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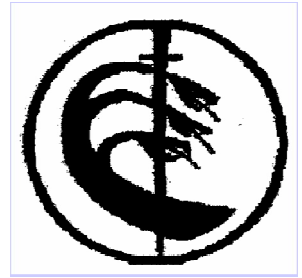
Evaluation of Fibroblast Growth Factor Receptors as Therapeutic Targets in Melanoma

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Zusammenfassung

Das Melanom ist die tödlichste Hautkrebsart und ist in einem fortgeschrittenen Stadium schwierig zu behandeln. Fibroblasten Wachstumsfaktoren (FGF1-23) und deren Rezeptoren (FGFR1-4) sind beteiligt an Tumorstadium, Angiogenese und Überleben von malignen Zellen bei verschiedensten Tumorarten. Eine krebstimulierende Rolle im speziellen beim Melanom, wurde für die Wachstumsfaktoren FGF-1 (aFGF) und FGF-2 (bFGF), sowie den FGF Rezeptor FGFR-1 nachgewiesen. Außerdem wurde eine Korrelation, zwischen der FGFR-4 Protein Expression und dem Überleben von Melanom-Patienten, beschrieben. Es wird auch angenommen, dass der kürzlich identifizierte Arg/Gly Polymorphismus von FGFR-4, die Progression und Zell Migration von einigen Krebsarten stimuliert.

Ziele: Die Ziele waren (A) eine Evaluierung des Potenzials von FGF-Rezeptoren als therapeutische "Targets" in der Melanombehandlung, und (B) die Untersuchung des FGFR-4 Arg/Gly Polymorphismus und seine möglichen Auswirkungen auf Melanom Zelllinien.

Methoden: Die Methoden umfassten, (I) eine Expressionsanalyse von allen FGF-Rezeptoren, sowie deren verschiedenen Isoformen und allen 22 humanen FGF-Liganden mittels Standard PCR und qRT-PCR, (II) Analyse der zellulären Effekte durch Blockierung des FGFR Signals mittels dominant-negativer Rezeptor Konstrukte, (III) Untersuchung der Effekte von FGFR Blockierung auf das Tumorstadium In Vivo mittels Xenotransplantationsexperimente in SCID Mäusen, (IV) Analyse der Auswirkungen von niedermolekularen FGFR Inhibitoren, im Vergleich zu FGF-2 Stimulation, auf den FGFR Signalweg, (V) Untersuchung von 41 Melanomproben hinsichtlich FGFR-4 Arg/Gly Expressionsmuster und Vergleich mit der Progressionsstufe des Tumors, (VI) Analyse der Effekte des FGFR-4 Arg Allels auf die Zellmigration und Proliferation.

Resultate: Die Expressionsanalyse zeigte, dass der FGFR-1 und der FGFR-4 in nahezu allen Melanom Zelllinien stark exprimiert werden, im Vergleich zu FGFR-2 und FGFR-3. Nimmt man jedoch primäre Melanozyten als Referenz, so scheint ausschließlich die Expression von FGFR-4 und FGFR-2 hinaufreguliert, während die Expression von FGFR-1 auf einem hohen Niveau bestehen, und FGFR-3 unverändert niedrig, bleibt. Im Allgemeinen, ist die Expression der FGF-Rezeptoren und deren Isoformen sehr heterogen ausgefallen, vergleicht man das

Expressionsmuster der Zelllinien untereinander. Verkürzte FGFR Isoformen und Isoformen ohne Transmembrandomäne, wurden nur in rund der Hälfte der untersuchten Zelllinien nachgewiesen.

Betrachtet man die Expression der FGF Liganden, so wurde gezeigt, dass FGF-2 in allen untersuchten Melanomzelllinien sehr stark exprimiert wird, im Gegensatz zu primären Melanozyten. FGF-5 wurde in der Mehrheit der Zelllinien (90%) exprimiert, während die Expression von FGF-18 in ungefähr 40% hinaufreguliert war und das Expressionsniveau von FGF-17 und FGF-8 in allen Zelllinien durchwegs niedrig ausfiel.

Eine Inhibierung der FGF-Rezeptoren durch dominant-negative FGFR Konstrukte zeigte, dass eine Blockierung des FGFR-1 Signalweges ein vielversprechender therapeutischer Ansatz beim Melanom ist. Eine „In Vitro“ Inhibierung wurde mittels, Proliferations-, Klonogenizitäts- und Soft Agar- Experimente, getestet. Resultate wurden anschließend „In Vivo“, durch Xenotransplantation von Melanomzellen in SCID Mäuse, bestätigt. Weiters wurde gezeigt, dass eine Inhibierung des FGFR-1 Signalweges in Melanomzellen Apoptose induziert. Dies konnte einerseits durch Fluoreszenz-Mikroskopie und Propidiumjodid Färbung verfolgt werden und wurde schließlich auch mittels Western-Blot Analyse bestätigt.

Es wurde gezeigt, dass die Zellvermehrung um ca. 20-40% zunahm, wenn Melanomzellen mit FGF-2 stimuliert wurden. Die Intensität stimulatorischer Effekte war abhängig vom endogenen Expressionsniveau. Zelllinien mit niedriger FGF-2 Expression konnten leichter mit zusätzlichem FGF-2 stimuliert werden. Auch die Migrationsfähigkeit der Melanomzelllinien konnte um 20-50% erhöht werden, wenn FGF-2 oder FGF-5 zugegeben wurde. Die Stärke der Stimulation durch FGF-2 oder FGF-5, war auch hier von der Eigenproduktion abhängig.

Weiters wurde demonstriert, dass der MAPK- und Akt- Signalweg durch FGF-2 stimuliert werden kann. Aber auch, dass eine vorhergehende Blockierung der FGF-Rezeptoren, durch „Small Molecule Inhibitors“ (PD166866 und SU5402), diese Stimulation verhindert. Eine Blockierung von Signalwegen durch diese Inhibitoren, ohne vorherige Stimulation durch FGF-2, konnte jedoch nicht gezeigt werden.

Eine homozygote Expression des FGFR-4 Gly388 Allels nahm mit zunehmender Krebsprogression deutlich ab (Primärtumore: 65%; Metastasen: 42% und maligne Effusionen: 20%). Ein Einfluss des FGFR-4 Arg388 Allels auf die Progression im Melanom ist daher naheliegend. Bei der Untersuchung von Zellmigration und

Proliferation, mittels stabiler Expression des FGFR-4 Arg388 versus Gly388 Allel in Melanomzelllinien, wurde gezeigt, dass die Zellproliferation zwischen den Allelen unverändert bleibt, die Migration in einer von zwei Arg388 Zelllinien aber 2.5 fach zunimmt.

Schlussfolgerungen: In Vivo, sowie In Vitro, wurde gezeigt, dass die Zellproliferation bzw. das Tumorwachstum um 50% reduziert wird, wenn der FGFR-1 Signalweg durch adenovirale dnFGFR1 Konstrukte inhibiert wird. Als Referenz wurden Zellen, anstatt mit dnFGFR1, mit GFP Konstrukten infiziert. Unsere Untersuchungen legen eine therapeutische Blockierung des FGFR-1 nahe. Weiters kann man daraus schließen, dass das FGFR-4 Arg388 Allel möglicherweise zur Progression von Melanomen beiträgt.

Abstract

Melanoma is the most deadly cutaneous malignancy and is difficult to treat at the advanced stage. Fibroblast Growth Factors (FGF1-23) and Fibroblast Growth Factor Receptors (FGFR1-4) are critically involved in tumour cell growth, survival and angiogenesis of several tumour types. In melanoma several reports have established an oncogenic role of acidic FGF (FGF-1), basic FGF (FGF-2) and FGFR1. Besides, a correlation between FGFR-4 protein expression and survival of melanoma patients has been described. The recently identified Arg/Gly polymorphism of FGFR-4 is suggested to influence cancer progression and cell migration of various tumour types.

Objectives: The purpose of the study was (A) to evaluate the potential of FGFRs as molecular therapy targets for melanoma and (B) to investigate putative cellular effects of the FGFR-4 Arg/Gly polymorphism in melanoma cell lines.

Methods: The thesis included (I) expression analysis in a panel of melanoma cell lines of all four FGFRs including different isoforms, and all 22 FGFs, by standard PCR and qRT-PCR, (II) analysis of cellular effects of blockade of FGFR signals by dominant-negative receptor constructs, (III) the effect of FGFR blockade on tumour growth in vivo by xenotransplantation experiments in SCID mice, (IV) the effect of small molecule FGFR inhibitors, versus FGF-2 stimulation on downstream signalling, (V) effects of FGF-2 on cell migration and proliferation, (V) investigation of 41 melanoma samples for FGFR-4 Arg/Gly expression and comparison with clinical stage, (VI) effects of the FGFR-4 Arg388 allele on cell migration and proliferation.

Results: Expression analysis revealed, that FGFR-1 and FGFR-4 are highly expressed in nearly all melanoma cell lines, compared to FGFR-2 and FGFR-3. Taking non-transformed melanocytes as reference, the expression of FGFR-4 and FGFR-2 seems to be highly up-regulated in nearly all melanoma cell lines, while expression of FGFR-1 is maintained on a high level and FGFR-3 stays low. In general, expression levels of all FGFRs and isoforms were heterogeneous. Truncated FGFR isoforms, especially those lacking the TM-domain, were not detected in around half of the melanoma cell lines. Considering expression of FGF ligands, FGF-2 was found to be highly up-regulated in all melanoma cell lines investigated while FGF-5 was up-regulated in the vast majority (90%). Expression of FGF18 was found up-regulated in 40% and FGF-17 and FGF-8 stayed low in all cell lines. Similar to FGF-Receptors, ligands were also expressed in a heterogeneous manner.

Inhibition of FGFRs by expression of dominant-negative FGFR constructs revealed, that blockade of the FGFR-1 pathway seems to be an effective therapeutic approach in melanoma cells. In vitro inhibition was tested by proliferation assays, clonogenic assays, and soft agar assays. Results have been confirmed in vivo, by xenotransplantation experiments in SCID mice. It has been shown, by blocking the FGFR-1 pathway, that melanoma cell lines induce apoptosis. This was monitored by fluorescence microscopy and propidium-iodide staining, and finally verified by immune-blotting determining protein levels of cleaved PARP1 and Caspase 3.

Cell proliferation was increased around 20-40% when cells were stimulated with exogenous FGF-2. Intensity of stimulatory effects depended on endogenous FGF-2 expression levels. Thus, cell lines with low FGF-2 expression levels were more easily stimulated. Migration of melanoma cells was also stimulated about 20-50%, either by exogenous FGF-2 or FGF-5. Migration of cells having low endogenous FGF-2 or FGF-5 expression was more easily stimulated.

The MAPK- and Akt- signalling pathways were shown to be stimulated by FGF-2. Prior incubation with FGFR small molecule inhibitors, PD166866 and SU5402, prevented stimulation by FGF-2. However, inhibition of constitutive activation of downstream signals by small-molecule FGFR inhibitors alone, could not be shown in this study.

Homozygous expression of the FGFR-4 Gly388 allele was found to be reduced with increasing cancer stage (primary tumours: 62%; metastases: 43%; malignant effusions: 20%), in melanoma cell lines. Therefore, the FGFR-4 Arg388 allele is suggested to be involved in cancer progression of melanoma. Investigating cell migration and proliferation, using stably expressing FGFR-4 Arg388 versus Gly388 cell lines, revealed no differences considering cell proliferation, but migration was increased 2.5 x in one of two FGFR Arg388 cell lines.

Conclusion: In Vivo as well as in vitro, cell proliferation or tumour growth is reduced to about 50%, when infected with adenoviruses bearing dnFGFR1 constructs, compared to GFP as control. Our investigation suggests that FGFR-1 inhibition might be a promising strategy for melanoma therapy. The FGFR-4 Arg388 allele might contribute to melanoma progression.

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1 Aims of the Study

Recently, so called “targeted therapies” have become an alternative to classical chemotherapies for the treatment of advanced tumours in cancer patients. The aim is to develop drugs targeting molecules specific for tumour cells. Therefore, targeted drugs have the potential for enhanced anti-cancer impact, and simultaneously avoid problems of severe adverse effects based on interactions with normal tissue. Thus, ideal targets need to have two important characteristics, they have to be specific for malignant cells and have to be “drug-able”, meaning that they are able to interact with and respond to the drug application.

Fibroblast Growth Factors (FGF1-23) and Fibroblast Growth Factor Receptors (FGFR1-4) are critically involved in tumour cell growth, survival and angiogenesis of several tumour types. In melanoma several reports have established an oncogenic role of acidic FGF (FGF-1), basic FGF (FGF-2) and FGFR-1. Besides, a correlation between FGFR-4 protein expression and survival of melanoma patients has been described. The recently identified Arg/Gly polymorphism of FGFR-4 is suggested to influence cancer progression and cell migration of various tumour types.

The purpose of the study was (A) to evaluate the potential of FGFRs as molecular therapy targets for melanoma, and included: Expression analysis in a panel of melanoma cell lines of all four FGFRs including different isoforms, and all 22 FGFs, by standard PCR and qRT-PCR; Analysis of cellular effects of blockade of FGFR signals by dominant-negative receptor constructs; Investigation of effects of FGFR blockade on tumour growth in vivo by xenotransplantation experiments in SCID mice; Analysis of effects of FGFR inhibitors (Small Molecule Inhibitors) versus FGF-2 stimulation on downstream signalling; Investigation of effects of FGF-2 on cell migration and proliferation.

And (B) to investigate putative cellular effects of the FGFR-4 Arg/Gly388 polymorphism in melanoma cell lines, involving: Investigation of 41 tumour samples for FGFR-4 Arg/Gly388 expression and comparison with the clinical stage of disease; Analysis of effects of the FGFR-4 Arg388 allele on cell migration and proliferation.

2 Introduction

2.1 Cellular and Molecular Biology of Melanoma

2.1.1 Melanocytes and Melanin

Melanocytes can be found in a variety of places in the human body. They are present in the middle layer of the eye called the uvea, in the inner ear, meninges, bones, heart and in the skin. Considering the skin, melanocytes are located in the bottom layer, called stratum basale, of the skin's epidermis and primarily impart skin colour (Barden & Koulish, 1983; Markert & Silvers, 1956; Mintz, 1971; Nichols, 1969; Theriault & Hurley, 1970).

In the human skin, melanocytes are distributed as single cells within the basal layer of the epidermis (Figure 2.1) and are embedded in a tissue environment consisting of multiple cell types, such as keratinocytes, fibroblasts, immune-regulatory cells and endothelial cells. They are present in a life-long stable ratio of 1:5 with basal keratinocytes and this balance is maintained by highly regulated induction of melanocyte division. As long as melanocytes are bound to the basement membrane they are not able to proliferate. They need to decouple from the basal lamina as well as from bound keratinocytes, retract their dendrites, divide and migrate along the basement membrane until they re-couple to the membrane and to keratinocytes. Melanocyte growth is controlled by surrounding keratinocytes, they divide quite slowly, compared to other cells of the human body (Haass & Herlyn, 2005; Haass, Smalley et al., 2005).

However, it is suggested that cell division can be accelerated significantly by various environmental factors, most importantly by ultraviolet (UV) light, which stimulates not only cell division but also the production of cytoprotective pigments, known as melanin (Herlyn, Ferrone et al., 2001; Satyamoorthy, Muyrers et al., 2001).

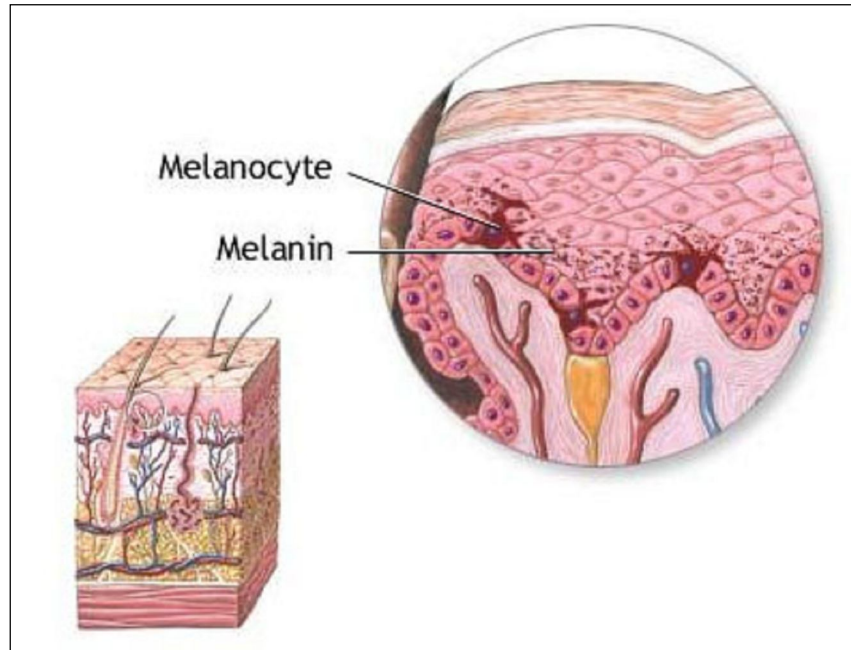


Figure 2.1: Location and distribution of melanocytes and melanin in the human skin (http://training.seer.cancer.gov/ss_module14_melanoma/unit02_sec02_anatomy.html; 26.07.2009).

Various studies of animal models suggest, that genetic as well as environmental cues might play a role in adult pigment patterning and thus prevalence of melanocytes and production of melanin (Meredith & Sarna, 2006). Melanin mainly functions as potent free radical scavenger in humans, protecting the DNA of skin cells from reactive oxygen species (ROS) that can be formed in high concentrations by UV light (Herrling, Jung et al., 2008; Jung, Seifert et al., 2008).

In the normal skin, a homeostatic balance between melanocytes and surrounding cells can be found. This fine-tuned balance is held upright by direct or indirect cell to cell interactions, activating or suppressing a great variety of signalling pathways, as well as by the extracellular matrix (ECM). For instance, melanocytes are under control of keratinocytes regulating their proliferation (Herlyn, Ferrone et al., 2001), while the basement membrane (basal lamina), which is a specialized, dense form of ECM, prevents melanocytes from migrating into the dermis. It is also known, that mature “non-transformed” melanocytes are not able to survive in the dermis, due to the completely different environment (Herlyn, Ferrone et al., 2001; Jung, Seifert et al., 2008).

