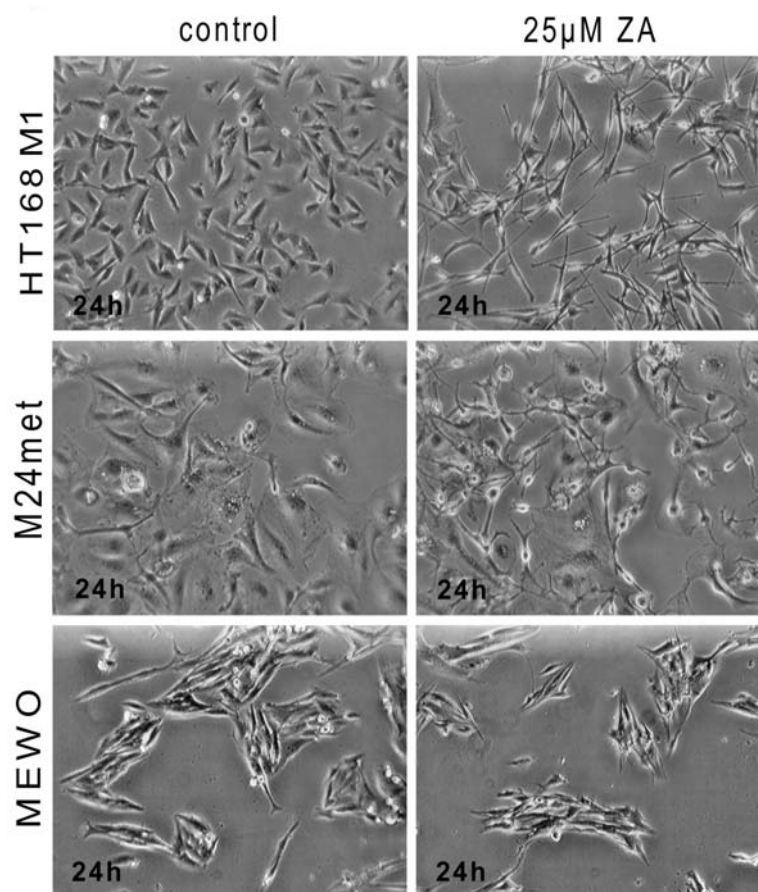

Figure 19. Representative pictures from melanoma cells treated with ZA for 24 h. Morphology of BRAF-mutant cells changed towards an essentially more elongated shape. In contrast only modest change was found in NRAS-mutant or in double wild-type cells.

Effect of the treatment with different concentrations of ZA on cell viability of melanoma cells was measured by SRB-assay (***Figure 20***). ZA treatment clearly decreased cell viability in NRAS mutant cells even in smaller doses. Cell viability of BRAF mutant and double wild type cells were decreased only to a smaller extent and at higher doses.
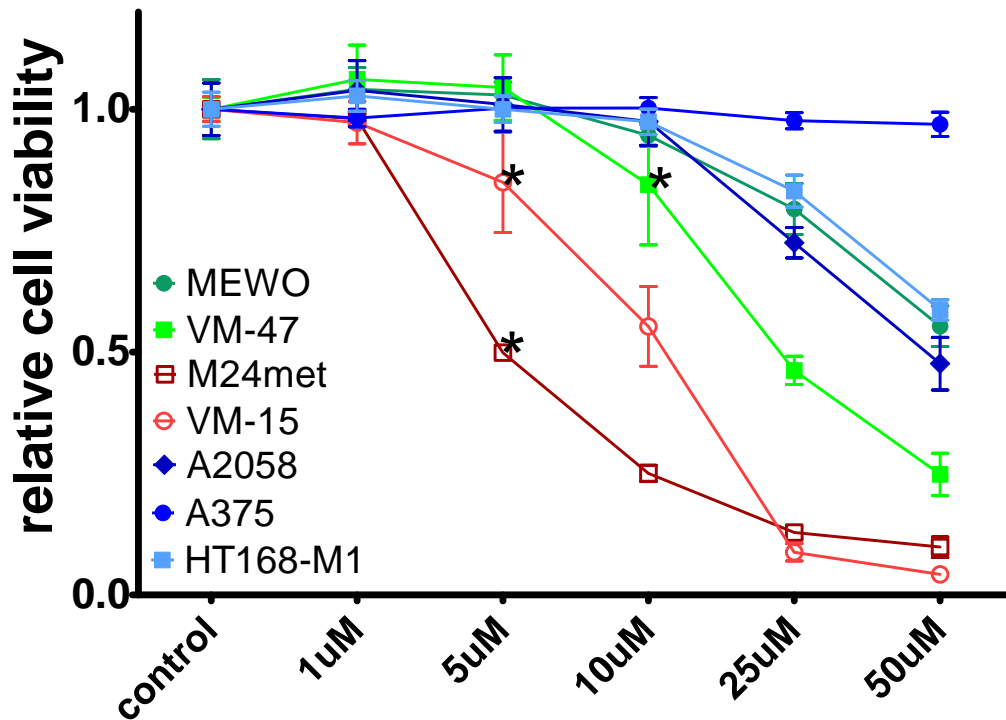

Figure 20. Dose-response analysis of cell viability of human melanoma cell lines with different mutations after 72h treatment with ZA. Asterisks indicate significant difference with $p < 0.05$ from control. Data shown as average ± SEM are from at least 5 repeats.

Videomicroscopy measurements were used to evaluate the effect of ZA treatment on migration of melanoma cells. Average migrated distance of the cell lines, grouped by their mutational types, were depicted as a function of time interval from 15 minutes to 20 hours (***Figure 21 A-C***). Furthermore, relative average migrated distance for 24 hours were calculated for each cell line and averaged for the three mutational groups (***Figure 21 D***). Despite the significant increase in the migration of one NRAS-mutant cell line and one double wild type cell line, ZA treatment increased the migratory activity of BRAF-mutant cells to a much higher extent compared to NRAS mutant and double wild type cells.
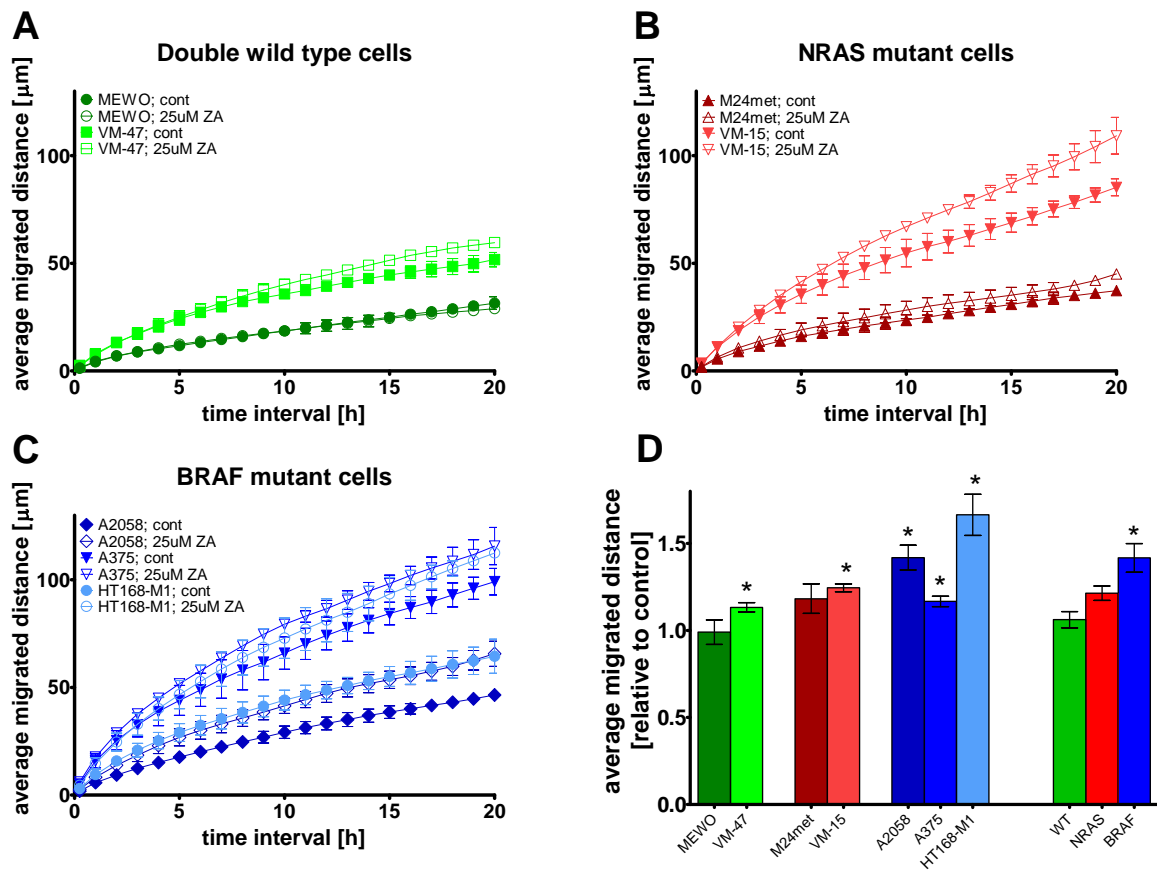
Figure 21. Changes in migrated distance as a function of time (***A-C***) and average migrated distance relative to control in each cell line and averaged by mutation (***D***) after ZA treatment in melanoma cells measured by videomicroscopy. Significant increase in migrated distance was found in VM-47 double wild type and VM-15 NRAS mutant cells. However, ZA treatment resulted in a significant increase of average migrated distance only in BRAF mutant cells whereas no significant alteration was found in average migrated distance of double wild-type and NRAS mutant cells. Colors blue, red and green indicate BRAF, NRAS mutation and wild type for these genes, respectively. Data shown as average ± SEM are from at least three independent measurements. Asterisks indicate significant difference of $p < 0.05$ from the respective control with unpaired two-tailed T test.

In order to characterize the pro-apoptotic effect of ZA, TUNEL staining was performed on melanoma cell lines treated with 25μM ZA. The proportion of TUNEL positive cells after the treatment was divided with the proportion of TUNEL positive cells detected under control conditions in order to quantify the pro-apoptotic effect (***Figure 22***). Though both NRAS-mutant cell lines were sensitive to ZA treatment, there was a huge difference in their

sensitivity level. In average, there was a more profound pro-apoptotic effect of ZA treatment in NRAS-mutant cells compared to BRAF mutant and double wild type cells. Of note, amount of apoptotic cells found in the TUNEL assay was in line with the measured cell viability.
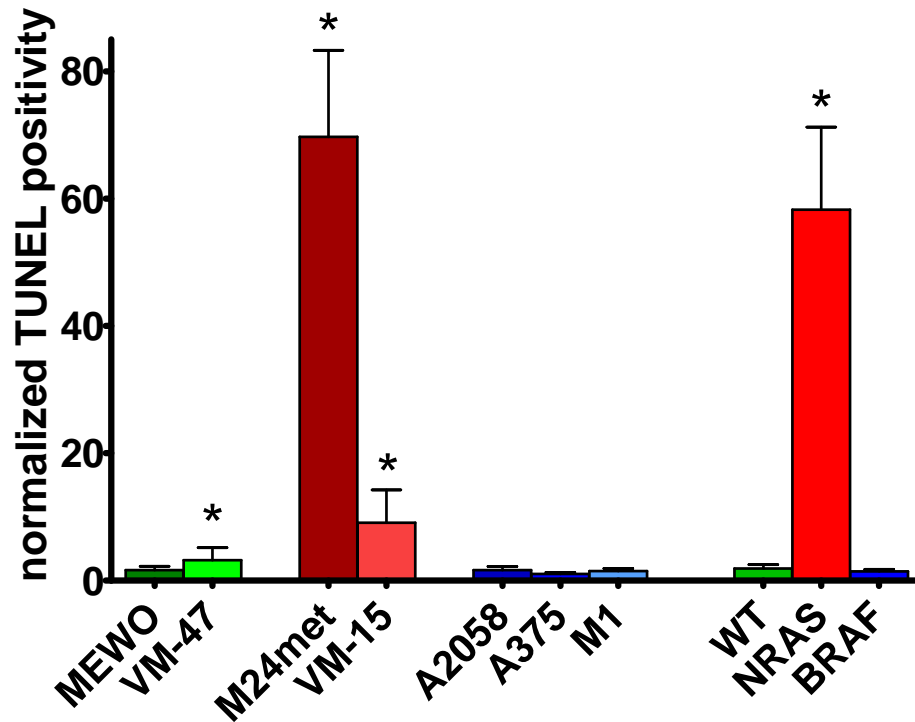

Figure 22. Proportion of TUNEL positive cells after treatment with ZA. Both NRAS mutant melanoma demonstrated increased apoptotic response to ZA compared to either BRAF or double wild-type cells. Data shown as average ± SEM are from at least 3 measurements. Asterisks indicate significant difference of $p < 0.05$ from the respective control by unpaired two tailed T test.