2.1.2 Melanomagenesis

In general, melanoma develops as a consequence of multifactorial perturbations (Figure 2.2) and its ability to invade and metastasize to distant organs suggests its adaptability to varying microenvironments. It can be considered as a disease of homeostatic imbalance in the skin, and its development can be influenced by a great variety of skin components (Satyamoorthy & Herlyn, 2002).

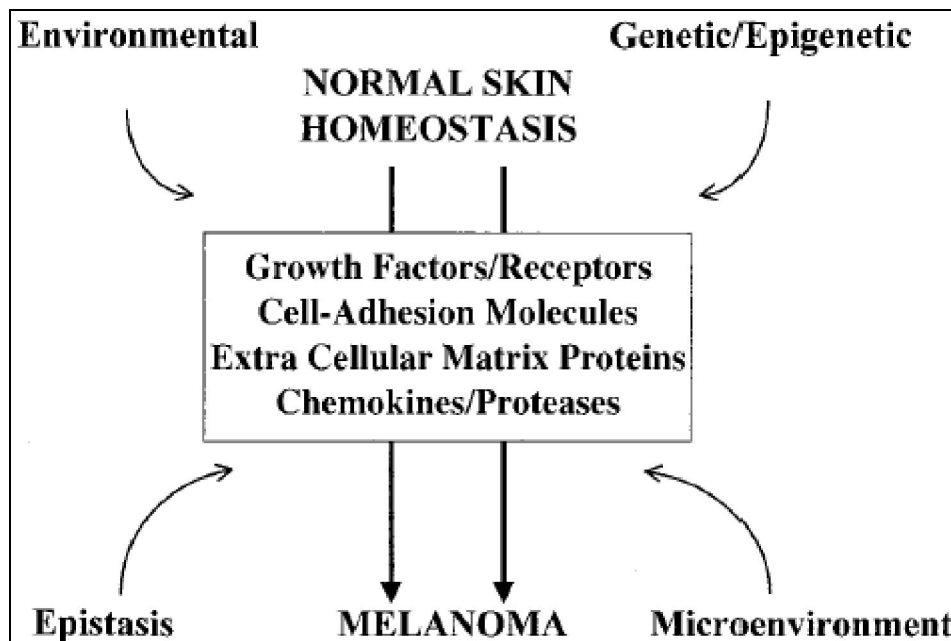


Figure 2.2: Factors suggested to influence melanoma development. A change of epistasis is thought to be the entry point of melanoma development and is achieved by environmental, genetic as well as by epigenetic alterations in the skin tissue. The homeostatic imbalance in the tissue can become more dynamic and irreversible, leading to melanoma progression (Satyamoorthy & Herlyn, 2002).

2.1.3 Homeostatic Imbalance and Dysplastic Nevi

The process of homeostasis determines whether cells remain quiescent, differentiate, proliferate or undergo apoptosis, in the normal environment. Considering melanocytes in the skin, homeostasis is mainly controlled by keratinocytes, controlling their growth and behaviour by cell-cell adhesion molecules as well as by paracrine growth factors.

Three major mechanisms are proposed to be involved in the process of escaping the control of keratinocytes, namely (a) down-regulation of receptors involved in communication with keratinocytes, (b) up-regulation of signalling molecules and receptors important for communication between melanoma-melanoma and melanoma-fibroblast cells, and (c) loss of anchorage to the basement membrane (Haass, Smalley et al., 2005).

During the first stage of melanoma development, melanocytes fail to distribute normally and remain in clusters instead, forming a dermal or dysplastic nevus. They may be able to break the basal lamina, the basement membrane barrier, however cells are prevented from aggressive cell division due to lack of transformation (Satyamoorthy & Herlyn, 2002). Melanocytes acquire the ability to express specific cell surface molecules allowing them to adhere, survive, grow and migrate. Examples of up-regulated receptors and signalling molecules are: N-cadherin, Mel-CAM and zonula occludens protein-1 (ZO-1) (Haass, Smalley et al., 2005). On the other hand, the cells stop expressing genes restricting migration, such as E-cadherin, which is known for suppressing invasion and is expressed by melanocytes and keratinocytes in the normal skin environment (Danen, Sonneveld et al., 2000; Gruss & Herlyn, 2001). Besides E-cadherin, also P-cadherin, desmoglein and connexins were found to be frequently down-regulated. Cell-matrix interactions are known to shift from lamin and collagen IV mediated adhesion to collagen I and vitronectin-binding receptors during early melanoma development (Gruss & Herlyn, 2001; Haass, Smalley et al., 2005). E-cadherin facilitates homophilic interaction and restricts cell division of melanocytes without killing them. In contrast, fibroblasts and endothelial cells express N-cadherin (Hsu, Andl et al., 2000; Hsu, Meier et al., 2000; Hsu, Wheelock et al., 1996). Cadherins are highly glycosylated cell surface proteins and are known to be an integral part of cell-cell adherens junctions, promoting calcium-dependent homotypic and heterotypic cell-adhesion as well as intercellular communication. E-, N- and P-cadherins, also considered as the classical cadherins, are known to be expressed during different stages of melanoma progression (Steinberg & McNutt, 1999; Vleminckx & Kemler, 1999).

The progressive loss of E-cadherin during melanoma development consequently leads to the disruption of adherens junctions and may impart the loss of terminal differentiation to melanocytes (Herlyn, Padarathsingh et al., 2002; Satyamoorthy & Herlyn, 2002). These complex formations convey cell to cell interactions and adhesion by indirect linkage of their cytoskeleton. Besides cadherins and several adaptor proteins, also catenins are part of these complexes and are known to be potent regulators of gene expression (Figure 2.3).

Transformed melanocytes lose the ability to express E-cadherin, but start to express N-cadherins instead. This cadherin subtype switching promotes interactions with endothelial cells as well as fibroblasts, subsequently leading to tumour-host cell interactions, migration and tumour cell invasion (Furukawa, Fujii et al., 1997). It was shown, that over-expression of E-cadherin in melanoma cells prevents their growth, invasion into the dermis and leads to cell apoptosis (Hsu, Andl et al., 2000; Hsu, Meier et al., 2000). However, it is still not known which molecular changes lead to the E- to N-cadherin switch. Mutations within the E-cadherin gene or epigenetic mechanisms, such as DNA methylation, are apparently not involved in human melanomas. There is evidence, that transcriptional silencing of the E-cadherin gene by transcription factors may be involved (Comijn, Berx et al., 2001).

N-cadherin is suggested to be the major inductor for the formation of a dysplastic nevus. It mediates the homotypic aggregation of melanoma cells and heterotypic interactions to vascular endothelial cells and fibroblasts (Utton, Eickholt et al., 2001) and is thought to facilitate cell migration into the dermis as well as the process of intra-vascularization (Satyamoorthy & Herlyn, 2002). It is also known, that N-cadherin is able to interact physically with the fibroblast growth factor receptor-1 (FGFR-1), which promotes growth and the formation of neurites (Utton, Eickholt et al., 2001).

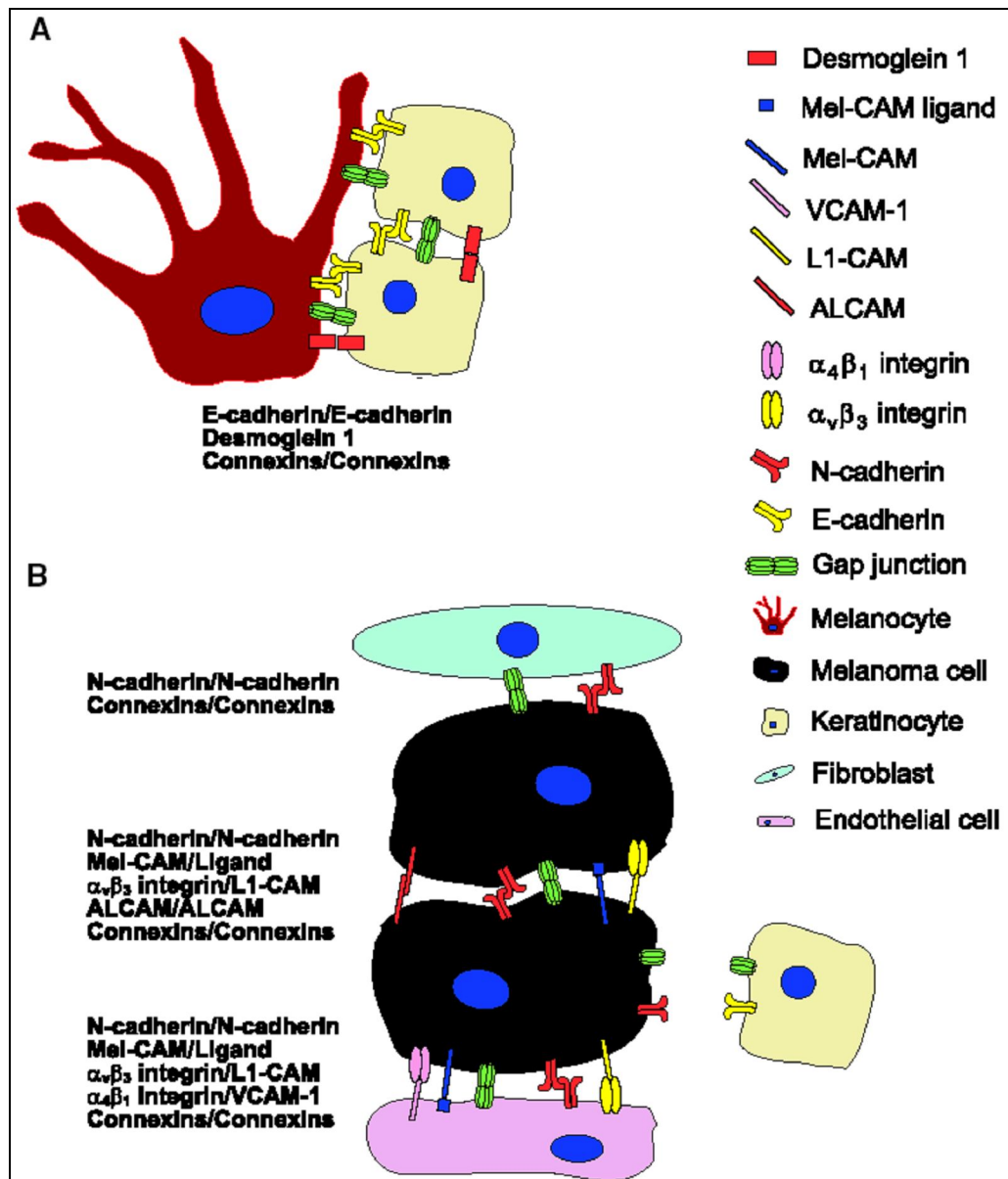


Figure 2.3: Cell-cell adhesion between normal melanocytes and melanoma cells. (A) In the normal skin melanocytes are surrounded by keratinocytes. E-cadherins and desmoglein adhere both cell types to each other and enable them to communicate via gap junctions. (B) In contrast, melanoma cells are present as clusters, they migrate into the dermis and are able to interact with three different cell types. They interact with each other through N-cadherins, Mel-CAM/ ligand, $\alpha_v\beta_3$ integrin/L1-CAM, ALCAM and connexins, with fibroblasts through N-cadherin and connexins, and with endothelial cells through N-cadherin, Mel-CAM/ligand, $\alpha_v\beta_3$ integrin /L1-CAM, $\alpha_4\beta_1$ integrin/VCAM-1 and connexins (Haass & Herlyn, 2005; Haass, Smalley et al., 2005).

2.1.4 Genetic Abberations and Melanoma Progression

Melanoma begins as a benign nevus, which consists of a clonal population of hyperplastic melanocytes locked in a state of cellular senescence and not displaying aggressive proliferation due to lack of transformation. It is suggested that cell senescence represents a true barrier to the progression of melanoma and once it is overcome, the nevus can progress to more lethal stages with increasing proliferation ability. Therefore, the investigation of the mechanism(s) by which melanoma evades senescence has been studied. In most cells senescence is regulated by combinational activities within Rb and p53 pathways. However, it seems that melanocyte senescence is regulated in a distinct manner (Ha, Merlino et al., 2008; Satyamoorthy & Herlyn, 2002), (Figure 2.4).

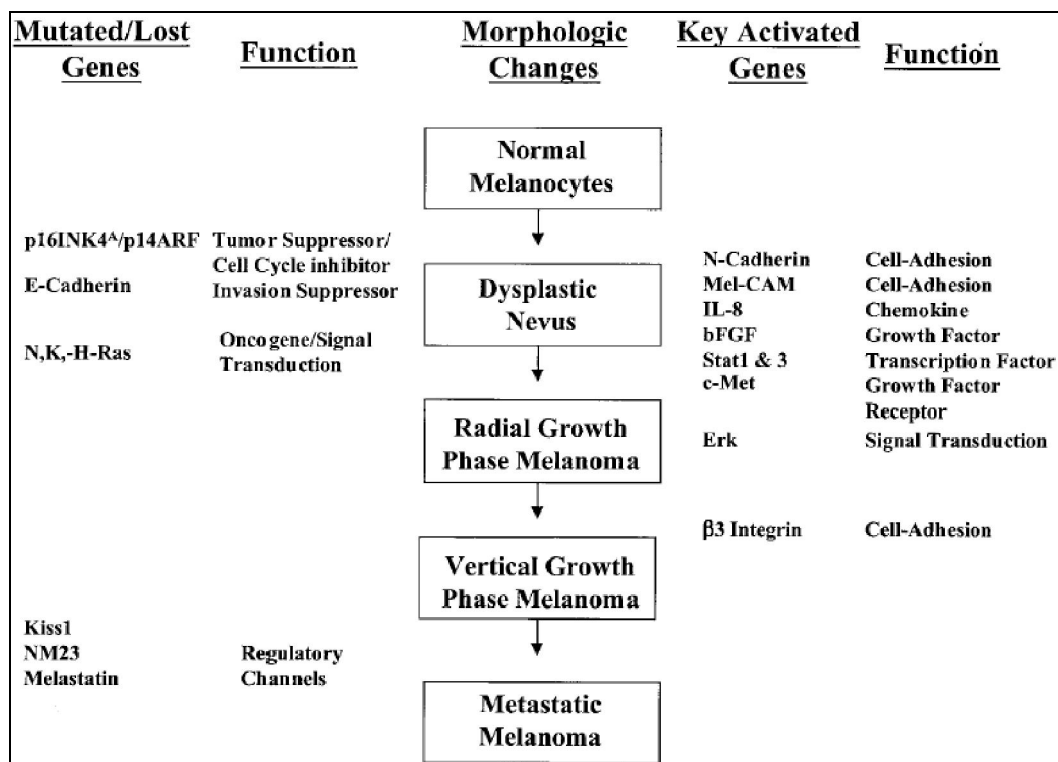


Figure 2.4: Oncogenes and Tumour-Suppressor Genes suggested to be involved in melanoma progression. Tumour progression is initiated by expression or down-regulation of molecules, which are affected by environmental, genetic and epigenetic events. The model also shows experimental stages of melanoma progression. A dysplastic nevus, also called multiple atypical nevus, develops more likely into melanoma than a melanocytic nevus. Melanomas in the stage of Radial Growth Phase (RGP) were shown to be less aggressive and non-tumourigenic in SCID mice. However, in the Vertical Growth Phase (VGP) and in the metastatic stage, melanomas are becoming more and more aggressive and tumourigenic due to several biological and molecular changes, some of these are indicated (Satyamoorthy & Herlyn, 2002). However, the recently identified BRAF oncogene is not shown in this figure. It has been shown, that around 60% of melanomas contain activating kinase mutations, concerning this member of the MAPK-pathway (Montagut, Sharma et al., 2008; Smalley, Nathanson et al., 2009).

Due to continuous and high exposure of melanocytes to environmental hazards, such as sunlight and chemicals, one might suggest, that melanomas solely demonstrate UV signature mutations. However, this is not the case. Compared to other tumours, mutations in the tumour suppressor gene p53 are found in less than 5% of melanomas, thus are very rare (Herlyn, Ferrone et al., 2001). In cases exposed to sunlight, mutations in the RAS oncogene contributed less than 21% (Herlyn & Satyamoorthy, 1996). Considering mutations in the tumour suppressor p16^{INK4A}, they were found in less than 25% of melanomas (Herlyn, Ferrone et al., 2001).

Mutations in genes like CDK4, PTEN or β -catenin were not found to be important in melanomas. Germ line mutations or deletions in chromosome 9p21 were found in about 20-30% of all cases, while genetic predisposition of familial melanoma did not exceed more than 10% (Satyamoorthy & Herlyn, 2002). The chromosome segment 9p21 harbours a variety of cell cycle inhibitors and tumour suppressors, such as p16^{INK4A} and p15^{INK4B}. Through the use of shared coding regions and an alternative reading frame the CDKN2A gene codes for two proteins, the cell cycle inhibitor p16^{INK4A} and p14/ARF (Becker, Rizos et al., 2001). The p14/ARF protein is known to stabilize p53 by interfering with MDM2, forming a p53/MDM2 protein complex (Rocco & Sidransky, 2001). However, the majority of mutations in this region affect the p16^{INK4A} locus, while 40% happen in both p16^{INK4A} and p14/ARF (Rizos, Darmanian et al., 2001; Rizos, Puig et al., 2001), (Figure 2.5).

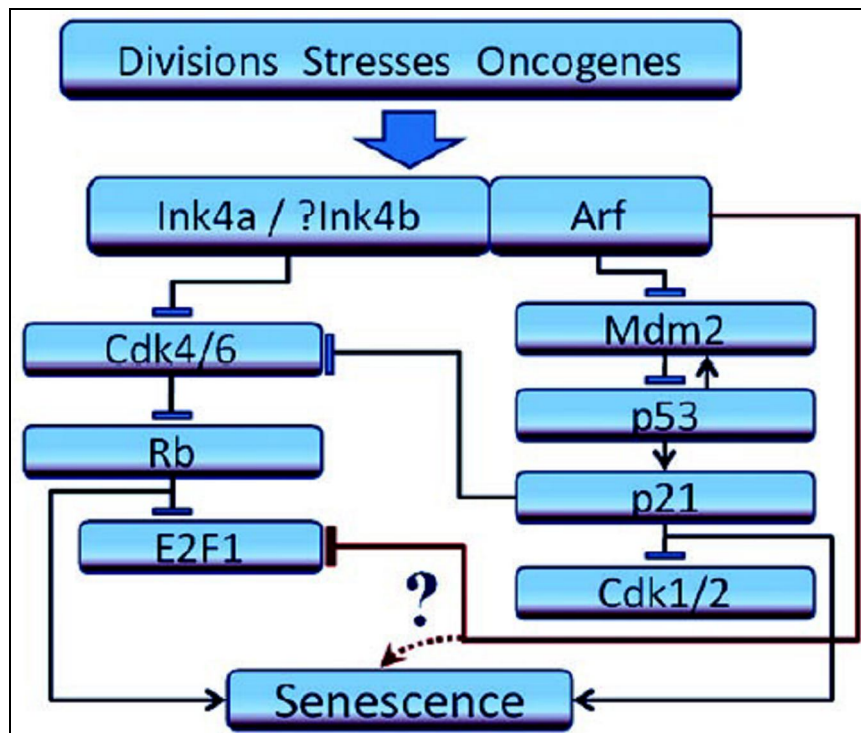


Figure 2.5: Suggested Arf regulation of melanocyte senescence, independent from p53. The Arf/p53 and Ink4a/Rb pathways are proposed to be both implicated in senescence. It was shown, that in mouse melanocytes Arf can promote p53 independent degradation of E2F1. These mechanisms, together with others (question mark), might trigger senescence in melanocytes (Ha, Merlino et al., 2008).

Two important questions are left open, (1) by which mechanism(s) melanoma cells evade senescence, if p53 is not involved and (2) why melanoma remains chemo-radiation resistant. It seems, that p53, despite its wild-type status, is not able to modulate melanoma cells, which is the case in nearly all other tumour cells. In melanoma cells, p53 accumulates in the nucleus, but its transcriptional activity is weak (Satyamoorthy, Chehab et al., 2000).

This indicates, that p53 is kept in an inactive state by other inhibitory factors or post-transcriptional mechanisms. It has been shown, that in melanoma cells, like in sarcomas, the MDM2 protein is over-expressed (Freedman & Levine, 1999; Freedman, Wu et al., 1999). MDM2 is known to inactivate p53 by regulating its degradation (Rocco & Sidransky, 2001).

In the case the p16^{INK4A} locus would be inoperative, the p14/ARF derived from the same locus might be less functional and MDM2 is able to inhibit p53. What's more, expression of Bcl-2, an anti-apoptotic factor, is known to inhibit not only the p53-dependent, but also the independent apoptotic mechanisms (Hsu & Hsueh, 2000). A further target, that lies downstream of p53, is Apaf-1 (apoptotic protease activating factor-1), which is known to initiate the proteolytic cascade by activating caspase 9. It has been shown, that Apaf-1 is frequently inactivated in melanoma cells (Soengas, Capodieci et al., 2001). In contrast, caspase 9 activation and initiation of apoptosis can be inhibited by Akt/PKB signalling (Soengas, Capodieci et al., 2001; Tang, Zhou et al., 2000).

Apart from regulating apoptosis, the Akt/PKB pathway is also thought to play an important role in cellular metabolism. A variety of growth factors have been found to activate the Akt/PKB signalling pathway, such as HGF/SF secreted by melanoma cells or IGF-I secreted by the surrounding stroma, known to function in an autocrine and paracrine manner (Delehedde, Seve et al., 2000; Satyamoorthy, Li et al., 2001) and leading to increased proliferation. It has been suggested, that constitutive activation of Stat1 and Stat3 together with members of the MAP kinase pathway might play an important role in melanoma cells (Kirkwood, Farkas et al., 1999).

In this context, it has been shown recently, that activating BRAF kinase mutations arise in around 7% of all human tumours and in the majority of melanomas (Montagut, Sharma et al., 2008; Smalley, Nathanson et al., 2009). The RAF-mitogen-activated protein / extracellular signal regulated kinase (ERK) kinase-ERK signalling cascade has been validated in preclinical studies as potential therapeutic target and selective RAF kinase inhibitors are in clinical development (Montagut, Sharma et al., 2008).

The investigation of the precise role of multiple factors in a multicellular environment is difficult and requires a lot of effort and time, but is essential to understand the cross-talk between several signal transduction pathways, their redundancies and finally the process of melanoma development.

2.2 Fibroblast Growth Factors (FGF) and FGF-Receptors

2.2.1 Evolution of the FGF and FGFR Gene Family

The FGF signaling pathway is conserved throughout metazoan evolution. Twenty-two FGFs have been discovered to date in mice and humans, FGF1-FGF23. FGF-19 is the human ortholog of mouse FGF-15. In total the family consists of 22 members (Ornitz & Itoh, 2001). The nomenclature does not imply, that all FGFs have a proliferative activity on fibroblasts, instead is based on biological characteristics of the first discovered family members as well as on the strong structural homology (Powers, McLeskey et al., 2000). If all members of a given group of genes have above 50% amino acid identity it is traditionally considered a gene family (Thornton & DeSalle, 2000). The human FGF gene family can be divided into seven subfamilies (Figure 2.6), members of these subfamilies share increased sequence similarity as well as biochemical and developmental properties (Ornitz & Itoh, 2001). Recently it has been shown, that FGF homologous factors (FGF11-14), although having high sequence identity with the FGF family, do not activate FGF receptors (FGFRs). Therefore, are not generally considered members of the FGF family (Beenken & Mohammadi, 2009).

Nearly all FGF genes are scattered throughout the genome, which indicates their generation by gene duplications and translocations during evolution (Ornitz & Itoh, 2001). The family seems to have expanded in two phases, namely during early metazoan evolution and during the evolution of early vertebrates. In the first phase the FGFs expanded by gene duplication from two or three to six genes, while in the second phase the expansion took place by two large gen(om)e duplications (Horton, Mahadevan et al., 2003; Sidow, 1996; Thornton & DeSalle, 2000).

The FGF-Receptor family by contrast consists of only four functional genes (FGFR1-4), human receptor genes contain 18 exons and have an identical exon-intron organization. (Powers, McLeskey et al., 2000). Alternative splicing has increased the functional diversity, and thereby also specificity (Table 2.1), significantly during evolution. Phylogenetic analysis indicates that mechanisms which regulate alternative splicing have been conserved during evolution (Itoh & Ornitz, 2004; Mistry, Harrington et al., 2003)

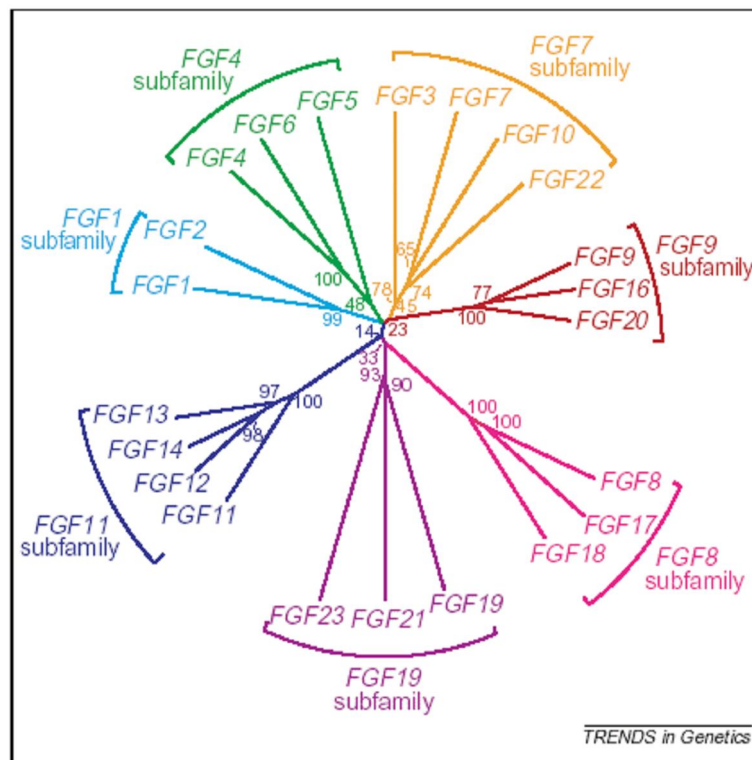


Figure 2.6: Members of the human fibroblast growth factor (FGF) gene family can be arranged into seven subfamilies that indicate evolutionary relationships. Evolutionary distance is proportional to the branch lengths. The human FGF-19 gene is thought to be the ortholog of the FGF-15 mouse gene (Ornitz & Itoh, 2001).

Table 2.1: FGF-Receptor genes, their chromosomal localizations, splice variants and ligand-specificities for FGF1-FGF10, are shown (Ornitz & Itoh, 2001).

<i>FGFR1</i>	8p12	<i>FGFR1b</i>	FGF1, 2, 3 and 10
		<i>FGFR1c</i>	FGF1, 2, 4, 5 and 6
<i>FGFR2</i>	10q26.12	<i>FGFR2b</i>	FGF1, 3, 7 and 10
		<i>FGFR2c</i>	FGF1, 2, 4, 6 and 9
<i>FGFR3</i>	4p16.3	<i>FGFR3b</i>	FGF1 and 9
		<i>FGFR3c</i>	FGF1, 2, 4, 8 and 9
<i>FGFR4</i>	5q35.2	<i>FGFR4</i>	FGF1, 2, 4, 6, 8 and 9

The genes are distributed throughout the genome similar to FGF genes, but without apparent relationship between the chromosomal locations. The distribution of the genes indicates the probability, that these are similarly derived from a common ancestral gene by two waves of gen(om)e duplication during early vertebrate evolution. Besides recent findings suggest that the expansions of the FGFR family and FGF subfamilies were linked in time. This kind of co-evolution presumably facilitated complex vertebrate evolution by increasing combinatorial specificities between ligand-receptor pairs (Itoh & Ornitz, 2004).

2.2.2 Structural and Functional Properties of FGFs and FGFRs

2.2.2.1 Fibroblast Growth Factors (FGFs)

Fibroblast growth factors are polypeptide growth factors and are able to induce chemotactic, mitogenic and angiogenic activity in cells of mesodermal and neuroectodermal origin (Basilico & Moscatelli, 1992). Members of the FGF family possess a central core of around 140 amino acids which is highly homologous between different family members. This core folds into twelve anti-parallel β -strands that form together a cylindrical barrel. The barrel is closed by the more variable amino- and carboxy-terminal stretches (Ago, Kitagawa et al., 1991; Zhang, Cousens et al., 1991). The historical nomenclature (FGFs) of the first of these proteins was based on their biological activity, however is not meant to imply that all of them have fibroblast stimulating activities but rather that they belong to the same family because of their structural relation (Powers, McLeskey et al., 2000).

The vast majority of FGFs (FGFs 3-8, 10, 17-19, 21 and 23) are secreted by the Endoplasmic Reticulum-Golgi pathway. These FGFs are readily secreted from cells and possess N-terminal signal peptides, which are cleaved in the ER according to the classical pathway. FGFs 9, 16 and 20 have uncleavable N-terminal hydrophobic sequences and maybe are secreted independent of the classical pathway. Others (FGFs 1 and 2) totally lack signal peptides and are not secreted, but can be released by damaged cells or by an exocytotic mechanism which is independent of the ER-Golgi pathway (Ornitz & Itoh, 2001), (Table 2.2).

FGFs 11-14 also lack signal peptides, but remain intracellular and function within cells in a receptor independent manner. FGF-22 remains attached to the cell surface and possesses a putative N-terminal signal peptide (Schoorlemmer & Goldfarb, 2001).

Table 2.2: Characteristics of the FGF family members (Miralles, Czernichow et al., 1999; Ornitz, Xu et al., 1996; Powers, McLeskey et al., 2000; Xu, Lawshe et al., 1999).

Name	Synonym(s)	High-Affinity Receptors	Comments
FGF-1	acidic FGF (aFGF)	FGFR1 (IIIb+IIIc) FGFR2 (IIIb+IIIc) FGFR3 (IIIb+IIIc) FGFR4	no signal sequence; nuclear localisation signal (intracrine stimulation of DNA synthesis); one mRNA form; strong heparin affinity
FGF-2	basic FGF (bFGF)	FGFR1 (IIIb+IIIc) FGFR2 (IIIc) FGFR3 (IIIc) FGFR4	no signal sequence; four protein isoforms (through alternating start codons); some possess nuclear localization signals; 55% sequence identity to FGF-1; phosphorylation by PKA/PKC (physiological role still unclear); strong heparin affinity
FGF-3	int-2	FGFR1 (IIIb) FGFR2 (IIIb)	signal sequence (loss leads to accumulation in nucleus and to inhibition of proliferation); nuclear localization motif; site of mouse mammary tumour virus (MMTV) integration; 44% homology to FGF-2; primarily expressed during development
FGF-4	kaposi FGF (kFGF); hst-1	FGFR1 (IIIc) FGFR2 (IIIc) FGFR3 (IIIc) FGFR4	signal sequence; identified by screening of stomach tumours and Kaposi's sarcomas; 42% homology to FGF-2
FGF-5		FGFR1 (IIIb+IIIc) FGFR2 (IIIb+IIIc) FGFR3 (IIIb+IIIc) FGFR4	signal sequence; identified by screening human tumour cell lines promoting the growth of 3T3 fibroblast growth without serum growth factors; glycosylation site
FGF-6	hst-2	FGFR1 (IIIc) FGFR2 (IIIc) FGFR4	signal sequence; glycosylation site (glycoprotein shows no change in biological activity similar to glycosylated FGF-5)
FGF-7	KGF	FGFR2 (IIIb)	signal sequence; glycosylation site; specific for epithelial cells
FGF-8	AIGF	FGFR1 FGFR2 (IIIc) FGFR3 (IIIc) FGFR4	all seven isoforms with signal sequences; alternate splicing using six exons; frequently activated in MMTV infections similar to FGF-3/4
FGF-9	GAF	FGFR2 (IIIc) FGFR3 (IIIb+IIIc) FGFR4	no signal sequence - although efficiently secreted; probably using alternative secretion pathway; not angiogenic
FGF-10	KGF-2	FGFR1 (IIIb) FGFR2 (IIIb)	signal sequence; high sequence similarity to FGF-7; both are mitogenic for keratinocytes and expressed in stromal cells, but FGF-10 has a much higher affinity to heparin which thereby potentiates activity
FGFs 11-14		Unknown?	no signal sequences; all contain nuclear localization signals; share 58% to 71% sequence homology between themselves, but less than 30% with other FGFs

FGF-15		Unknown?	suggested to play a role during embryogenesis; gene is activated by E2A-Pbx1
FGFs 16-19		FGF17; FGFR1 (IIIc), FGFR2 (IIIc)	all contain signal sequences; suggested to play a role during development
FGF-20	XFGF20	Unknown?	high sequence similarity to FGF-9; expressed in early stages of embryonic development

2.2.2.2 FGF-Receptors (FGFRs)

Different FGFs can be specific for diverse target cells and are able to induce a variety of diverse effects, like chemotaxis, angiogenesis or mitogenesis for example. To achieve this amount of diversity the signalling system needs a great variety of different receptors. In general there exist two mechanisms to achieve this FGFR diversity, namely by different expression of FGFR genes and alternative splicing of the same gene (Figure 2.7). Three out of four existing FGFR genes, if expressed, can give rise to a variety of receptor isoforms by using different splice variants (Powers, McLeskey et al., 2000).

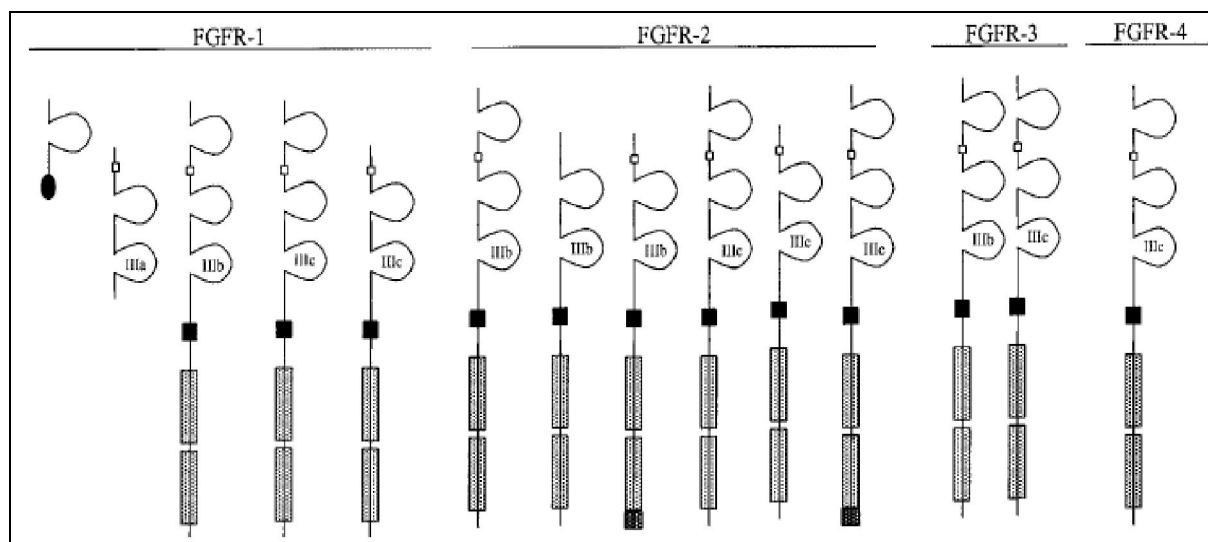


Figure 2.7: Variety of FGF-Receptors by using the mechanism of alternative splicing. Concerning the FGFR-2 gene, Ig domain IIIb is pre-dominantly expressed in the epithelial lineage, while Ig IIIc domains are only expressed in the mesenchymal lineage (Orr-Urtreger, Bedford et al., 1993; Powers, McLeskey et al., 2000; Yan, Fukabori et al., 1993).

Comparing the mRNAs of the FGFR genes 1, 2 and 3, one finds a remarkable conservation of the exon/intron arrangement (Ornitz, Xu et al., 1996). Variations in using different splice sites or the introduction of early stop codons may results in secreted receptors that do not possess transmembrane domains (Johnson & Williams, 1993). Alternatively, it may lead to the loss of the IgI domain, which alters the binding affinity of FGF-1 and FGF-2 to FGFR-1 significantly, or in different IgIII domains (Johnson, Lee et al., 1990; Johnson & Williams, 1993), (Figure 2.8 and 2.9).