Since a robust apoptotic effect in NRAS mutant melanoma was found the activation of the ribosomal protein S6 a downstream target of RAS involved in the regulation of survival was investigated via immunoblot assay (**Figure 23**). Interestingly, the treatment with ZA resulted in the increased activation of ribosomal protein S6 in M24met NRAS mutant cells and in a decreased activation in the other NRAS mutant cell line, in VM-15 cells. In accordance with the results seen in the TUNEL assay, modest number of ZA induced apoptosis was accompanied by modest changes in S6 phosphorylation.
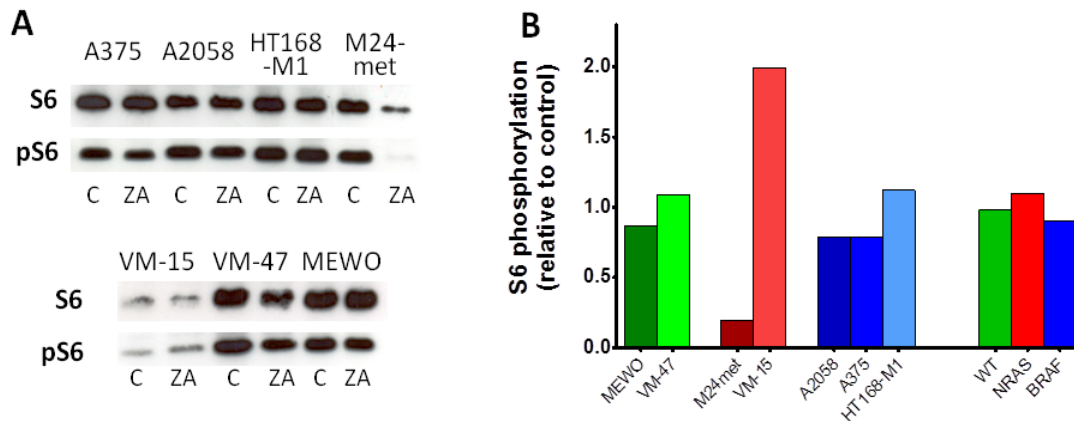
Figure 23. Activation of downstream elements of the RAS/RAF pathway in melanoma cells after ZA treatment. (*A*) Representative blots of the effect of ZA treatment on the activation of S6. (*B*) Quantification of the effect of ZA treatment on the activation of S6. After the treatment with ZA, decreased activation of S6 proteins was found in M24met cells. In contrast, following the same treatment an increase in the phosphorylation of S6 was measured in VM-15 cells.

To examine the effect of ZA on primary tumor growth, human melanoma cells were injected subcutaneously into the flank region of NSG (NOD scid gamma) mice (*Figure 24 A, C and E*). BRAF-mutant HT168-M1 cells showed a very rapid growth rate after the initial lag phase that led to an early termination of the experiment. In addition, ZA treatment failed to show an inhibitory effect on subcutaneous tumor growth of NRAS-mutant and double wild type melanoma. In order to investigate the metastasis related effects of ZA, colonization experiment of spleen to liver was performed in NSG mice (*Figure 24 B, D and F*). As a result, ZA failed to inhibit the metastatic growth of NRAS-mutant or double wild-type melanoma cells and it exerted only modest inhibition on metastatic growth of BRAF-mutant melanoma cells. Importantly, the lower dose (50μg/kg) of ZA resulted in a significantly higher metastasis formation in double wild-type cells ($p < 0.05$ by ANOVA and post hoc Tukey's multiple comparison test).
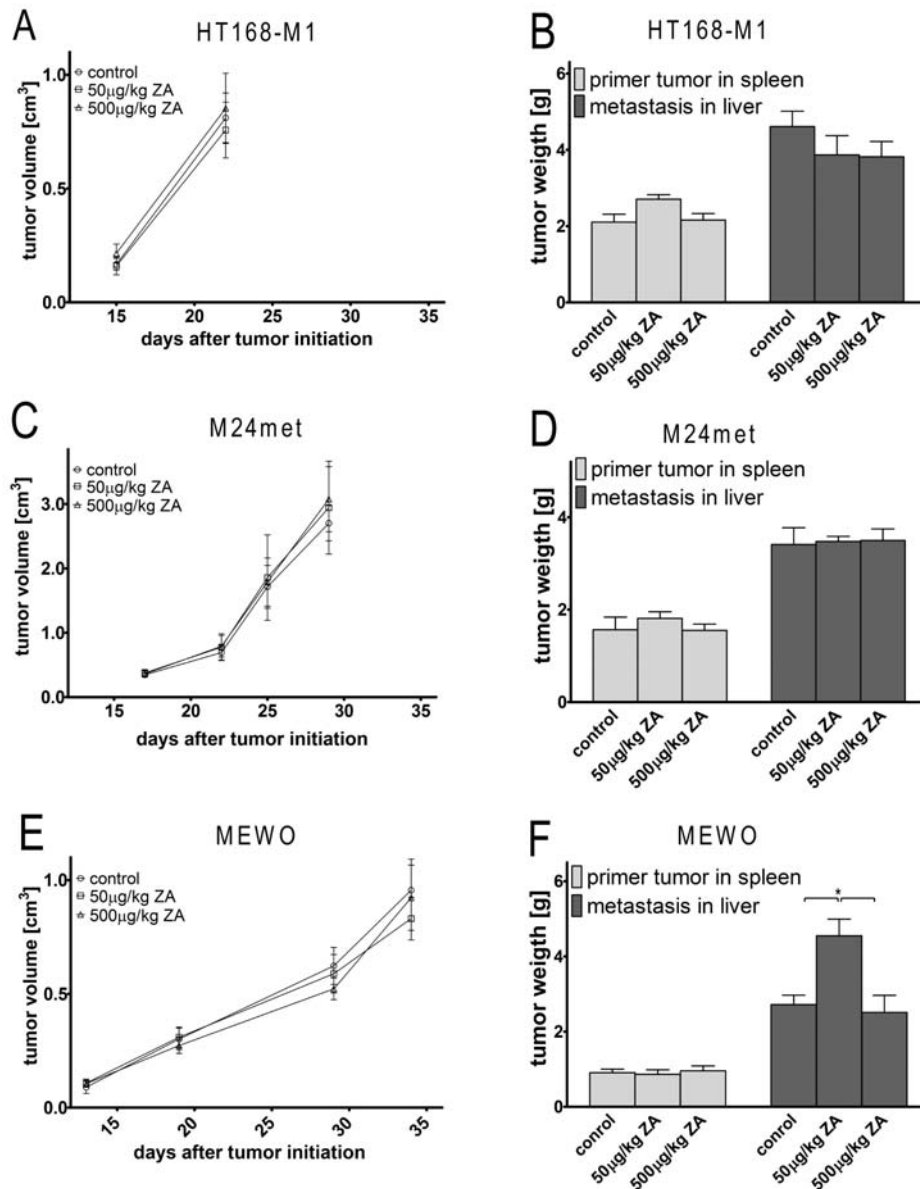
Figure 24. *In vivo* effects of ZA treatment. (***A, C, E***) Effect of ZA treatment using *in vivo* subcutaneous xenograft model of human melanoma cells in NSG mice. ZA treatment failed to show suppressive effect in the subcutaneous growth of melanoma cells with either of the mutations. Moreover, the rapid growth of xenografts led to the early termination of mice injected with BRAF-mutant cells. (***B, D, F***) Weight of primary tumor in the spleen and that of metastasis in the liver applying the spleen to liver colonization model. In the liver, ZA modestly inhibited the metastatic growth of BRAF- but not that of NRAS-mutant melanoma cells. Importantly, the lower dose of ZA treatment resulted in significantly higher metastasis formation in double wild-type cells. Data is shown as average ± SEM of at least seven independent measurements. Asterisk indicates significant difference $p < 0.05$ by ANOVA and post hoc Tukey's multiple comparison test.