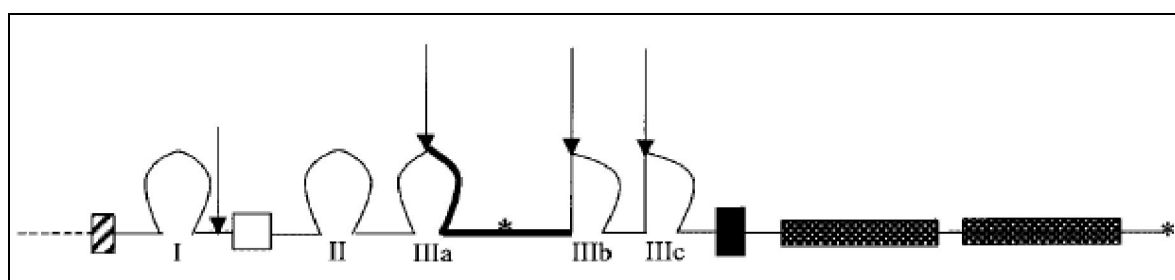


Figure 2.8: Coding exons of the human FGFR-1 gene. The 5' untranslated region is the dashed line at the beginning, followed by the signal sequence shown as striped box, IgI domain, the acidic sequence as open box, IgII and possible IgIII domains. The arrows indicate alternative splice sites, while asterisks are stop codons, thus the usage of Ig IIIa consequently leads to a stop of translation. The black box indicates the transmembrane domain, which is followed by a split kinase domain, shown as two stippled boxes (Johnson, Lu et al., 1991; Powers, McLeskey et al., 2000).

It is suggested that the usage of different IgIII domains (IIIa, IIIb or IIIc) is most important for the FGF-FGFR specificity. Coding exons of these three possible domains are situated in exactly the same order in FGFR-1, FGFR-2 and FGFR-3. The exception is FGFR-4 with only one possible IgIII domain (Chellaiah, McEwen et al., 1994; Johnson, Lu et al., 1991; Vainikka, Partanen et al., 1992). The usage of the IgIIIa splice variant leads to a truncated protein that lacks the TM- and kinase domains, thereby is secreted and probably acts to sequester released FGFs thus inhibiting their binding to functional receptors (Powers, McLeskey et al., 2000).

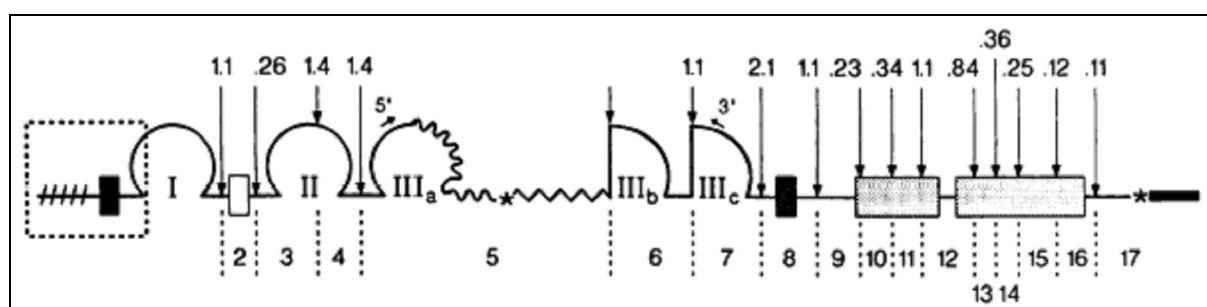


Figure 2.9: Arrangement of exons and introns of the human FGFR-1 gene. Arrows and vertical dashed lines indicate the positions of intron sequences, while the numbers above indicate the size of the intron in kilobases. Assigned exon numbers are indicated by numbers between vertical dashed lines below the coding region (Johnson, Lu et al., 1991).

Concerning the FGFR-2 gene, Ig domain IIIb is exclusively expressed in the epithelial lineage, while Ig IIIc domains are only expressed in the mesenchymal lineage (Orr-Urtreger, Bedford et al., 1993; Powers, McLeskey et al., 2000; Yan, Fukabori et al., 1993). As IgIIIb and IgIIIc domains are more homologous between different FGFR genes, one might suggest that specificity of binding is regardless of the expressed FGFR gene. This is not the case, FGF-7, for instance binds to FGFR-2 (IIIb) but not to the IIIb domain of FGFR-1 or FGFR-3 (Table 2.3). Thus, there have to be other FGFR domains that confer binding specificity and differ between FGFR genes (Ornitz, Xu et al., 1996; Powers, McLeskey et al., 2000). It has been shown, that the loss of Igl alters the binding of FGF-1 and FGF-2 to FGFR-1 significantly (Johnson & Williams, 1993).

Table 2.3: Affinities of FGFs 1-9 to FGF-Receptor isoforms, indicating ligand-receptor specificity determined by mitogenic stimulation (Ornitz, Xu et al., 1996).

FGFR	FGF-1	FGF-2	FGF-3	FGF-4	FGF-5	FGF-6	FGF-7	FGF-8	FGF-9
1, IIIb	100	60	34	16	4	5	6	4	4
1, IIIc	100	104	0	102	59	55	0	1	21
2, IIIb	100	9	45	15	5	5	81	4	7
2, IIIc	100	64	4	94	25	61	2.5	16	89
3, IIIb	100	1	2	1	1	1	1	1	42
3, IIIc	100	107	1	69	12	9	1	41	96
4	100	113	6	108	7	79	2	76	75

2.2.2.3 The FGF-Heparin Interaction and its Role in FGFR Activation

A defining feature of FGFs is their interaction with heparin or glycosamino-glycan heparan sulfate. Heparin is a polymer composed of a disaccharide chain of alternating iduronic acid and glucosamine moieties and each unit may contain a total of three sulfate groups that convey a strong negative charge. The glycosaminoglycan heparan sulfate by contrast, also contains glucuronic acid moieties, but otherwise is structurally similar to heparin (Faham, Hileman et al., 1996). Specific heparin-binding domains in FGFs are required for a strong binding to heparin. It is suggested that these binding sites are composed of groups of basic amino acids and are either already existent in the primary sequence or brought together by the secondary structure of the folded polypeptide (Baird, Schubert et al., 1988; Faham, Hileman et al., 1996).

The binding of Fibroblast Growth Factors to these extracellular polysaccharides may have several functional consequences. It was shown that the interaction stabilizes FGFs by preventing thermal denaturation and proteolysis and besides creates a local reservoir of growth factors which is required for effective activation of FGF-receptors (Damon, Lobb et al., 1989; Gospodarowicz & Cheng, 1986; Ornitz & Itoh, 2001). FGF binding proteins in general, increase the bioavailability of FGFs and might facilitate the receptor activation (Tassi, Al-Attar et al., 2001). The formation of a local reservoir implies that the signalling pathway could be spatially regulated and besides a large supply of FGFs may be mobilised from it.

The mobilization from the ECM may itself be highly regulated thereby allowing an indirect control of signalling. For the presentation of the FGFs to a particular population of cells, the growth factors have to be localized in order to be useful in organised development and prevention of diffusion (Cohn, Izpisua-Belmonte et al., 1995). This kind of regulation may be specifically important in the process of angiogenesis, which is known for its complex regulation due to a variety of positive and negative factors. For the release of the FGFs from the ECM there are currently two proposed mechanisms, namely the enzymatic cleavage of ECM components or by the binding to a carrier protein (FGF-BP), which then can deliver bound growth factors to their receptors.

Concerning the FGF-Receptor activation, the current understanding is that FGFs induce receptor dimerization thereby activating their receptors, and that this dimerization is highly facilitated by glycosaminoglycan heparan sulfates and heparin (Powers, McLeskey et al., 2000). This facilitation may occur by the recruitment of FGFs to the cell surface thereby increasing their concentration and by inducing a conformational shift in either FGFs or FGF-Receptors (Baird & Klagsbrun, 1991; Klagsbrun & Baird, 1991). Recently very similar models have been established to explain the HLGAG (Heparin-like Glycosaminoglycan) -FGF-FGFR complex.

In these independently proposed models a (2xFGF:2xFGFR) tetramer is stabilized by HLGAG (heparin-like glycosaminoglycan) binding across this tetramer (Figure 2.10). The IgII and IgIII domains wrap around one FGF molecule in each of the FGF-FGFR pairs.

Together with the other pair, a highly positive canyon at the IgII-FGF interface is formed, in which a single HLGAG can bind. The models differ in a few possible interactions, all three however, have in common the interactions between HLGAGs and both FGFs and FGFRs, as well as the numerous interactions between FGFs and their receptors (Davis, Venkataraman et al., 1999; Plotnikov, Schlessinger et al., 1999; Stauber, DiGabriele et al., 2000; Venkataraman, Raman et al., 1999; Venkataraman, Shriver et al., 1999).

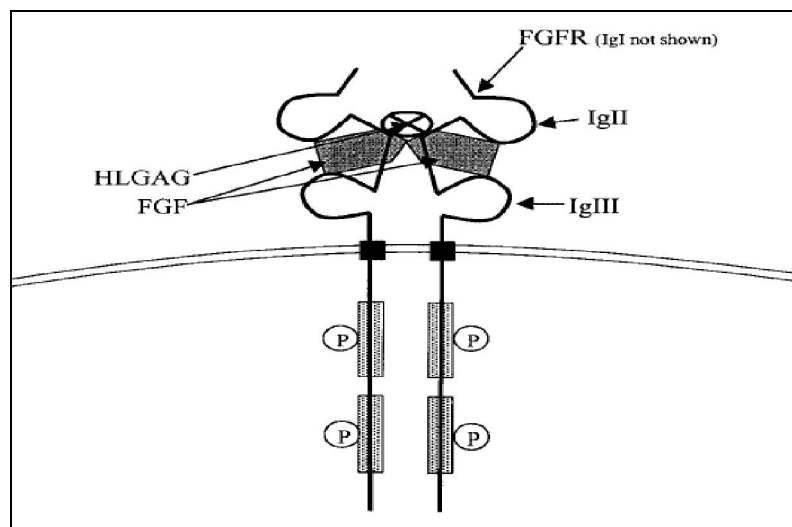


Figure 2.10: Shows the proposed model for the HLGAG-FGF-FGFR complex (Powers, McLeskey et al., 2000).

2.2.2.4 FGF-Receptor Activation, Auto-Inhibition and Signal Transduction

After the ligands are bound and have induced dimerization, the receptors are capable of phosphorylating specific tyrosine residues on their cytoplasmic tails. The phosphorylation can take place either on their own cytoplasmic tail or on the neighbouring one, which can be seen as auto- and transphosphorylation respectively (Lemmon & Schlessinger, 1994; Schlessinger & Bar-Sagi, 1994).

FGF-Receptor dimerization and subsequent transphosphorylation is not restricted to homodimers but can take place also between heterodimers (Bellot, Crumley et al., 1991). This increases complexity of the FGF signalling significantly and opens further opportunities for signal regulation.

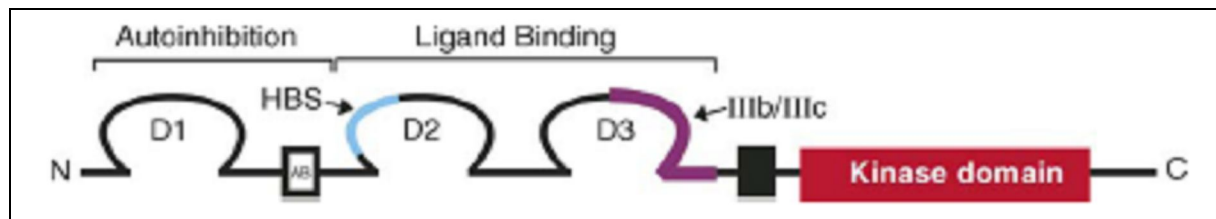


Figure 2.11: Schematic diagram of the FGFR. Regions involved in the mechanism of auto-inhibition as well as ligand binding are indicated. IgI-IgIII are shown as D1-D3 (Mohammadi, Olsen et al., 2005).

FGF signalling components are mainly co-localized which implicates the existence of a tight auto-inhibitory control. It is suggested, that this mechanism is conveyed by the IgI domain as well as by the IgI/IgII linker region containing the acidic box (AB), a stretch of acidic amino acids (Figure 2.11). In this proposed model, the flexible nature of the IgI/IgII linker region allows electrostatic interaction between the AB region and the highly basic heparin binding site (HBS) of IgII, thereby occluding heparin binding (Figure 2.12). This interaction results in a disposition of IgI, which then starts interacting with the IgII and IgIII region, leading to occlusion of the FGF binding interface. It has been shown, that the progression of pancreatic cancers towards a more malignant state correlates with an alternative splicing switch of FGFR-1, which leads to the expression of isoforms lacking the IgI domain as well as the AB region (Mohammadi, Olsen et al., 2005).

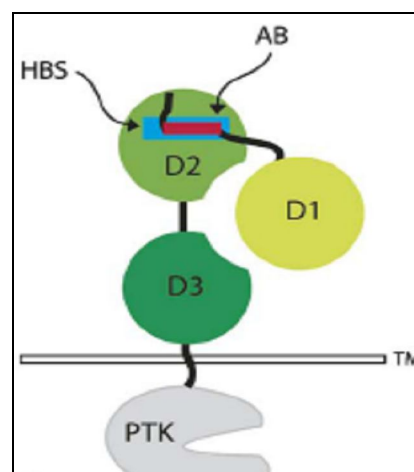


Figure 2.12: Model for FGFR auto- inhibition. IgI-IgIII are shown as D1-D3 (Mohammadi, Olsen et al., 2005).

Once tyrosine residues are phosphorylated, the signal is passed on by the recruitment of proteins that recognize and bind to phosphotyrosine residues through their Src-homology 2 (SH2) domains.

These proteins can either function as adaptors, thus recruiting further target proteins by their own specific binding sites, or they become phosphorylated themselves by the receptor kinase. In general the propagation of the signal is achieved by induction of a conformational change in target proteins which results in the activation of a variety of catalytic sites within these proteins (Pawson, 1995a; Pawson, 1995b).

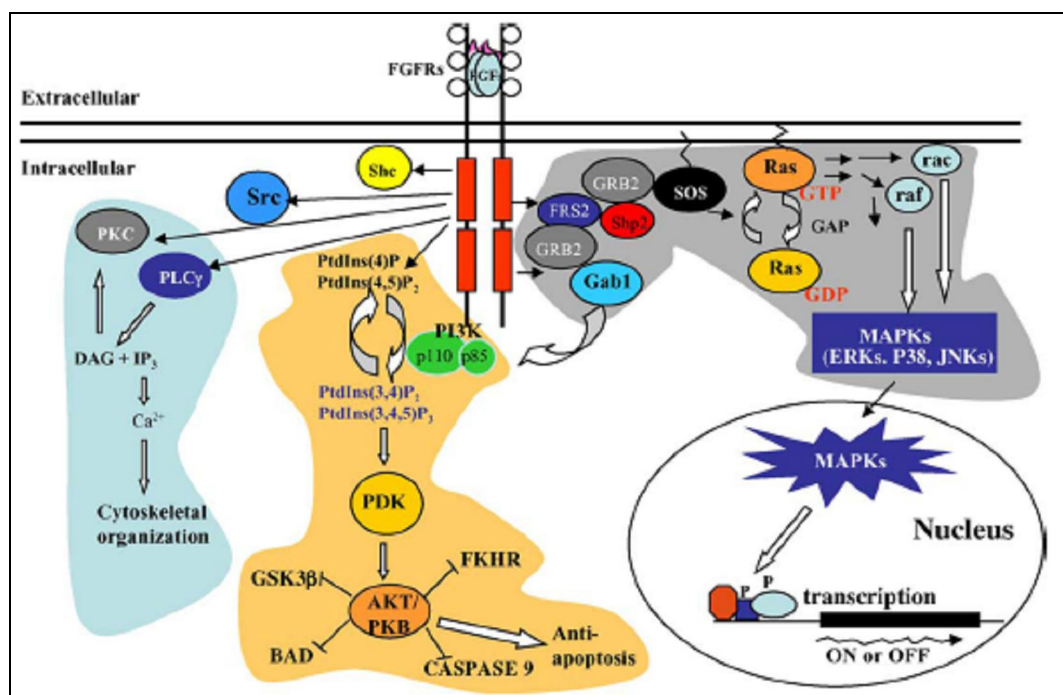


Figure 2.13: Suggested transduction pathways by the cytoplasmic tails of activated FGF-Receptors (Dailey, Ambrosetti et al., 2005).

Activated tyrosine-kinase substrates, often kinases themselves, can then pass on the signal to further targets (Figure 2.13). There are quite a lot of different downstream targets and adaptors of many possible transduction pathways which are frequently linked to each other thereby leading to various cell responses (Pawson, 1995a; Pawson, 1995b; Powers, McLeskey et al., 2000). It is suggested that different FGFRs activate quite similar signalling pathways (Table 2.4 and Figure 2.14), because of the high degree of homology at the amino acid level (Johnson & Williams, 1993).

Table 2.4: Possible FGFR-mediated pathways.

PLCγ signaling pathway	Phospho-lipase C γ (PLC γ) gets activated by binding to Tyr766 of FGFR-1 with its SH2-domain (Mohammadi et al 1992). Then cleaves Phosphatidyl Inositol-4,5-Bisphosphate (PIP2) to Inositol-Triphosphate (IP3) and Diacylglycerol, that remains membrane bound. IP3 is able to diffuse to the endoplasmic reticulum, where it facilitates calcium release by binding to specific calcium channels. Both, DAG and released calcium, are activating Protein-Kinase C (PKC) (Burgess, Dionne et al., 1990).
Src signaling pathway	Src is thought to link FGFR signalling to cortactin, which is a focal adhesion-associated protein that binds filamentous actin. Src itself is a non-receptor tyrosine kinase. This suggested pathway would be an alternative to the PLC γ signalling for FGFR mediated alterations of the cytoskeleton (Wu, Ma et al., 1994; Zhan, Plourde et al., 1994).
Crk-mediated signaling	Crk is suggested to link FGF-Receptors to downstream signalling molecules like Shc, C3G and Cas. Crk possesses SH2/SH3-domains and functions as adaptor protein. Its recruited proteins are thought to propagate a mitogenic signal from FGFRs. However, may not have effects concerning cell motility (Larsson, Klint et al., 1999).
SNT-1/FRS-2 signaling pathway	It was also shown, that FGF-Receptors lacking all non-catalytic tyrosine residues were able to phosphorylate a novel protein identified as SNT-1 or FRS-2. This findings provide an alternative pathway to the phosphotyrosine recruitment of SH2-containing proteins. Two groups, Whang and Kouhara, independently showed the linkage to the Ras/MAPK pathway by SNT-1/FRS-2. Activation of SNT-1/FRS-2 leads to the recruitment of Grb-2/Sos adaptor protein, which in turn recruits Ras (Kouhara, Hadari et al., 1997).

In the absence of FGFs there must be also a mechanism that prevents FGFR dimerization and activation. A proposed model suggests that the binding of divalent cations and HLGAGs to an extracellular receptor sequence, a region which promotes self-association, may inhibit the dimerization by preventing direct interaction between these regions. These extracellular domains, that are thought to promote receptor dimerization, are located between IgII and IgIII and range from Glu¹⁶⁰ to Lys¹⁷⁶ (Kan, Wang et al., 1996; Wang, Kan et al., 1997).

	FGFR-2	FGFR-3	FGFR-4
Signal peptide	43%	17%	20%
Ig domain I	40%	27%	19%
Acidic region	43%	33%	27%
Ig domain II	79%	64%	61%
Ig domain III	78%	81%	74%
Transmembrane domain	62%	33%	24%
Kinase 1	88%	83%	75%
Kinase 2	92%	91%	84%
C-tail	62%	46%	42%

Figure 2.14: The human FGF-Receptors 2-4 compared to the FGFR-1 at the amino acid level (Johnson & Williams, 1993). Note the high degree of homology concerning the kinase domains.

2.2.2.5 Biological Functions of the FGF Signalling Pathway

It has been shown that FGF signalling plays a significant role in wound healing and angiogenesis. The progress of wound repair can be classified into four phases, namely inflammation, contraction, repair and regeneration. It is suggested that FGFs play a role in at least inflammation, repair and regeneration. The initial inflammatory response is characterized by an invasion of blood platelets and other cells from the hematopoietic cell lineage that play a role in immune response. This is followed by an accumulation of myofibroblasts, which are suggested to differentiate from pericytes or mesenchymal stromal cells. The myofibroblasts bind to the injured site and start to contract, significantly reducing the area that has to be repaired. Afterwards a granulation tissue starts to form, that can be seen as a chief hallmark of the repair phase. The granulation tissue is a richly vascularized connective tissue and is characterized by excessive migration of fibroblasts and endothelial cells through a non-collagenous extracellular matrix that leads to the formation of new capillaries. At last collagen will be deposited and newly formed blood vessels will become organized. The repair phase is then followed by regeneration, where lost epithelial cells are replaced (Powers, McLeskey et al., 2000).

It has been shown, that various growth factors, including FGFs, are released due to mechanical force from endothelial cells. Thus the release of FGFs and tissue repair is in fact stimulated by the wound itself (McNeil, Muthukrishnan et al., 1989). FGFs are stored in fibroblasts (Werner, Roth et al., 1991) and endothelial cells (McNeil, Muthukrishnan et al., 1989), but also in the extracellular matrix. However, there have to be some other signals, except for mechanical stress, for the release of FGFs from the stroma and endothelium, because angiogenesis does not exclusively happen due to mechanical injury (Powers, McLeskey et al., 2000).

Some FGFs are able to stimulate the proliferation of lymphocytes (Byrd, Ballard et al., 1999), may regulate the production of blood platelets and are suggested to play an important role in the migration of inflammatory cells (Huber, Zhou et al., 1998; Konishi, Ochiya et al., 1996). It is thought to be mainly the powerful proliferative effect, that FGFs have on fibroblasts and endothelial cells, which stimulates tissue repair (Powers, McLeskey et al., 2000). However, facilitation of cell migration through the extracellular matrix by regulating proteolysis and adhesion molecules may also facilitate the repair process (Besser, Presta et al., 1995; Coltrini, Gualandris et al., 1995; Gualandris & Presta, 1995; Presta, Urbinati et al., 1995). Considering the last step of wound healing, namely regeneration of the damaged tissue. FGF-7 was found to be highly expressed in interstitial cells and the corresponding FGFR-2 (IIIb) in the tubular epithelium, after chemically induced renal tubule damage (Ichimura, Finch et al., 1996). This suggests, that FGFs are probably facilitating epithelial regeneration by a paracrine loop (Powers, McLeskey et al., 2000).

FGFs play a major role in development, particularly in gastrulation, the formation of all three germ layers (Hebert, Boyle et al., 1991; Paterno, Gillespie et al., 1989; Sun, Meyers et al., 1999) and in organogenesis. Their importance concerning organogenesis, was shown especially in that of the lung, limb and the nervous system (Crossley, Martinez et al., 1996; Crossley, Minowada et al., 1996; Sekine, Ohuchi et al., 1999; Ye, Shimamura et al., 1998).

It is known, that FGFs can have diverse effects on different cell types. In some cells, activation of FGFRs leads to proliferation, migration or differentiation, while in other cell types leads to cell cycle arrest, apoptosis or inhibition of differentiation (Boilly, Vercoutter-Edouart et al., 2000). Moreover, the cellular response to FGFs can also differ for a specific cell type in a stage-dependent manner. Immature osteoblasts, for instance, respond to FGF stimulation by proliferation, while in a later stage of development it leads to apoptosis (Mansukhani, Bellosta et al., 2000). The phenomenon, in which signals can have different effects, depending on cell type and maturational stage, is called “competence”. The molecular reasons for that, are thought to be differential presence or activity of FGF signalling components, which can be regulated also by other signalling pathways, making the mechanism more dynamic as well as more complex (Dailey, Ambrosetti et al., 2005).

2.2.3 The FGF Signalling Pathway in Cancer

The binding of FGFs to their receptors induces a variety of signal transduction pathways, that regulate many different transcription factors involved in cell cycle progression and inhibition of apoptosis. This consequently leads to excessive cell growth and development of neoplasms. In general, FGFs promote cell growth by at least one of three mechanisms, by mitogenesis, promotion of angiogenesis or by inhibition of apoptosis (Powers, McLeskey et al., 2000). The FGF signalling system also plays an important role in development of metastatic cancer by changing cell adhesion, migration and invasiveness (Cavallaro, Niedermeyer et al., 2001; Cavallaro, Tenan et al., 2001; Suyama, Shapiro et al., 2002), mainly by interaction with adhesion molecules.

2.2.3.1 Overexpression of FGFs

In general, there are two possibilities of sources over-expressing FGFs, surrounding stromal cells or the tumour cells themselves. At the same time both sources may also act as target cells, thus FGFs can function in an autocrine or/and in a paracrine manner (Table 2.5). The tumour may secrete FGFs due to mutation, leading to over-expression, or due to induced over-expression by signals secreted from non-transformed stromal cells (Takahashi, Fukumoto et al., 1992; Takahashi, Mori et al., 1990; Takahashi, Hoshimaru et al., 1992). On the other hand, FGFs may be secreted by stromal cells, where their expression is activated by signals from tumour cells (Samaniego, Markham et al., 1998). Thus surrounding non-transformed stromal cells may also change their expression levels, however not due to mutations but as response to tumour secreted factors. What's more, FGFs may also act in an intracrine manner. Some do not possess a signal sequence for the classical secretion pathway, thereby remain in the cytoplasm or may be secreted by an alternative pathway as already mentioned. Others possess a nuclear localisation sequence instead, mediating their nuclear translocation. FGFs are probably able to directly act on transcription factors or their regulators, without secretion and binding to cell surface receptors (Delrieu, 2000; Joy, Moffett et al., 1997; Stachowiak, Maher et al., 1997).

Table 2.5: Examples of known cancer related functions of FGF-2 and FGF-5. These two are shown, because they are among the best investigated and are highly up-regulated in melanoma, see also expression analysis in the Results and Discussion sections.

FGF-2	Its expression has been shown in over 94% of human gliomas, but has not been detected in normal brain (Takahashi, Fukumoto et al., 1992; Takahashi, Mori et al., 1990); when released by tumour cells, it may function as autocrine growth factor and paracrine angiogenic factor, by binding to FGF receptors on the tumour itself as well as on surrounding cells; correlates with the degree of malignancy and vascularity in human gliomas (Takahashi, Fukumoto et al., 1992; Takahashi, Hoshimaru et al., 1992); in Kaposi's sarcoma the expression in transformed cells seem to be induced by surrounding non-transformed stromal cells by release of TNF- α , IL-1 and Interferon- γ (Samaniego, Markham et al., 1998); its translocation to the nucleus is suggested to stimulate S-phase entry (Stachowiak, Maher et al., 1997); appears as 18 kDa protein, localising in the cytoplasm and shown to be efficiently secreted, as well as high molecular weight (HMW) isoforms, mainly accumulating in the nucleus and having different effects on the phenotype (Delrieu, 2000);
FGF-5	Has not been detected in fibroblasts of healthy pancreatic tissue, however in cancer-associated macrophages and fibroblasts (Kornmann, Ishiwata et al., 1997); the expression can be activated in fibroblasts due to action of other growth factors, like EGF, PDGF and TGF- α , which are indeed present in human pancreatic cancer tissues (Ebert, Yokoyama et al., 1995; Korc, Chandrasekar et al., 1992; Werner, Werner-Felmayer et al., 1991; Werner, Roth et al., 1991);

2.2.3.2 Increased Availability of FGFs

Apart from FGF over-expression by tumour cells or surrounding non-transformed cells, it may also be an increased mobilization of FGFs from the ECM (Figure 2.15), that leads to a dysregulated signalling pathway in cancer. The release may take place by an enzymatic cleavage of ECM components, by heparanases or proteases, or by binding to carrier proteins, which are then able to deliver freed factors to their receptors (Rak & Kerbel, 1997).

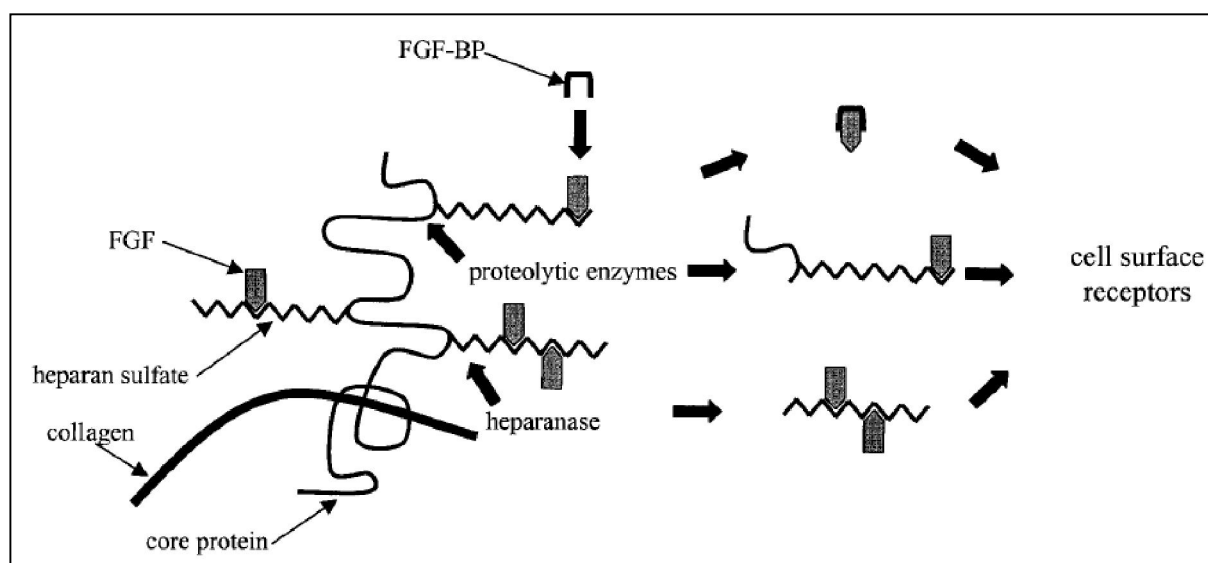


Figure 2.15: Possible mechanisms for FGF mobilization from the ECM (Powers, McLeskey et al., 2000).

The expression of the heparanase gene was shown at the mRNA and protein levels in samples of human liver, colon and breast carcinomas as well as in metastatic human and rat cell lines. Melanoma cells, transfected with heparanase cDNA, were able to metastasize to liver and lung (Hulett, Freeman et al., 1999; Vlodavsky, Friedmann et al., 1999), compared to untransfected cells. Heparanases seem to play an important role in metastasis and angiogenesis due to mobilisation of FGFs from the ECM (Powers, McLeskey et al., 2000).

The same effects are thought to be achieved by the action of proteases. It has been shown *in vitro*, that FGF-2 bound to the ECM can be mobilized by plasmin, which is secreted by bovine capillary endothelial (BCE) cells. In turn, FGF-2 is able to increase the plasminogen-activator activity in cultured BCE cells providing a positive feedback loop (Saksela & Rifkin, 1990). In contrast to enzymatic degradation of the ECM, bound FGFs may be also mobilized by FGF-binding proteins (FGF-BPs).

The 17kDa FGF-BP was originally isolated from a human epidermoid carcinoma cell line, and was shown to bind covalently and reversibly to FGF-1 and FGF-2 (Wu, Kan et al., 1991). Over-expression of FGF-BP in human adrenal adenocarcinoma cell lines resulted in a malignant phenotype (Czubayko, Smith et al., 1994).

The binding protein is known for its tight regulation during development, it is downregulated in adult tissues and was shown to be frequently up-regulated in early stages of carcinogenesis (Czubayko, Liaudet-Coopman et al., 1997; Kurtz, Wang et al., 1997). Concerning cancer development, these observations indicate, that regulation of the FGF mobilization from the ECM might be as important as regulation of FGF production.

2.2.3.3 Alterations of FGF-Receptors

Deregulation of the FGF signalling pathway may also result from alteration of FGFRs. This receptor alterations can happen in different ways, namely due to point mutations, change in expression, alternative splicing and genomic alterations. However, not all alterations could be shown to be associated with cancer development.

Activating point mutations in FGFRs have been found in various developmental defects (Chun, Siegel-Bartelt et al., 1998; Gripp, Stolle et al., 1998; Li, Mangasarian et al., 1997; Mangasarian, Li et al., 1997; Webster, D'Avis et al., 1996; Webster & Donoghue, 1996) and have been implicated in the development of human cancers. These point mutations result in a ligand-independent activation, and are thought to occur in the intracellular kinase domains as well as in extracellular and transmembrane domains of FGFRs (Neilson & Friesel, 1996). Alternative splicing has been shown to occur with FGFR-3, leading to a receptor protein lacking its transmembrane domain, but retaining an intact kinase domain and accumulating in the nucleus (Johnson & Allen, 1995). However, the role in human cancers is unknown, a novel autocrine pathway in the nucleus has been suggested (Powers, McLeskey et al., 2000). Beside point mutations, also gene rearrangements have been shown to lead to ligand-independent activation of FGFRs. Gene fusion of FGFR-2 with a novel gene, called FGFR activating gene 1 (FRAG-1), leads to a constitutively active form of FGFR-2, found in a rat osteosarcoma cell line.

The fusion protein is thought to form constitutive dimers, which results in auto-phosphorylation of FGFR-2 kinase domains (Lorenzi, Horii et al., 1996). In human myeloid cells, a chromosomal translocation has been shown to lead to increased expression of FGFR-3 resulting in the activation of an FGF-4 dependent autocrine loop (Otsuki, Yamada et al., 1999).

3 Materials and Methods

3.1 Cell lines and Media

The cell culture unit of the Institute of Cancer Research supplied all melanoma cell lines. Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum (FBS) was used for all cell lines. RPMI medium was purchased from Sigma Aldrich and uses, like most other media, a hydrogen-carbonate buffer system in a solution of glucose, salts, amino acids and vitamins. For indicating the pH-value phenol-red was added. FBS is used to supply cells with a variety of growth factors, hormones and trace elements to make cell survival in culture possible (Sigma Aldrich). Penicillin/streptomycin solution was added to the medium only during selection of stable transfectants, was bought from PAA and used at an end-concentration of 1%.

Table 3.1: Used cell lines, including VM (Vienna Melanoma) numbers, origin^a and histology^b.

Cell Line	Origin	Histology	Cell Line	Origin	Histology
Mela	PM		VM-37 Xfoa	SC	NM
VM-10 Jmum	PT	SSM	VM-18 Pnjc	SC	ALM
VM-19 Rall	PT	SSM	VM-34 Xbne	SC	Unknown
VM-30 Wcre	PT	SSM	VM-35 Xdbc	SC	Unknown
VM-36 Xewl	PT	SSM	VM-9 Gyk	BN	SSM
VM-44 Xapg	PT	SSM	VM-22 Rima	BN	NM
VM-2 Gcrf	PT	NM	VM-46 Huma	BR	NM
VM-7 Gtbs	PT	NM	VM-47 Host	BR	NM
VM-21 Rhtp	PT	NM	VM-48 Kaka	BR	NM
VM-23 Rktj	PT	NM	VM-50 Hoin	BR	NM
VM-25 Tpck	PT	NM	VM-28 Tmfi	BR	Unknown
VM-32 Wpza	PT	NM	VM-41 Ydfr	BR	Unknown
VM-33 Wubi	PT	NM	VM-31 Wltj	ME	NM
VM-38 Xiba	PT	NM	VM-11 Jtst	ME	NM
VM-5 Grbd	LN	SSM	VM-13 Krfm	ME	NM
VM-1 Ftsl/a	LN	SSM	VM-16 Myah	ME	Unknown
VM-20 Rgoh	LN	NM	VM-3 Gesp	ME	Unknown
VM-4 Glj	LN	NM			
VM-6 Gsta	LN	NM			
VM-8 Gubs/a	LN	NM			
VM-12 Jwmw	LN	NM			
VM-14 Lcwc	LN	NM			
VM-15 Mjzj	LN	Unknown			
VM-17 Nra	LN	Unknown			
VM-24 Shtj	LN	Unknown			

^aEstablishment of cell lines from primary tumours (PT), lymph node (LN), subcutaneous (SC), bone (BN), brain (BR) metastases, malignant effusions (ME) of malignant melanoma and primary melanocytes (PT), from which cDNA was used as control and was obtained from primary culture only. ^bHistology of the primary tumour: ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.

3.2 Standard Growth Conditions

All melanoma cell lines were grown in a humidified incubator (Forma Scientific) at 37°C and 5% CO₂. Tissue culture flasks (T25 (=25cm²), T75 (=75cm²) and T175 (=175cm²)) were bought from Greiner Bio-One as well as from BD Falcon. 6-/12- and 24-well plates were bought from IWAKI, while 15cm tissue culture dishes were bought from Sarstedt and 96 well-plates from TPP. The medium used for cell culture always contained 10% FBS, except where indicated otherwise. Generated cell lines stably expressing the *TetR* protein, used for generation of inducible expression by the Tet-Off Advanced System (Clontech), were always held under doxocyclin (100 ng/ml), thereby preventing expression of cell toxic genes of interest (See Selection of Stable Transfectants).