4.7. Modulation of the activin signaling in mesothelioma

Activin signaling can be associated with both inhibition and promotion of tumor progression in various types of tumors. However, the role of this signaling pathway in mesothelioma has not been investigated yet. Videomicroscopy measurements were performed to investigate the effect of activin A (20 ng/ml), the inhibitor SB431542 (20 μM) or the combination of these two agents on cell proliferation (***Figure 25 A***) and cell migration (***Figure 25 B***). Treatment with the activin receptor inhibitor SB-431542 alone or in combination decreased the proliferation of M38K mesothelioma cells significantly compared to control ($p < 0.05$ by Kruskal-Wallis test and post hoc Dunn's multiple comparison test). In contrast, there were no differences in the proliferation of P31wt cells upon inhibition or activation of the activin receptors (***Figure 25 A***). Unlike cell proliferation result, migration of M38K cells was increased after activin treatment, whereas migration of P31wt cells remained unaffected (***Figure 25 B***).
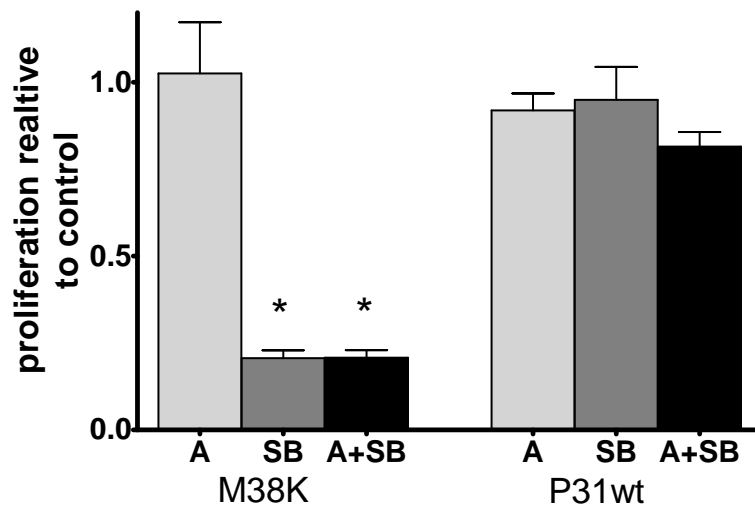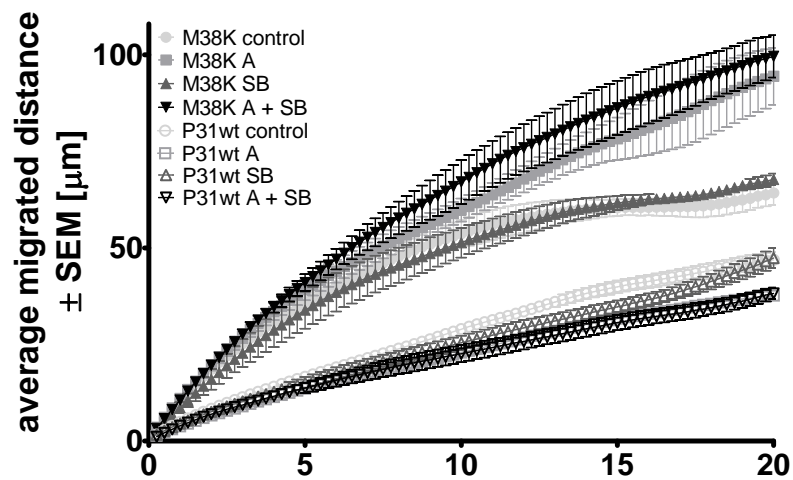
Figure 25. Effect of activation and/or SB-431542 treatment on the proliferation and migration of mesothelioma cells. (**A**) Proliferation relative to control. In M38K cells, the treatment with SB-431542 alone or in combination with activin decreased cell proliferation significantly. (**B**) Changes in migrated distance as a function of time. Treatment with activin alone or in combination with the activin receptor inhibitor SB-431542 resulted in an increase of migrated distance of M38K cells. Data shown as average ± SEM are from at least three independent measurements. Asterisks indicate significance of $p < 0.05$ with ANOVA and Dunnett's multiple comparison test. (A=activin; 20 ng/ml, SB=SB-431542; 20 μM)

Videomicroscopy provides a unique opportunity to identify aberrant cytokinesis, which was observed in P31wt mesothelioma cell line even under control conditions. Examples of cell divisions resulting in three daughter cells are demonstrated in *Figure 26 A*. Almost 2 percent of mitoses were multipolar even in untreated cell populations (*Figure 26 B*). Interestingly, treatment with activin receptor inhibitor SB-431542 alone or combined with activin resulted in a significantly higher proportion of multipolar (mostly tripolar) cytokineses as compared to control ($p < 0.05$ by Kruskal-Wallis test and post hoc Dunn's multiple comparison test).
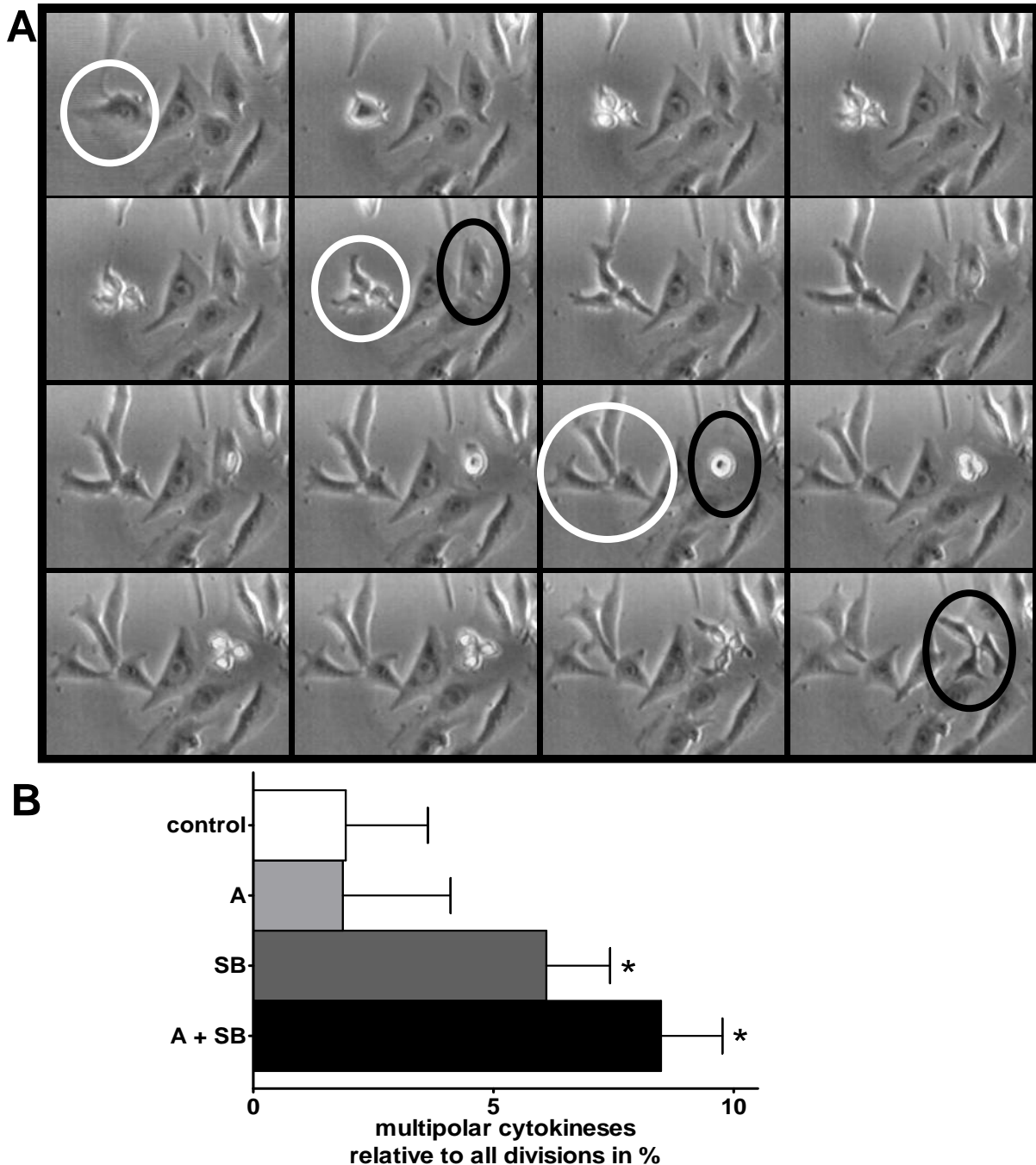
Figure 26. Representative pictures (***A***) and incidence (***B***) of multipolar cytokineses in P31wt cells relative to all cell divisions. Aberrant cytokineses were detected in the mesothelioma cell line P31wt even under control conditions. Interestingly, treatment with SB-431542 alone and combined with activin resulted in a significant higher proportion of multipolar (in majority tripolar) cytokineses as compared to control. Data shown as average ± SEM are from at least three independent measurements. Asterisks indicate significance of $p < 0.05$ by Kruskal-Wallis and Dunn's multiple comparison test. (A=activin; 20 ng/ml, SB=SB-431542; 20 µM)