3.3 Splitting

All melanoma cells in culture grew adherent, thus the medium was aspirated and cells were washed once with PBS (2.7 mM EDTA (Merck), 137 mM NaCl (Merck) and 10 mM Na₂HPO₄ (Merck); pH 7.4). Trypsin/EDTA (T/E; 0.01% EDTA (Fluka), 0.1% Trypsin (Difco)) was added onto the cell layer and incubated at 37°C for about 5 minutes, the progress of detachment was monitored using the microscope. Cells were resuspended in medium and the desired number of cells was transferred into a new dish or bottle, containing fresh medium.

3.4 Cell Counting

Cells were counted by using either a Casy Cell Counter (Schärfe) or a Neubauer Chamber (Phelan, 2007; Paul Marienfeld GmbH).

3.5 Freezing

Cells were grown in 15 cm tissue culture dishes. When dishes were nearly confluent, cells were trypsinized and resuspended in 4 ml medium plus 10% FBS. If cells were on selection for stable transfectants, the medium also contained the corresponding selection reagent as well as penicillin and streptomycin. 1ml of cell suspension was transferred into cryotubes (Greiner) and DMSO (dimethylsulfoxid) was added to a final concentration of 50 µg/ml. The suspension was immediately put on ice and cooled down in a styrofoam container at –80°C for at least 24 hours, but not longer than 72 hours. Afterwards, cells were transferred into a liquid nitrogen tank for long term storage.

3.6 Isolation of RNA

Medium was discarded, and cells were washed with PBS once. Subsequent steps were done on ice. Trifast (Peglab) or Trizol (Invitrogen) was added onto the cell layer or cell pellet, if cells were trypsinized and centrifuged before harvest (1.5 ml volume was used for 25 cm² confluent cell layer or for 20 x 10⁶ cell pellet). In case of isolating RNA from a cell layer, the cells were scraped into suspension.

Cells were transferred into 2 ml Eppendorf tubes. For lysis, cells were vortexed and incubated in Trifast solution for about 5 minutes. For RNA extraction, chloroform/isoamylalcohol (50:1; 0.2 x vol. Trifast) was added, vortexed and incubated again for around 5 min. Cell waste was pelleted at 11.000 g at 4°C for 11 min. Aqueous supernatant, containing solubilized RNA, was transferred into new Eppendorf tubes and isopropanol (0.5 times vol. Trifast) was added, vortexed and again centrifuged at 11.000 g at 4°C for 11 min. Supernatant was discarded and 70% EtOH/DEPC (diethylpyrocarbonate-treated ethanol) was added onto the pellet (~500 µl), vortexed and centrifuged at 7.500 g at 4°C for 7 min. In general, DEPC treatment is used for RNase enzyme inactivation by covalent modification of histidine residues. The alcohol was removed, the pellet was air dried and taken up in EDTA solution in DEPC water (3 mM; 10-30 µl volume). As quality control, purified RNA was loaded onto agarose gel (1 µg RNA; 1% agarose gel) and separated. RNA was frozen at –20°C, or at –80°C for long term storage.

3.7 Determination of RNA concentration

The concentration of purified RNA was determined by OD₂₆₀ measurement on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

3.8 Synthesis of cDNA

1 µg RNA was diluted with DEPC water to 11 µl and incubated for denaturation at 70°C for 10min. It was put on ice and 9 µl cDNA Synthesis Master Mix (MM) was added to a total volume of 20 µl. Afterwards, the reaction was incubated for 1.5 hours at 37°C. The quality of cDNA was tested by performing standard RT-PCR of a “housekeeping gene” followed by agarose gel electrophoresis. cDNA was stored frozen at –20°C.

cDNA Synthesis Master Mix (1 x)

Solution (Concentration)	Volume (µl)
M-MLV RT buffer (5 x; Promega)	4
Hexanucleotides (20 ng/µl)	1
dNTPs (10 mM)	1
DTT (100 mM)	2
MMLV-RT (200 u/µl; Promega)	1
RNA (1 µg / 11 µl)	11
	20
	total volume

3.9 Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

The RT-PCR Master Mix (MM) was prepared on ice. 1 µl cDNA solution was pipetted into 0.2 ml PCR Tube Strips (Bio-Rad), then the MM was added and mixed. MyCycler™ Thermal Cycler (Bio-Rad) was used for RT-PCR. For PCR conditions, primer sequences and PCR fragments see tables below, Table 3.2 and Figure 3.1.

RT-PCR Master Mix (1 x)	Solution (Concentration)	Volume (µl)
	GoTaq colourless buffer (5 x; Promega)	5
	dNTPs (10 mM)	0.5
	Primer I (20 µM)	0.5
	Primer II (20 µM)	0.5
	GoTaq polymerase (5 u/µl; Promega)	0.125
	Water bidest.	17.375
	<i>cDNA</i>	1
		25
		<i>total volume</i>

RT-PCR Conditions	Gene of Interest	Dissociation	Annealing	Elongation	
	GAPDH	94	50	72	Temperature (°C)
		Time			Number of Cycles
		3'			1x
		50''	50''	50''	33x
				2	1x
	FGFRs	94	56	72	Temperature (°C)
		Time			Number of Cycles
		3'			1x
		50''	50''	50''	40x
				2	1x
	FGFR-4 (Arg/Gly SNP)	95	50	72	Temperature (°C)
		Time			Number of Cycles
		3'			1x
		30''	30''	30''	40x
				2	1x

Table 3.2: Primer sequences and expected sizes of FGFR PCR products, including identified receptor isoforms and their NM numbers. Alpha splice variants contain IgG-I domains, while beta variants do not possess extracellular IgG-I domains. IgG-III domains may differ in sequence in their C-terminal part, thus can be sub-classified into IgG-IIIb and -IIIc. Other splice variants are enlisted as associative names.

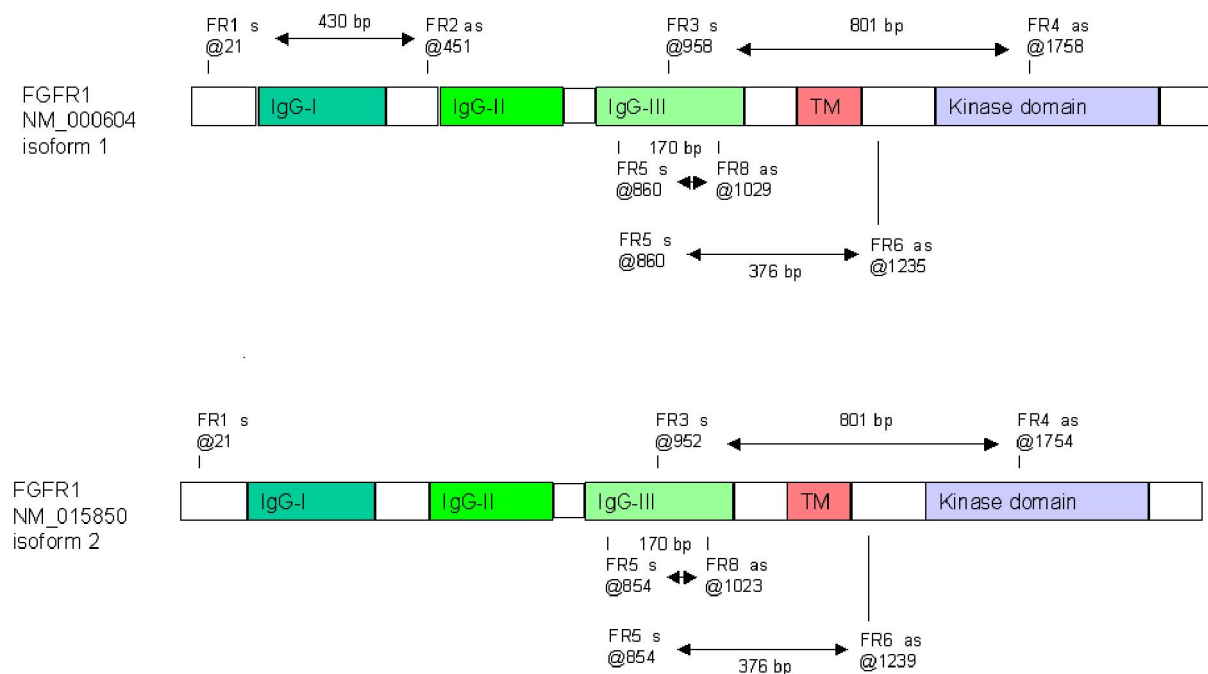
GENES	Primer-Names	Exons	Amplicon Length (bp)	NM Numbers	Splice Variants	Sequences
FGFR 1	FGFR 1 (A) s	2				CCTCTTCTGGGCTGTGCT
	FGFR 1 (A) as	4/5	164	NM 023105	beta	CGGGCATACGGTTTGGTT
			164	NM 023107	beta	
			431	NM 023109	alpha	
			431	NM 023111	alpha	
	FGFR 1 (B) s	9 IIIc				GACAAAGAGATGGAGGTGCT
	FGFR 1 (B) as	14	801	NM 015850	IIIc	GTTGTAGCAGTATTCACGCC
			801	NM 023105	IIIc	
			801	NM 023106	IIIc	
			801	NM 023109	IIIc	
			801	NM 023111	IIIc	
	FGFR 1 (B) Ex7 s	7				TCCAGTGGCTAAAGCACATC
	FGFR 1 (B) Ex10 as	10	376	NM 000604	IIIb+c	ATCTGGCTGTGGAAGTCACTC
			376	NM 015850	IIIb+c	
			376	NM 023105	IIIb+c	
			376	NM 023106	IIIb+c	
			376	NM 023109	IIIb+c	
			376	NM 023111	IIIb+c	
	FGFR 1 (B) Ex8 IIIb as	8 IIIb	~ 125	?	IIIb	CCGCATCCGAGCTATTAATC
	FGFR 1 (B) Ex9 IIIc as	9 IIIc	170	NM 000604	IIIc	CGCCAAGCACGTATACTC
				NM 015850	IIIc	
				NM 023105	IIIc	
				NM 023106	IIIc	
				NM 023109	IIIc	
				NM 023111	IIIc	

FGFR 2	FGFR 2 Ex2 s	2				GTCACCATGGCAACCTTGTC
	FGFR 2 Ex6 as	6	711	NM 000141	alpha	CACAACATCCAGGTGGTACG
			711	NM 022969	alpha	
			711	NM 022970	alpha	
			711	NM 022971	alpha	
			711	NM 022972	alpha	
			711	NM 022973	alpha	
			711	NM 022974	alpha	
			444	NM 022975	beta	
			711	NM 022976	alpha	
			711	NM 023028	alpha	
			444	NM 023029	beta	
			366	NM 023030	beta -Acidic box	
			366	NM 023031	beta -Acidic box	
	FGFR 2 s	5				AACGGGAAGGAGTTTAAGCAG
	FGFR 2 as	9 IIIc	504	NM 000141	IIIc	TGGCAGAACTGTCAACCATGC
			513	NM 022972	IIIc	
			504	NM 022973	IIIc	
			513	NM 023028	IIIc	
			504	NM 023029	IIIc	
	FGFR 2 (B) Ex8 IIIb as	8 IIIb	417	NM 022969	IIIb	CTCGGTACATTGAACAGAG
			417	NM 022974	IIIb	
			417	NM 022975	IIIb	
			417	NM 022976	IIIb	
			417	NM 023030	IIIb	
			417	NM 023031	IIIb	
	FGFR 2 (B) Ex10 as	10	598	NM 000141	IIIb+c	CCACCATACAGGCGATTAAG
			601	NM 022969	IIIb+c	
			607	NM 022972	IIIb+c	
			598	NM 022973	IIIb+c	
			601	NM 022974	IIIb+c	
			601	NM 022975	IIIb+c	
			607	NM 023028	IIIb+c	
			598	NM 023029	IIIb+c	
			601	NM 023030	IIIb+c	
			601	NM 023031	IIIb+c	

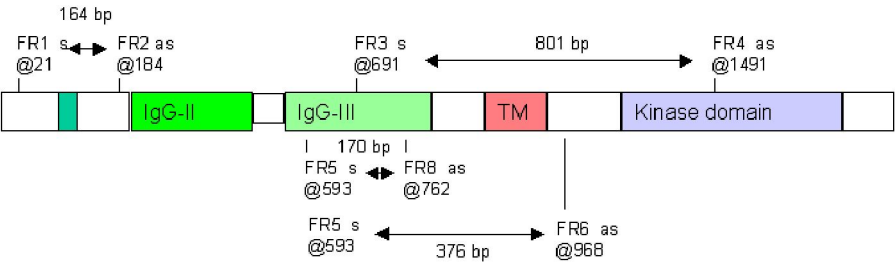
FGFR 3	FGFR 3 Ex1 s	1				AGTCCTTGGGGACGGAGCA
	FGFR 3 Ex5 as	5	672	NM_000142	alpha	CCAGCACGTCCAGCGTGTA
			672	NM_022965	alpha	
	FGFR 3 s	4				AACGGCAGGGAGTTCCGCGGC
	FGFR 3 as	9	531	NM_000142	IIIb+c	GTCAGCCTCCACCAGCTCCTC
	FGFR 3 (B) Ex10 as	10	720	NM_000142	all variants	GGACGCGTTGGAAGTCCAGGG
			384	NM_022965	del.Ex9 TransDom.	
	FGFR 3 (B) Ex7 IIIb as	7 IIIb	429	Gudrun Vari.	IIIb	CCCGTCCCCTCCGACACATTG
	FGFR 3 (B) Ex8 IIIc as	8 IIIc	435	NM_000142	IIIc	CCCGGCGTCCTCAAAGGTG

FGFR 4	FGFR 4 (A) Ex2 s	2				TGCCTGGGCCTCCAGTCTTG
	FGFR 4 (A) Ex5 as	5	486	NM_002011	Isoforms 1-3	CTGCAGCTGGACAGCGGAAC
			486	NM_213647		
			486	NM_022963		
			597	AF_359241	Ezzat soluble	
	FGFR 4 (B) Ex6 s	6				AGCACTGGAGTCTCGTGATG
	FGFR 4 (B) Ex10 as	10	766	NM_002011	Isoforms 1-3	CATAGTGGGTCGAGAGGTAG
			766	NM_213647	Isoforms 1-3	
			766	AF_359246	ptd del Ex1-6	
			646	NM_022963	soluble del Ex9	
	FGFR 4 s	5				GATGGACAGGCCTTTCATGG
	FGFR 4 as	9	525	NM_002011	Isoforms 1-3	TGCTGCGGTCCATGTGGGGTCCTC
			525	NM_213647		

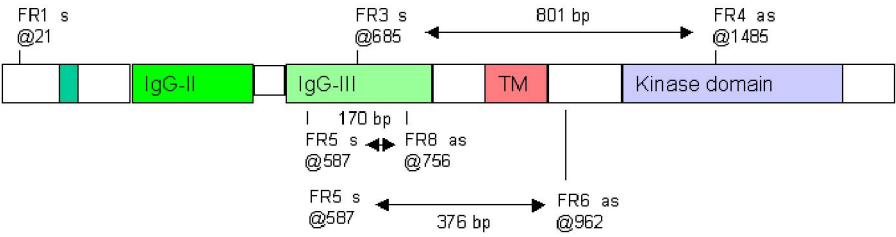
3.10 FGFR Splice Variants and PCR Fragments



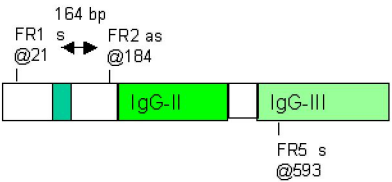
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isoform 3



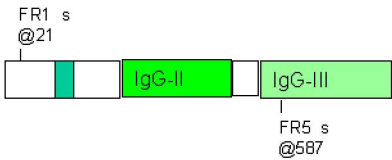
FGFR1
NM_023106
isoform 4



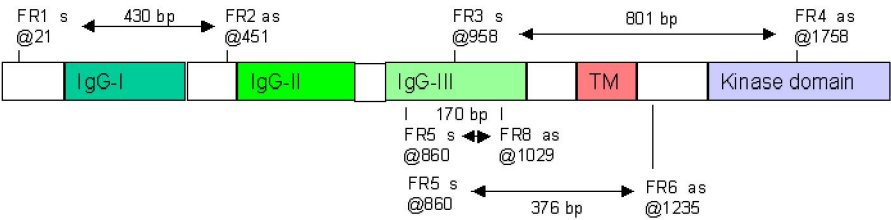
FGFR1
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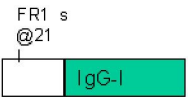
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isoform 6



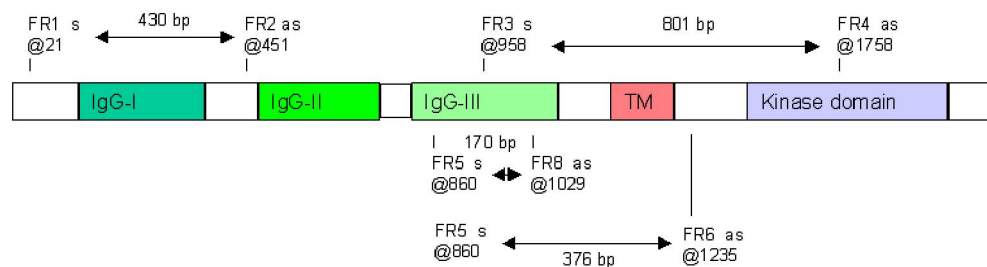
FGFR1
NM_023109
isoform 7
no tk function