5. DISCUSSION

5.1 The migration/proliferation dichotomy in cancer

The coordinated interplay of proliferation and migration in tumor cells is of outmost interest in terms of tumor progression and metastasis. The "go or grow" hypothesis postulates the "opposition" between migration and proliferation. This hypothesis was mainly studied in neuroectodermal cells and literature addressing this issue is rather conflicting, with studies querying (Lund-Johansen et al. 1990; Penar et al. 1997; Zhang et al. 1997; Corcoran and Del Maestro 2003) and supporting (Merzak et al. 1994; Giese et al. 1996; Tamaki et al. 1997; Khoshyomn et al. 1999; Roth et al. 2000) the inverse connection between migration and proliferation. Accordingly, the "go or grow" hypothesis was revisited on a large series of tumor cell lines with different origin. The experiments presented in this thesis failed to prove the "go or grow" hypothesis and negative correlation between migration and proliferation could not be verified in any of the tested cancer types. Moreover, a significant positive correlation was found between proliferation and migration in lung cancer and malignant melanoma cells at single cell level and also when comparing populations of cells deriving from different cases.

The "go or grow" dichotomy is addressed in several recent theoretical studies (Fedotov and Iomin 2007; Wang et al. 2009; Bauer et al. 2010; Hatzikirou et al. 2012). In most of these studies, a molecular agent is considered, which regulates the cells' phenotype and constrains it either in a motile but non-proliferative or in a proliferative non-motile state. If the dynamics of the tumor cell population also influences this molecular agent being responsible for phenotype switching than the feedback is predicted to result in spatiotemporal cell density fluctuations and uneven growth dynamics. The applied single cell studies in low density cell cultures tested for the presence of postulated dichotomy under the simplest possible experimental settings without the interference of specific molecular agents. The chosen statistical methods are sensitive enough to detect alterations in motility parameters if some generic mechanism – such as the contact inhibition of cell motility – were to exert a significant effect. Thus, as no negative correlations between proliferation and cell motility

were found, the generic "go and grow" dichotomy is most likely specific for tumors of glial origin.

In line with former studies (Maiuri et al. 2012)., the migratory capacity of examined tumor cells showed a large variance. Interestingly, there were significantly higher average migrated distances in malignant mesothelioma compared with malignant melanoma and lung cancer cells. Similarly to migration, the highest proliferation was found in mesothelioma followed by malignant melanoma and lung cancer, although with no significant differences. The finding that cells with higher migration potential showed higher proliferation intensity indeed challenged the "go or grow" hypothesis, and this cannot be explained with the difference in the duration of cytokinesis, as no significant difference in the average duration of the latter process was found. Interestingly, duration-of-cytokinesis parameter had a considerably lower variation in the panel of the examined cell lines when compared to variations in migration or proliferation.

From the clinical point of view, increased mitotic activity of cancer cells (often measured via Ki67/MIB1 immunohistochemistry) was considered as a sign for poor prognosis in a number of tumors. In a variety of brain tumors (Torp 2002; Preusser et al. 2008; Habberstad et al. 2011) and in subsets of breast cancers (Luporsi et al. 2012; Milde-Langosch et al. 2013) high mitotic activation is a sign for poor prognosis. In melanocytic tumors, the mitotic index is considered as the most useful instrument in distinguishing between benign and malignant alterations (Ohsie et al. 2008) and a useful prognostic factor (Hazan et al. 2002; Vereecken et al. 2007). Correspondingly, high mitotic activation of tumor cells measured by Ki67 immunostaining is considered as an adverse prognostic marker in lung cancer (Martin et al. 2004). Likewise melanoma and lung cancer, a high mitotic index in mesothelioma indicates poor prognosis (Kadota et al. 2012). Considering the fact that most patients die from metastatic disease, the negative prognostic value of proliferation markers per se challenge the "go or grow" hypothesis and support our finding that enhanced overall proliferation also supports migration/invasion and thus metastasis.

The locomotion activity displays a universal exponential distribution in 2D cell cultures that may be explained with the limited amount of available energy (Czirók et al. 1998). This could be an explanation for a negative correlation between proliferation and migration as both processes require cellular energy and the fast and dynamic reorganization of the cytoskeletal apparatus. However, there was no evidence found that could support this type of negative correlation. The importance of the ability for this fast reorganization is also supported by the

observation that in melanoma cells a significant negative correlation was found between the duration of cytokinesis and cell migration.

Since the strongest correlation between cell migration and proliferation as well as a significant correlation between cell migration and duration of cytokinesis were found in melanoma, next the possible underlying molecular mechanism was investigated. Activation of FAK (but not Src) – pivotal components of the focal adhesion complexes – showed a correlation with migratory potential. Importantly, recent studies demonstrated that FAK is not only involved in the regulation of the migratory cytoskeletal apparatus but also in the regulation of proliferation (Cox et al. 2006; Schaller 2010). Effect of FAK on the cell migration may be exerted through a reciprocal linkage with ERK1/2 (Provenzano et al. 2009; Srinivasan et al. 2009)

In summary, the "go or grow" hypothesis could not be proven in the examined sets of mesothelioma, melanoma and lung cancer cells. On the contrary, a significant positive correlation between proliferation and migration was found in human malignant melanoma and lung cancer cell lines. Considering one single cell, cytokinesis and migration were separated temporally but on the level of cell population – as this is the case in tumors – cell migration and proliferation occurs simultaneously. Of note, tumor cells deriving from various organs may differ in the molecular mechanism regulating cell migration and cell proliferation. Additionally, our findings are in line with the general observation of pathologists that highly proliferative tumors often display significant invasion of the surrounding normal tissue.

5.2 Proliferation independent invasion

Interplay between proliferation and migration is an inherent characteristic of tumor progression and invasion. Furthermore, though not proven, a great number of theoretical works anticipates proliferation as a prerequisite of the 3D invasion of tumor cells in the extracellular matrix (Khain and Sander 2006; Rubenstein and Kaufman 2008; Poplawski et al. 2010). Therefore the proliferation-dependency of extracellular matrix invasion was studied in glioblastoma cells. Accordingly, the invasion pattern from a multicellular aggregate into a surrounding ECM was compared between proliferation-inhibited and untreated cells. In addition, parallel to this evaluation a novel mathematical model that describes the invasion of

tumor cells into the surrounding matrix without assuming their proliferation was elaborated. The invasion patterns in the first 24 hours were essentially the same in division inhibited and control cells indicating that cell proliferation does not play an important role in the early stages of the invasion process in 3D cell cultures.

5.3 Oncogenic mutation-dependent response to EGFR-FGFR signaling

A large number of novel targeted therapies are available to inhibit the GF receptor pathways that are affected by major oncogenic mutations in malignant melanoma. Accordingly, the mutation dependent activation and inhibition of GFR signaling cascades were investigated. Reflecting the prevalence of these oncogenic mutations three BRAF-, two NRAS-mutant and two double wild-type cell lines were investigated.

In concordance with earlier findings that the majority of melanoma cell lines are EGFR positive (Gordon-Thomson et al. 2001), in this thesis work, all of the examined cell lines expressed EGFR. In recent studies, expression of a variety of FGFR receptors has been demonstrated on melanoma cell lines (Easty et al. 2011; Metzner et al. 2011). Interestingly, in our study none of the NRAS-mutant cell lines showed FGFR2 and FGFR3 expression, and wild type cell lines showed the lowest levels of GF receptor expression in average.

In line with the findings of clinical studies that BRA-F or NRAS-mutant melanomas may have a worse prognosis (Jakob et al. 2012; Safaee Ardekani et al. 2012), higher proliferation and migration was found in BRAF- and NRAS-mutant cells compared to melanoma cells lacking these driver mutations. It suggests that *in vitro* biological characteristics may correspond to a dismal clinical course.

Although the correlation of BRAF mutation and downstream activation of the GF receptor pathway have not been demonstrated yet in clinical studies (Houben et al. 2008; Yazdi et al. 2010), a higher phosphorylation of the two downstream effectors Erk1/2 and S6 – surrogate markers of the activation of the RAS/RAF/MEK and PI3K/AKT/mTOR pathways – were found in BRAF- and NRAS-mutant cells. The higher baseline phosphorylation measured in BRAF-mutant cells indicates crosstalk to the PI3K pathway possibly through feedback mechanisms or other concomitant mutations of that particular signaling cascade. Loss of PTEN is often found in melanoma (Matunis and Guzzo 2012; Mehnert and Kluger 2012), and

,in our series of cell lines, both A2058 and A375 BRAF mutant melanoma harbor PTEN mutations (Pollock et al. 2002; Lopez-Bergami et al. 2010) suggesting that there is a cooperation between BRAF mutations and mutations affecting the PI3K pathway in malignant melanoma (Tsao et al. 2004). In line with the high baseline phosphorylation of ERK and S6 in BRAF- and NRAS-mutant cells, the treatment with GFs resulted in only a modest activation of these downstream targets. In wild type cells, in contrast, although poor in GFRs compared to the investigated mutant cell lines, a notably higher level of phosphorylated Erk1/2 and S6 was detected after the treatment with GFs.

The responsiveness of wild type melanoma cells towards GF treatment was seen not only in the phosphorylation of the downstream targets of the GF pathway but also in cell migration. Significant increase in cell migration was found in double wild type cell lines following treatment with both GFs. The increase in migratory activity was higher after EGF and combined EGF and FGF2 treatment in both wild type cell lines when compared to the FGF2 only treatment. Of note, FGF2 treatment increased slightly the migration of NRAS mutant cells, whereas BRAF mutant cells failed to show changes in cell migration after either GF treatment. Interestingly, more profound response in cell migration was found when compared to proliferation. The results from the videomicroscopy and protein measurement-based cell viability assay were comparable demonstrating that cell proliferation can be estimated by viability assays in this setting. Overall, there was a modest increase of cell proliferation in double wild type cells upon FGF2 treatment. In line with the lack of further downstream activation of the EGFR and FGFR pathway, there was no remarkable increase in the proliferation or migration of cells with BRAF or NRAS oncogenic mutations.

Inhibition of GF signaling with a single compound treatment showed minimal effect and no mutation dependence on the examined melanoma cell lines. These finding is in line with earlier clinical studies where EGFR and FGFR-VEGFR inhibitors failed to reach significant effect in unselected patients with metastatic melanoma (Kim et al. 2011; Patel et al. 2011). Our experiments, however, could not recapitulate the recently shown *in vivo* proliferation inhibiting effect of the EGFR inhibitors gefitinib and CI-1033 on BRAF-, NRAS-mutant and wild type melanoma cells (Djerf et al. 2009; Djerf Severinsson et al. 2011). Importantly, supporting our observations, treatment with EGFR inhibitor erlotinib as a single agent failed to decrease proliferation in earlier *in vitro* and *in vivo* investigations (Schicher et al. 2009). Interestingly, EGFR inhibition in combination with VEGFR-A or FGFR inhibition combined

with multikinase/BRAF inhibitor sorafenib showed anti-melanoma effect in a number of melanoma cell lines (Schicher et al. 2009; Metzner et al. 2011).

In summary, we provided evidence that increased proliferation, migration and activation of downstream effectors ERK and S6 is present in melanoma cells harboring BRAF or NRAS mutation. Furthermore, we demonstrated that, while activation of EGFR and FGFR is NRAS or BRAF mutation dependent, the inhibition of the EGFR and FGFR does not follow the same oncogene mutation dependency.