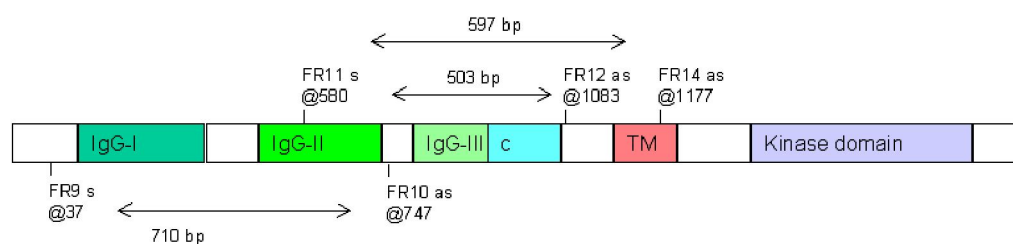
FGFR1
NM_023110
isoform 8



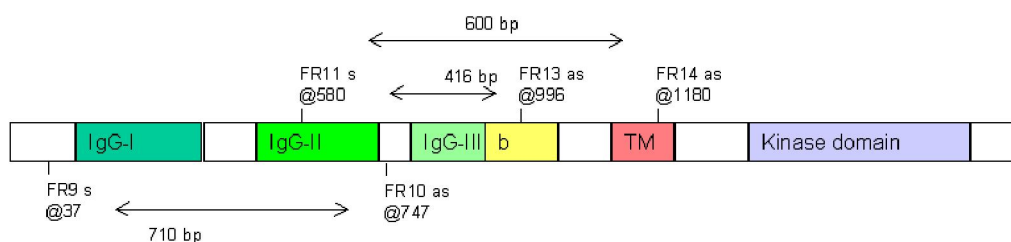
FGFR1
NM_023111
isoform 9
no tk function



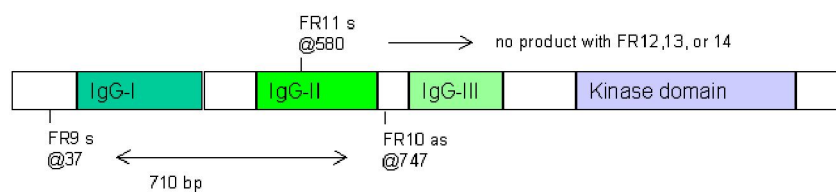
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isoform 1
NM_000141



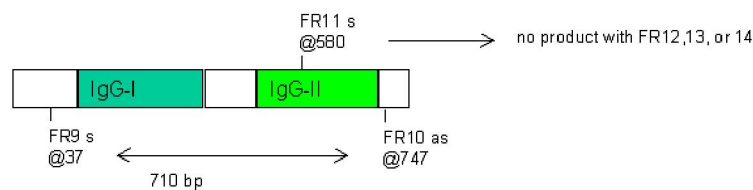
FGFR2
isoform 2
NM_022969

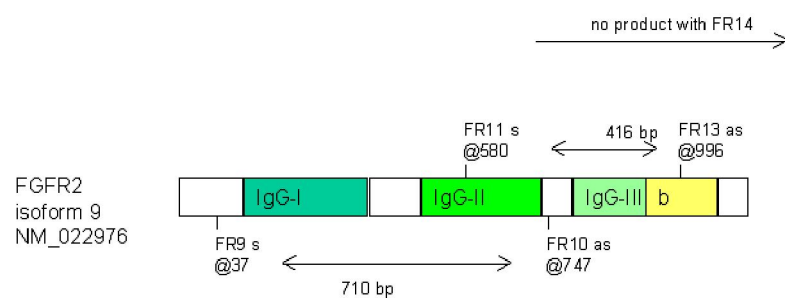
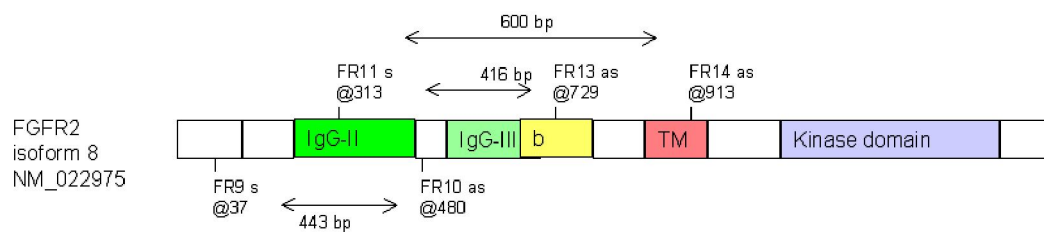
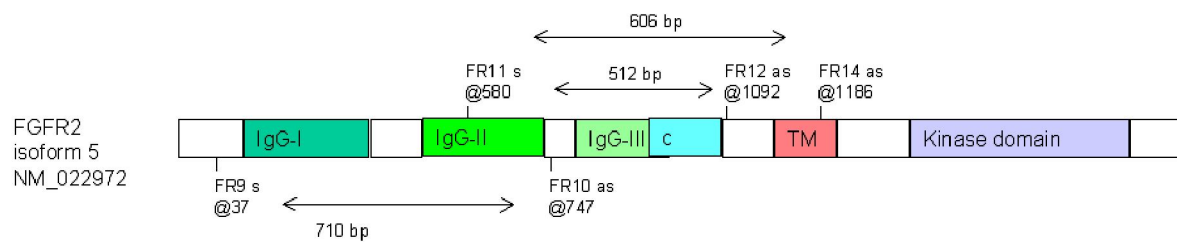


FGFR2
isoform 3
NM_022970



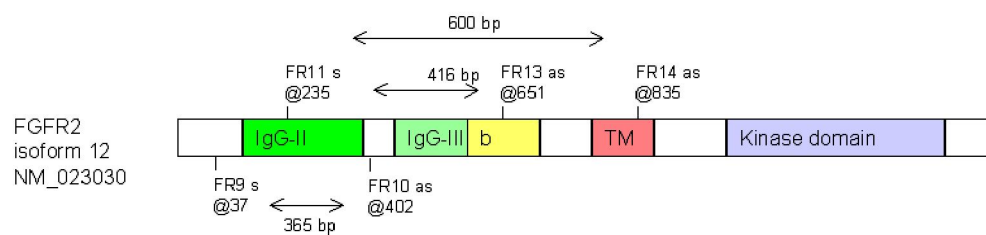
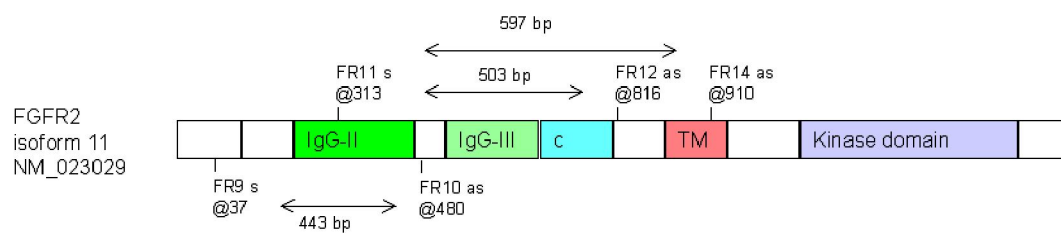
FGFR2
isoform 4
NM_022971



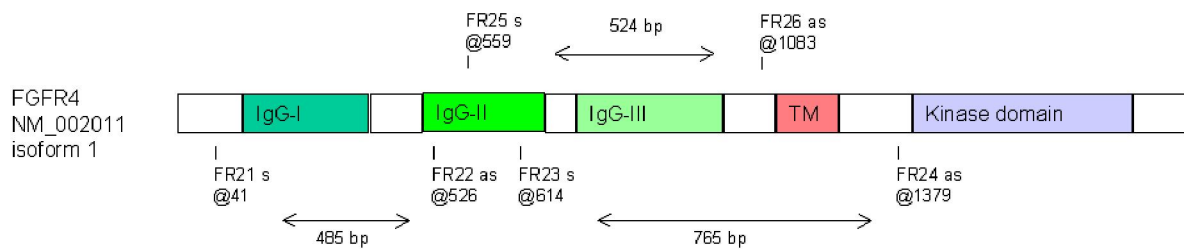


FGFR2 isoform 10
NM_023028

same as isoform 5 except a few nucleotides at C-terminus



same as isoform 12 except for deletion of GTAACA at position 939



uses alt splice site for exon 1 but encodes the same protein as NM_002011

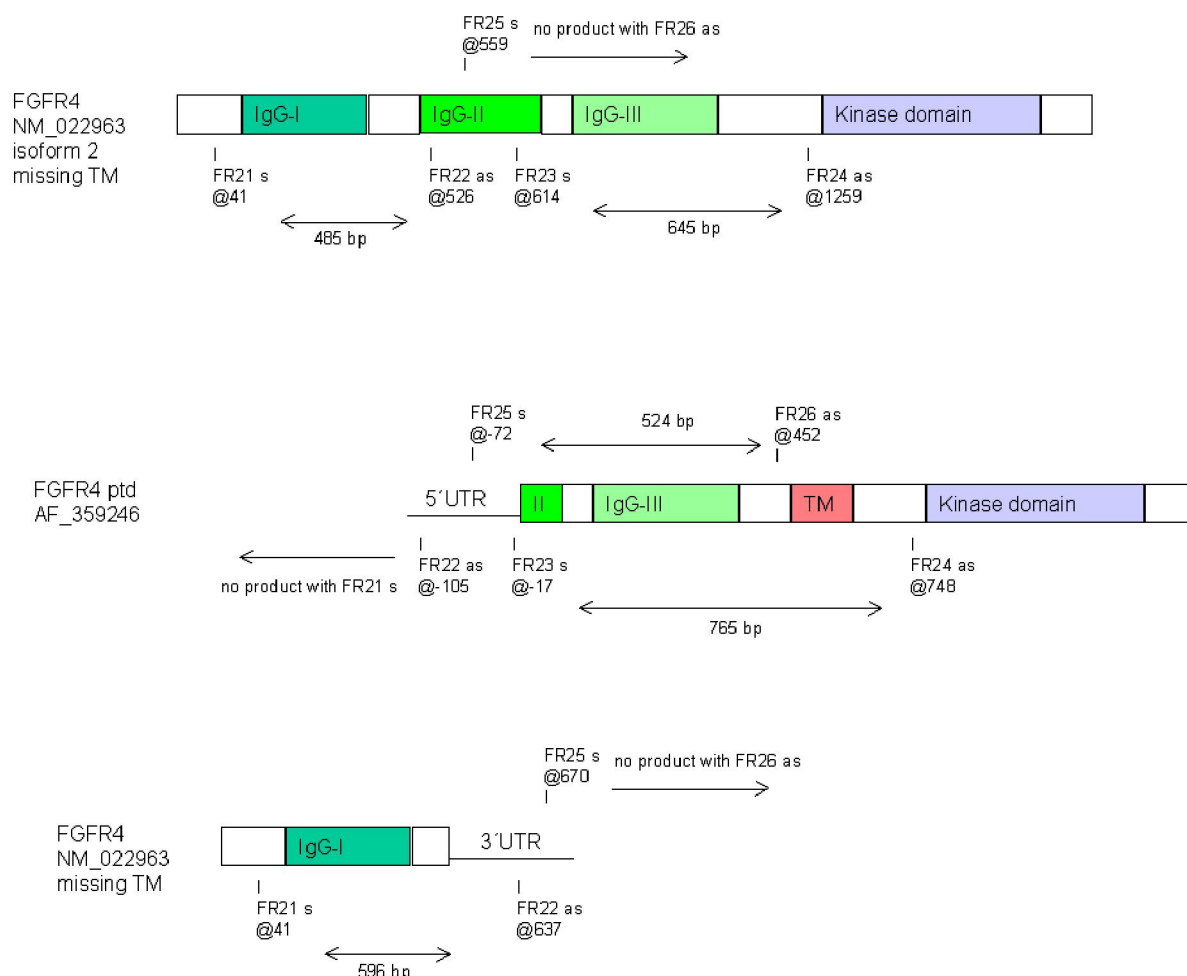


Figure 3.1: Schematic Representation of splice variants of all four FGF-Receptors, including binding sites of used primers and expected PCR products.

3.11 SYBR-Green quantitative RT-PCR (qRT-PCR)

SYBR-Green Master Mix was prepared on ice. 1 µl cDNA was pipetted into two wells (per sample) of an Optical 96-Well Reaction Plate (Micro Amp^R; Applied Biosystems). The master mix was added, and fluorescence was measured during PCR amplification by using an ABI Prism 7000 SDS thermocycler (ABI), according to ABI manuals.

SYBR-Green qRT-PCR Master Mix (1 x)	Solution (Concentration)	Volume (µl)
	Sensi Mix Plus (2 x Mix; Quantace)	10
	Primer I (20 µM)	0.2
	Primer II (20 µM)	0.2
	Water bidest.	8.6
	cDNA	1
		20
		total volume

3.12 TaqMan - “Real Time” quantitative RT-PCR (qRT-PCR)

TaqMan Master Mix was prepared on ice. 2 µl cDNA was layered into two wells (per sample) of an Optical 96-Well Reaction Plate (Micro Amp^R; Applied Biosystems), in case of 18S-rRNA, used for normalization, the cDNA was diluted 1:10. 10 µl Master Mix was added to a total volume of 12 µl, and mixed. PCR was performed and fluorescence measured after each cycle using an ABI Prism 7000 SDS thermocycler (ABI), according to ABI manuals. Taqman probes used in this study are listed in Table 3.3.

TaqMan qRT-PCR Master Mix (1 x)	Solution (Concentration)		Volume (µl)
	TaqMan Probes (Applied Biosystems)		0.625
	TaqMan Master Mix (Applied Biosystems)		6.25
	water bidest.		3.125
	cDNA		2
			12
			total volume

Table 3.3: Used Taqman-Probes for FGFR 1-4. Binding sites and recognized isoforms are described on the company webpage (Applied Biosystems).

Taqman-Probes	FGFR1	Hs00915135_m1
	FGFR2	Hs01552926_m1
	FGFR3	Hs00179829m_1
		Lot: 529030
		Hs00997397m_1
	FGFR4	Hs00608751g_1
		Hs00608744g_1
		Hs00242558_m1

3.13 Restriction Digest

Restriction digestion was performed in a total volume of 20µl. Master Mix, including restriction enzyme and corresponding buffer (Fermentas), was prepared on ice. 15 µl of the PCR product was added first, followed by 5 µl Master Mix and mixing. Incubation time and temperature were usually one hour and 37°C, unless different conditions were required for the enzyme as stated by the supplier (Fermentas).

Master Mix	1 µl Restriction Enzyme 2 µl Buffer 2 µl Water dest.
Restriction Digestion	15 µl PCR Product 5 µl Master Mix 20 µl (Tot.Vol.)

3.14 Gel Electrophoresis (Nucleic Acids)

Nucleic acids, thus RNA and DNA, were separated on agarose gels of 0.5-2% (w/v), depending on fragment sizes. Typical sizes of 1-3 kilobases (kb) were separated on gels consisting of 1% agarose (Biozyme).

Agarose was solubilised in 0.5 x TBE buffer (5.4 g/l Tris (Fluka), 2.75 g/l boric acid (Sigma) and 1 mM EDTA). In cases of desired high separation quality, nucleic acids were separated on poly-acrylamide gels ranging from 6-10% and run in 1 x TBE buffer. Samples were mixed with 6 x loading buffer (333 µl/ml 6 x loading dye (Fermentas), 250 µl/ml 80% glycerol (Merck), 66.5 µl/ml 0.5 M EDTA, 0.5 µl/ml 10 000 x Vistra Green (GE Healthcare)) and loaded onto the gel. Depending on expected fragment sizes, different markers were loaded.

Appropriate amounts of water and loading buffer were mixed with 2 µl of the respective marker. For agarose gels, 0.5 x TBE was used as running buffer and 1 x TBE for poly-acrylamide gels. Gels were run at constant voltage of 50 V for 10 min and raised afterwards to 120 V until the desired separation was achieved (gel apparatus: Bio-Rad). Fragment bands were visualized by *FluorImager 595 Scanner* from Molecular Dynamics.

Poly-Acrylamid Gel Electrophoresis (Nucleic Acids)	8.5% (1 gel)	
	2.4 ml	5x TBE
	3.45 ml	30% Acrylamid/Bis; 29:1 (Bio-Rad)
	6.84 ml	Water bidest.
	100 µl	APS
	10 µl	TEMED

3.15 Isolation of Proteins

For SDS-PAGE and Western-Blot analysis, all non-secreted proteins were isolated by using Lysis Buffer II (LBI). Cells were trypsinized and washed with cold PBS. Centrifugation was done at 800 rpm for 5 minutes. All successive steps were done on ice. Cells were lysed by adding LBI onto the cell pellet. Volume of lysis buffer depended on cell amount and usually ranged between 30 μ l for around 500 000 cells and 120 μ l for 2 million. The pellet was resuspended by pipetting up and down for several times, then the suspension was incubated for some minutes. Additionally the cell suspension was sonicated 3 times for 3 seconds (Bandelin). Insoluble components were removed by centrifugation at 4°C and 20 800 g for 10 minutes. Aqueous supernatant was transferred and stored at -20°C, for longer periods at -80°C.

Protein Lysis Buffer II (FGFR3-IP):

Stocksolution	Final Concentration	1 Liter (+ bdH ₂ O)	10 ml (+ bH ₂ O) + 1 Complete Tab. (Roche)
100 mM EGTA	1 mM EGTA	10 ml	100 μ l
5 M NaCl	150 mM NaCl	30 ml	300 μ l
50 mM Na ₃ VO ₄	1 mM Na ₃ VO ₄	20 ml	200 μ l
100% Triton X	1% Triton X	10 ml	100 μ l
100 mM NaF	10 mM NaF	100 ml	1 ml
1M Tris pH8	50 mM Tris	50 ml	0.5 ml

3.16 Determination of Protein Concentration

Protein isolations were subjected to Bradford Assay (*Bio-Rad Protein Assay*) to determine protein concentration (Simonian and Smith, 2006). Bradford solution (*Bio-Rad*) was diluted 1:5 with water. Samples, as well as BSA for the calibration curve were placed (Table and Figure) into *PS microplate 96-well* (Greiner). Following this, 190 μ l of diluted Bradford solution was added and mixed, then incubated for around 10 minutes at RT. Absorption of monochromatic light of 562 nm was determined using a *SynergyHT* plate reader (BioTEK) and *Gen5* software (BioTEK). Protein concentration of samples was finally calculated from the calibration curve (Figure 3.2 and Table 3.4). Obtained concentrations had to be multiplied by 10, because of the 1:10 dilution of samples.

Table 3.4: Pipetting Scheme of BSA standards and protein samples.
All concentrations were calculated by taking the average of double determinations.