5.4 Selective growth inhibition of zoledronic acid in NRAS mutant melanoma

Prenylation − a critical posttranslational modification of Ras proteins − is one of the major regulators of its activity. In earlier investigations, despite great promises, inhibition of prenylation via farnesyl-transferase inhibitors (FTIs) showed limited efficacy in monotherapy clinical trials (Downward 2003; Appels et al. 2005; Nikolaou et al. 2012). Although several studies have investigated why targeting major posttranslational molecular mechanism is not effective (Smalley and Eisen 2003; Buzzeo et al. 2005; Raz et al. 2007), the mechanism of FTI-resistance achieved through the alternative prenylation enzyme geranylgeranylase could not be ruled out in case of K-Ras and possibly N-Ras (Lerner et al. 1997; Rowell et al. 1997; Whyte et al. 1997). Due to this alternative mechanism in order to efficiently prevent RAS activation, the dual inhibition of farnesyl-transferase and geranylgeranylase seems to be necessary (Sebti and Hamilton 2000). Antitumor effect of ZA is due to the inhibition of the key enzyme of the mevalonate pathway, namely farnesyl-diphosphate synthase, which is responsible for the production of farnesyl-diphosphates. The lack of farnesyl-diphosphates, substrates of geranylgeranyl-transferase and farnesyl-transferase effectively impairs the posttranslational modification of Ras (Amin et al. 1992; van Beek et al. 1999). Accordingly, the response of melanoma cells with different oncogenic mutations to ZA treatment was investigated.

ZA treatment induced profound morphological changes and increased migratory activity in BRAF-mutant cells. Furthermore, in line with earlier evaluations, a modest decrease in proliferation and a slight increase in apoptosis were found in BRAF-mutant cells upon treatment with ZA (Forsea et al. 2004). In general, a strong correlation was observed between

the decrease of cell viability and increase of TUNEL positivity. Of note, ZA treatment caused remarkably decreased cell viability and increased apoptosis in both examined NRAS-mutant cell lines compared with BRAF-mutant and double wild type cells *in vitro*. Interestingly, large differences were found between the two NRAS-mutant cell lines in the induction of apoptosis and reduction of cell viability. Similar differences were found in the activation of the ribosomal protein S6, a downstream target of Ras, being involved in cell survival, between the two NRAS-mutant cell lines. Besides additional tumor-specific genetic alterations (such as p53 or PTEN status), another possible explanation might be that the substituting amino acids are different in the two cell lines. However, there is no data currently available that describes amino acid substitution-specific differences in NRAS mutant melanoma. ZA treatment increased the *in vitro* migration of almost all examined cell lines. In contrast, no major inhibitory effects of ZA on either the subcutaneous primary tumor growth or on the metastatic capacity of human melanoma cells were found *in vivo*. Surprisingly, in the spleen to liver colonization experiment, the lower-dose-treatment of double wild-type cells with ZA resulted in an increased growth of liver metastases in NSG mice. These observations suggest that targeting of a non-hyperactivated pathway in melanoma cells may lead to adverse effects. Our findings suggest that benefit from therapy targeting the prenylation may be strongly dependent on the oncogenic mutations.

5.5 Activation and inhibition of activin signaling in mesothelioma

The role of activin (and of TGF-β) signaling in tumor progression is dependent on the type of malignancy (Antsiferova and Werner 2012). Previously, antisense RNA against TGFβ inhibited tumor growth and cell proliferation in malignant mesothelioma (Fitzpatrick et al. 1994). However the role of activin signaling in mesothelioma has not been investigated yet. Therefore, the activation of activin signaling with recombinant activin and the inhibition of activin receptors by the treatment with activin receptor kinase inhibitor SB-431542 was investigated. Activin stimulated migration of one of the investigated mesothelioma cells and did not decrease migration or proliferation, which is similar to the findings described in esophageal and lung adenocarcinoma (Seder et al. 2009; Seder et al. 2009). Collectively, these data confirm the suggestion that activin has a pro-tumorigenic effect in thoracic

malignancies, in contrast to hepatocellular carcinoma and breast cancer (Deli et al. 2008; Katik et al. 2009). In order to validate the tumor promoting role of activin, the effect of activin receptor inhibitor SB-431542 on mesothelioma cells was evaluated. Treatment with SB-431542 decreased the proliferation of M38K mesothelioma cells significantly, which supports the tumor promoting effect of activin signaling in mesothelioma.

Interestingly, we found multipolar cytokineses in P31wt cells and treatment with SB-431542 increased significantly their incidence. Aberrant cytokineses in tumor sections were originally described at the end of the 19th century (Krompecher 1895). One of the biological consequences of this process is the generation of tumor cells with trisomy that is an often observed genomic alteration in malignancies (Gisselsson et al. 2010). The loss of p53 in P31wt mesothelioma cells may contribute to this phenomenon, as the absence of p53 is permissive for multipolar asymmetric divisions of polyploid cells (Vitale et al. 2010). Of note, our finding that mesothelioma cells with type p53 showed a response in migration and proliferation whereas cells harboring mutant p53 entered aberrant cytokinesis at a higher rate suggest that cancer related mutations may determine the differences in migratory and proliferative response in mesothelioma, as well.

In summary, these results support the protumorigenic role of activin signaling in mesothelioma and suggest that activins may be candidates for further evaluation as potential targets for the treatment of mesothelioma.

6. CONCLUSIONS

Considering the results of this thesis the following main conclusions can be drawn in order to answer the questions formulated as the aims of the thesis.

1.   Revisiting the "go or grow" hypothesis, no negative correlation between proliferation and migration and thus no supporting evidence for the hypothesis was found. In contrast, positive correlation was found between migration and proliferation in melanoma and lung cancer cells.

2.   Our experiments using collagen embedded multicellular brain tumor cell spheroids demonstrated that the invasion process in 3D matrices did not require concurrent cell proliferation.

3.   Investigation of the mutational status dependence of EGF and FGF response revealed a lower baseline activity and higher inducibility of proliferation and migration in double wild-type melanoma cells compared to cells with oncogenic BRAF or NRAS mutation. In contrast, response to GF receptor tyrosine kinase inhibitors was oncogenic mutation independent.

4.   Investigation of the mutation dependence of prenylation inhibition resulted in a decrease of proliferation in NRAS mutant cells but in the increase of migration *in vitro* and increased metastatic potential *in vivo* in BRAF mutant and double wild type cells, respectively. The apoptosis induction in NRAS mutant melanoma suggests that prenylation targeting treatment modalities may be effective in this molecular subgroup of melanoma.

5.   In certain mesothelioma cells, response in migration and proliferation to both the induction and inhibition of activin signaling suggested a pro-tumorigenic effect of activin activation. The presented results suggest that activin may be a valuable candidate for therapeutic interference.

7. SUMMARY

The high mortality of solid tumors can be attributed to their invasive and metastatic potential. These two processes require a fine spatiotemporal interplay between cell migration and proliferation. The "go or grow" hypothesis postulates that tumor cells defer proliferation to cell migration. Furthermore, proliferation is considered to be a prerequisite for invasion to the extracellular matrix. Importantly, growth factor receptor signaling pathways are pivotal regulators of proliferation and migration and they are often affected by oncogenic mutations and are very important targets for novel antitumor therapeutic approaches.

The "go or grow" hypothesis was revisited by measuring proliferation and migration of 35 lung cancer, melanoma and mesothelioma cell lines via videomicroscopy, and no supporting evidence was found. In contrast, positive correlation was found between migration and proliferation in melanoma and lung cancer cells. Experiments using collagen embedded multicellular brain tumor cell spheroids demonstrated that the invasion process in collagen type I matrices did not require concurrent cell proliferation.

Investigation of the influence of BRAF or NRAS mutations on EGF and FGF response in human melanoma cells revealed a lower baseline activity and higher inducibility of proliferation and migration in double wild-type melanoma cells compared to cells with oncogenic BRAF or NRAS mutation. In contrast, response to GF receptor tyrosine kinase inhibitors was oncogenic mutation independent. Prenylation inhibition resulted in a decrease of proliferation in NRAS mutant cells but in an increase of migration *in vitro* and increased metastatic potential *in vivo* in BRAF-mutant and double wild type cells, respectively.

In certain mesothelioma cells, induction and inhibition of activin signaling resulted in increased migration and decreased proliferation, respectively. The shown pro-tumorigenic effect of activin in mesothelioma suggests that activin may be a valuable candidate for therapeutic interference.

In summary our findings demonstrate that tumor cells do not defer proliferation for migration and that proliferation is not a prerequisite for tumor cell invasion. Furthermore, the specific oncogenic mutations may differentially regulate migration and proliferation of tumor cells. Finally, these mutations may provide promising targets or profoundly influence the effect of molecularly targeted therapies but there is a need to establish these correlations for the respective oncogenic mutations.

8. ÖSSZEFOGLALÁS

A szolid tumorok nagy mortalitása elsősorban inváziós és áttétképző képességüknek tulajdonítható. Ez a két folyamat igényli a sejtmozgás és sejtosztódás nagyfokú tér- és időbeli összehangoltságát. A „go or grow" hipotézis kimondja, hogy a tumorsejtek elhalasztják osztódásukat, hogy vándorolhassanak. Továbbá, a sejtosztódást az extracelluláris mátrixba történő invázió előfeltételének tekintik. A növekedési faktor (GF) receptor jelátvitelei útvonalak meghatározó szabályozói a sejtmozgásnak és sejtosztódásnak. Ugyanakkor sok onkogén mutáció érinti ezeket a jelátviteli útvonalakat és gyakran támadáspontjai molekulárisan célzott terápiáknak.

A „go or grow" hipotézist vizsgáltuk harmincöt tüdődaganat, mesothelioma és melanóma sejtvonal migrációjának és proliferációjának videomikroszkópos elemzésével, de nem sikerült a hipotézist bizonyítani. Ugyanakkor szignifikáns korrelációt találtunk melanóma és tüdő daganat sejteken a sejtmozgás és sejtosztódás között. Kollagén gélbe ágyazott többsejtű agydaganat szferoidokon végzett kísérleteinkkel bemutattuk, hogy ezekben a sejttenyészetekben az osztódás nem előfeltétele az inváziónak.

A BRAF és NRAS mutációk hatását melanóma sejtek EGF és FGF kezelésre adott válaszát vizsgálva megállapítottuk, hogy kezelés nélkül kisebb aktivitás, ugyanakkor kezelés hatására nagyobb mértékben fokozódó migráció és sejtosztódás jellemzi a vad típusú BRAF és NRAS gént hordozó sejteket. Ezzel szemben a GF-receptorok tirozinkináz aktivitásának gátlása nem mutatott mutáció-függést. A preniláció-gátlás csökkentette az osztódás mértékét NRAS-mutáns sejtekben, ugyanakkor *in vitro* növelte a migrációt BRAF-mutáns és *in vivo* az áttétképző képességet dupla vad típusú melanóma sejteken.

Az aktivin jelátvitel gátlása csökkent osztódást, serkentése megnövekedett migrációt okozott mesothelioma sejteken. A megfigyelt tumor-serkentő hatása miatt az activin jelátvitel ígéretes célpontja lehet célzott terápiás beavatkozásoknak.

Összefoglalva elmondható, hogy a tumorsejtek nem szüneteltetik sejtosztódásukat migrációjuk miatt, valamint a sejtosztódás nem előfeltétele a sejtek inváziójának. Továbbá, a különféle onkogén mutációk eltérően befolyásolhatják a tumor sejtek migrációját és proliferációját. Végezetül pedig ezek az onkogén mutációk ígéretes támadáspontot jelenthetnek célzott terápiák számára, ugyanakkor nagymértékben befolyásolhatják azok hatékonyságát, azonban ezt minden mutáció esetében külön-külön vizsgálni kell.

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LIST OF PUBLICATIONS

Publications related to the thesis

1.   Garay, T., E. Juhasz, E. Molnar, M. Eisenbauer, A. Czirok, B. Dekan, V. Laszlo, M. A. Hoda, B. Dome, J. Timar, W. Klepetko, W. Berger and B. Hegedus (2013). "Cell migration or cytokinesis and proliferation?--revisiting the "go or grow" hypothesis in cancer cells in vitro." Exp Cell Res 319(20): 3094-3103.

2.   Hoda, M. A., J. Munzker, B. Ghanim, K. Schelch, T. Klikovits, V. Laszlo, E. Sahin, A. Bedeir, A. Lackner, B. Dome, U. Setinek, M. Filipits, M. Eisenbauer, I. Kenessey, S. Torok, T. Garay, B. Hegedus, A. Catania, S. Taghavi, W. Klepetko, W. Berger and M. Grusch (2012). "Suppression of activin A signals inhibits growth of malignant pleural mesothelioma cells." Br J Cancer 107(12): 1978-1986.

3.   Szabo, A., K. Varga, T. Garay, B. Hegedus and A. Czirok (2012). "Invasion from a cell aggregate--the roles of active cell motion and mechanical equilibrium." Phys Biol 9(1): 016010.

Publications not related to the thesis

1.   Lotsch, D., E. Steiner, K. Holzmann, S. Spiegl-Kreinecker, C. Pirker, J. Hlavaty, H. Petznek, B. Hegedus, T. Garay, T. Mohr, W. Sommergruber, M. Grusch and W. Berger (2013). "Major vault protein supports glioblastoma survival and migration by upregulating the EGFR/PI3K signalling axis." Oncotarget **4**(11): 1904-1918.

2.   Illyés Z., Halász K., Rudnóy Sz., Ouanphanivanh N., Garay T., Bratek Z. (2009) "Changes in the diversity of the mycorrhizal fungi of orchids as a function of the water supply of the habitat." Journal of Applied Botany and Food Quality 83, 28-36.

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