	Water (µl)	Lysis-Buffer (µl)	BSA (1 µg/µl)	Conc. (µg/µl)	Samples (µl)
blank	9	1	0	0	
1	8	1	1	0.1	
2	7	1	2	0.2	
3	5	1	4	0.4	
4	3	1	6	0.6	
5	1	1	8	0.8	
6	0	1	9	0.9	
	9				1

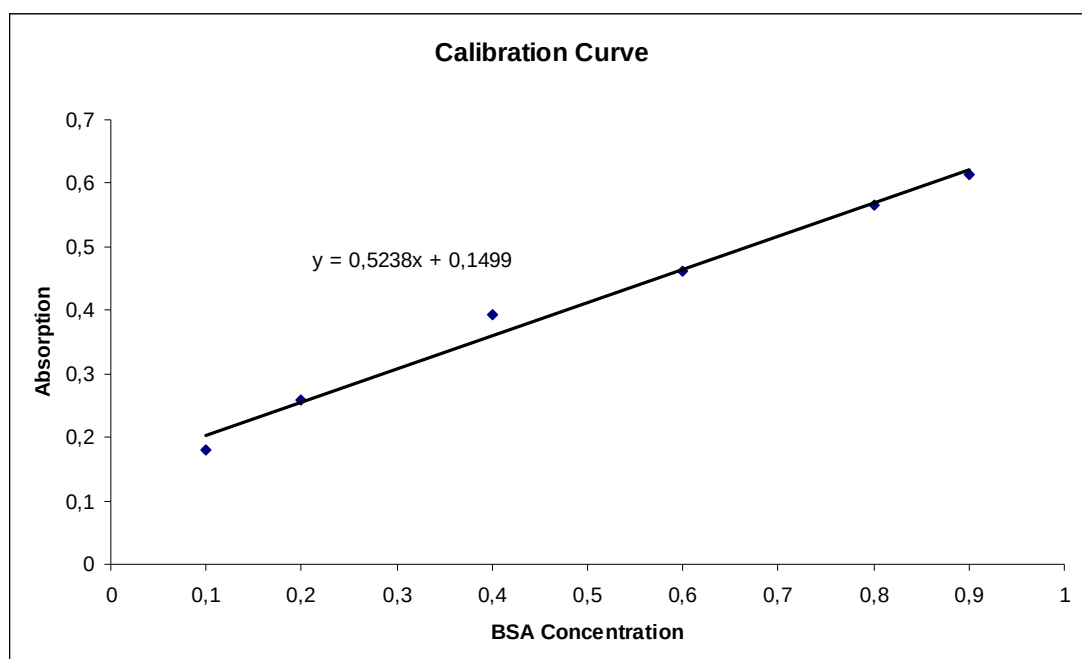


Figure 3.2: Example of calibration curve used for determination of protein concentration by Bradford Assay.

3.17 SDS-Polyacrylamid Gel Electrophoresis

For Western Blot (Gallagher, Winston et al., 2008) analysis, protein samples were subjected to SDS-PAGE (Gallagher, 2007) first. The *Mini Protean 3* system (Bio-Rad) was used for protein separation. Discontinuous poly-acrylamide gels were generated (Table 3.5), with densities between 7.5 and 15% according to protein sizes desired to be investigated. The high density separation gel was cast and topped with isopropanol. After polymerization, the isopropanol layer was rinsed off with water and removed with filter (Schleicher and Schuell). Afterwards, the stacking gel was cast on top and sealed with a ridge. Following polymerization, the ridge was removed and pockets were cleaned with SDS Running Buffer (25 mM Tris, 192 mM glycine and 0.1% SDS), which was also used as running buffer for SDS-PAGE.

Usually 20 µg of protein lysate was mixed with 5 x SDS-PAGE Loading Buffer, denaturated for 5 minutes at 95°C and loaded into the gel pocket. Additionally, 5 µl of *Page Ruler Plus Prestained Protein Ladder* (Fermentas) was loaded as marker. Gels were run at a voltage of 50 V, until tracking dye (bromphenolblue) reached the separation gel, then was increased to 110 V.

Table 3.5: Recipes for PAGE gels.

Separation Gels	7,50%	10%	12%	15%	Volume
Water	2.379	1.960	1.625	1.122	ml
1.6 M Tris pH 8.8	1.250	1.250	1.250	1.250	ml
20% SDS	50	50	50	50	µl
30% Acrylamid/Bis; 29:1(Bio-Rad)	1.256	1.675	2.010	2.513	ml
10% APS (Merck)	25	25	25	25	µl
TEMED (Amresco)	5	5	5	5	µl

Stacking Gel	4%	Volume
Water	1.5	ml
0.5 M Tris pH 6.8	0.625	ml
20% SDS	12	µl
30% Acrylamid/Bis; 29:1(Bio-Rad)	0.325	ml
10% APS (Merck)	25	µl
TEMED (Amresco)	2.5	µl

3.18 Western Blot

Proteins separated by SDS-PAGE were transferred onto a PVDF membrane (*Hybond-P*, GE Healthcare) for immunodetection with specific antibodies. The *Mini Protean 3* system was used for blotting. The transfer procedure was done according to the tank transfer protocol (Gallagher, Winston et al., 2008). First, the PVDF membrane was activated with methanol, rinsed with water and equilibrated in Towbin Buffer (25 mM Tris, 192 mM glycine, 5% MeOH), also used as transfer buffer. The blotting sandwich was assembled, and transfer was performed at a voltage of 18 V, overnight in the fridge (4°C). In rare cases, the transfer was done for 2 hours at 4°C and with an additional -20°C cooling pack in the tank, at a current of 200 mA. The PVDF membrane was rinsed with water. Quality of transfer was controlled by staining protein bands with *Ponceau S Solution* (0.5 g/l Ponceau S (Sigma) and 1 ml/l glacial acetic acid (Gallagher, Winston et al., 2008). The membrane was incubated in the staining solution for about 5-20 minutes, then the background was removed by washing with distilled water, on the shaker. To increase quality, water was changed several times. Afterwards, the staining was documented using a photocopier.

For immune-staining, the membrane was re-activated by short incubation in methanol, was washed in water and blocked in TBST-M (TBS: 8 g/l NaCl, 0.2 g/l KCl, 3 g/l Tris, pH 7.4; T: 0.1% Tween 20 (Sigma); M: 5% Skim Milk Powder (Fluka) for 1 hour at RT, on the shaker. Blocking solution was washed off 3 x 5 minutes with TBST. The membrane was placed into a 50 ml Falcon tube, protein bands oriented towards the inside, and incubated in at least 5 ml of Primary Antibody Solution (Table 3.6), overnight on a rotator in a 4°C room. Next day, the membrane was washed 3 x with TBST and incubated in the Secondary Antibody Solution, for 1 hour on the shaker at RT. PVDF membrane was washed again, 3 x with TBST and 2 x with TBS, then incubated upside down on a parafilm with *Immune-Star WesternC* reagent (Bio-Rad) and visualized using *Hyperfilm ECL* (GE Healthcare).

Table 3.6: List of antibodies used for immune-detection. Concentration of BSA as diluent was 3%, but for milk powder (M) was 5%.

Primary Antibodies	Dilution	Supplier	Diluent	Size of Target
Mouse-anti-GFP	1:2000	Roche; 11814460001	M	27 kDa
Rabbit-anti-pAkt (Ser 473)	1:1000	Cell Signaling; 9271	BSA	60 kDa
Rabbit-anti-pAkt (Thr 308))	1:1000	Cell Signaling; 4056	BSA	60 kDa
Rabbit-anti-Akt	1:1000	Cell Signaling; 9272	BSA	60 kDa
Rabbit-anti-pFRSalpha (Tyr 196)	1:1000	Cell Signaling; 3864	BSA	85 kDa
Mouse-anti-pERK	1:1000	Sigma; M8159	BSA	44/42 kDa
Mouse-anti-ERK	1:1000	Cell Signaling; 9101	BSA	44/42 kDa
Rabbit-anti-pS6	1:1000	SAB; 11232-2	BSA	32 kDa
Rabbit-anti-S6	1:1000	SAB; 21225-2	BSA	32 kDa
Rabbit-anti-FGFR4 (C16)	1:1000	Santa Cruz; sc124	M	125 kDa
Mouse-anti-Cytochrom C (A-8)	1:1000	Santa Cruz; sc13156	BSA	16 kDa
Rabbit-anti-PARP-1	1:1000	Cell Signaling; 9542	BSA	24, 89, 116 kDa
Cleaved PARP-1	1:1000	Cell Signaling; 9541	BSA	89 kDa
Rabbit-anti-Caspase-3	1:1000	Cell Signaling; 9662	BSA	17, 19, 35 kDa
Rabbit-anti-pSTAT-3	1:1000	Cell Signaling; 9134	BSA	79, 86 kDa
Mouse-anti-β-actin	1:5000	Sigma	M	45 kDa
Secondary Antibodies	Dilution	Supplier	Diluent	Size of Target
Rabbit-anti-Mouse-HRP	1:10 000	Dako; P0260	M	n/a
Goat-anti-Rabbit-HRP	1:10 000	Dako; P0448	M	n/a

3.19 Lipofection

3 x 10⁵ cells were seeded into 6-well plates with 2 ml RPMI medium (+10% FBS) and incubated overnight. Next day, cells were adherent and around 50-80% confluent. As lipofection reagent either *FuGENE 6* or *FuGENE HD* was used (both from Roche). Considering toxicity and lipofection efficiency, both reagents showed similar results.

Transfection reagent (3 μ l) was added directly to 97 μ l Serum Free Medium (SFM; RPMI without FBS) and mixed. After 5 minutes incubation time, 1 μ g plasmid was added, mixed and incubated for another 25 minutes. In case the plasmid did not contain a desired selection marker, a second plasmid possessing the specific marker was added, thereby performing a co-transfection.

The total volume of transfection suspension was dropped onto the medium containing adherent cells (2 ml/well of a 6-well plate) and gently mixed. The following day, lipofected cells were expanded into 10 cm tissue culture dishes, thereby medium was changed, and incubated for another 24 hours. Then the standard growth medium was changed to selection medium containing antibiotics, according to the used resistance gene (See Selection of Stable Transfectants).

3.20 Selection of Stable Transfectants

Cells integrating selection marker and hopefully the gene of interest into their genome, were seen as stably expressing cell lines. All plasmids used, contained either a neomycin resistance gene or a puromycin resistance gene, therefore stable transfectants were selected with G418 / neomycin or puromycin (Table 3.7). To reduce the risk of the fake clone phenomenon, where only selection markers are expressed but not the gene of interest, some plasmids coded for bicistronic mRNA with these two genes separated only by an IRES (Internal Ribosomal Entry Site; Gurtu and Yan et al., 1996). However, all generated cell lines were tested for expression of the respective genes of interest by Western Blot analysis or Immunohistochemistry (IHC) and qRT-PCR.

Selection medium was changed several times, facilitating perfect growth conditions. When resistant clones appeared, usually after 1-2 weeks, clones were picked and re-seeded. New cell lines were frozen and tested for expression, as soon as possible, to reduce the risk of losing clones due to contamination. Besides, all selection media contained also penicillin and streptomycin to prevent bacterial infections.

Table 3.7: End-concentration of additives during selection and cultivation.

	G418 (PAA)	Puromycin (PAA)	Doxocyclin
Concentration (μg/ml)	300	0,4	0,1

3.21 Adenoviral Transduction

3×10^5 cells were seeded into 6-well plates with 2 ml RPMI medium (+10% FBS) and incubated overnight. The following day viral suspension was added drop-wise onto the medium and the plates shaken gently. Cell lines were transduced at different MOI (Multiplicity Of Infection). Adenoviruses were diluted and the appropriate volume was calculated according to the ifu / μ l (infectious units per μ l) of viral stocks.

3.22 Viability Assay

Cell lines (10^5 cells per 6-well) were transduced by adenoviruses containing different dominant-negative receptor constructs (dnFGFRs). The day after transduction, cells were trypsinized and counted. Following different incubation times, varying between one and six days, cells were trypsinized and counted again (Casy Cell Counter). Additionally, the proliferation was monitored by microscopy and Höchst 33258/propidium iodide staining. For cell staining, all cells of a well (6-well plate), including those in the supernatant, were collected and centrifuged. The cell pellet was taken up in 99 μ l medium and 1 μ l staining solution was added, thereby diluted 1:100. After 30 minutes of incubation, numerical ratio of viable versus apoptotic cells was counted under the microscopy.

3.23 Soft Agar Assay

Cells were washed (PBS), trypsinized and counted. Appropriate volume, containing 30000 cells, was centrifuged (800 rpm for 5 minutes) and the pellet was taken up in 750 μ l *Soft Agar Medium*, having a temperature of 37°C. 750 μ l 1% *Agar Solution* (40-41°C) was added to the cell suspension and mixed. A total volume of 1.5 ml was added on top of solid soft agar and resulted in a total volume of 3 ml (0.5% solid soft agar medium) per well, of a six well plate. Solutions were kept on temperature in the water bath. To reduce velocity of temperature reduction, solutions were put into a tray containing warm water during pipeting. After the desired clone size was reached (>30 μ m), the number of clones above a specific threshold level was counted under the microscope, e.g.: clones larger than 30 μ m. The clone size was calculated using Metamorph software.

Soft Agar Medium, as well as *1% Agar Solution*, were prepared the day before, as follows. Considering the *Soft Agar Medium*, 2ml NaHCO₃ (110 mg/ml, in water bidest.), 1 ml glutamine, 17 ml water (bidest.) and 50 µl folic acid were added to 10 ml (10 x) RPMI medium (Sigma). The solution was brought to a pH value of 8 and sterile filtered. Then, 20 ml FCS and 1ml penicillin-streptomycin were added under sterile conditions. For subsequent generation of the soft agar bottom, the medium was brought to a temperature of 37-40°C. For preparation of *1% Agar Solution*, 1.2 g agar was added to 80 ml water (bidest.) and solubilised by boiling. Simultaneously another 30 ml of water (bidest.) were heated up and used to fill up the 80 ml suspension to 100 ml. At the end the suspension was boiled again. Afterwards, the solution was brought to a temperature of about 40°C, for subsequent generation of the soft agar bottom.

Soft Agar Medium and *1% Agar Solution* were mixed 1:1. 1.5 ml were subsequently pipetted into each well of a six well plate, constituting the soft agar bottom. Air bubbles were prevented by leaving a low amount of solution in the pipette. Afterwards, plates were put in the fridge until the agar became solid, then were put into the incubator over night.

3.24 Clonogenic Assay

1000-3000 cells were seeded into one well of a six well plate containing 2 ml RPMI medium (10%). After the desired clone sizes (>1 mm) was reached, depending on specific clonogenicity of the cell line used, cells were stained with crystal-violet. The number of clones above a specific threshold size was counted, e.g.: all clones larger than 1mm in diameter.

3.25 Crystal-Violet Staining

Supernatant was discarded, cells were washed in PBS and fixed by incubation in methanol : acetone (1:1) for about 20 minutes. Then cells were exposed to crystal-violet solution, diluted 1:1000 in PBS (stock: crystal violet in PBS and 10% ethanol) and incubated for another 20 minutes. Staining solution was discarded and cells were air dried over night.

3.26 Migration Assay

To test for cell migration and invasion, transwell-chambers in a 24 well-plate format were used. 40000 cells in 200 µl medium were seeded onto porous membranes, 800 µl medium were added into the well resulting in a total volume of 1 ml. The RPMI medium used, contained 4% FCS. As control, migration of untreated cells was tested in RPMI medium including 10% FCS. Migration through the membrane pores was visualized by crystal-violet staining. Incubation times ranged from 24-72 hours.

3.27 MTT Assay

To test cell proliferation, 3000-5000 cells were seeded in 100 µl RPMI medium (10% FBS) into one well of a 96 well-plate. Suggested inhibitor or stimulator molecules were added as (2 x) concentrations in 100 µl serum-free medium, resulting in a total volume of 200 µl (5% FBS). Inhibitors were added 24 hours after seeding, while putative stimulators were added already after around 3 hours. Incubation times ranged between 3-5 days. For determination of cell concentration the MTT kit EZ4U from Biomedica was used according to the manufacturer's protocol. Cells were incubated at 37°C and 5% CO₂ for 2-5 hours. Absorption was measured at 450 nm and 620 nm as reference using a Synergy spectrophotometer.

3.28 Tumour Growth in SCID Mice

Cell lines were seeded in 10% RPMI and incubated over night. The following day, cells were transduced with adenoviruses bearing dominant-negative FGFR constructs, according to suggested MOIs. After 24h incubation, 10⁶ cells in 50 µl serum free medium were injected subcutaneously into the flanks of SCID mice.

4 Results

4.1 Expression Analysis of FGF-Receptors and Isoforms

RT-PCR of the major isoforms of FGFR 1-4 was performed in 12 cell lines derived from primary tumours or metastases of different histological types of melanoma. Primary melanocytes were included for comparison. Considering the investigated splice variants, alpha isoforms possess IgG-I domains, while beta variants do not. IgG-IIIb and -IIIc isoforms differ in sequence within the second, C-terminal part of the IgG-III domain. Additionally it has been shown, that the IIIb domain is predominantly expressed in cells derived from the epithelial cell lineage, while mesenchymal derived cells tend to express the IIIc domain. The isoform named beta acidic box possesses an alternative amino acid sequence within the IgG-II domain. Soluble stands for isoforms not containing transmembrane domains, thus will not integrate into the cell membrane. Isoform-1 and -3 of FGFR-4 encode the same protein and differ only in the used splice site for exon 1. Isoform 2-soluble has no transmembrane domain, in contrast to isoform-1 and -3. The isoform named ezzat soluble consists only of the IgG-I domain and a 3'UTR region (Table 4.1).

Table 4.1: Expression analysis of FGF-Receptors, including different splice variants, by standard Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). From left to right: FGFR 1-4, Splice Variants, 12 Vienna Melanoma Cell Lines (Number and Trivial-Name) and Primary "Non-transformed" Melanocytes (PM) as reference.

		VM1	VM7	VM8	VM10	VM21	VM23	VM24	VM28	VM30	VM31	VM47	VM48	
FGFR	Splice Variant	FTSL/A	GTBS	GUBS/A	JMUM	RHTP	RKTJ	SHTJ	TMFI	WCRE	WLTJ	HOST	KAKA	PM
1	alpha	+	+	+	+	+	+	+	+	+	+	+	+	+
	beta	+	+	+	+	+	+	+	+	+	+	+	+	+
	IIIb	+	+	+	+	+	+	+	+	+	+	+	+	+
	IIIc	+	+	+	+	+	+	+	+	+	+	+	+	+
2	alpha	+	+	+	+	+	+	+	-	-	+	+	+	+
	beta	-	+	+	+	-	+	+	-	-	+	+	+	-
	beta acidic box	+	+	-	-	+	-	-	-	+	+	+	+	+
	IIIb	+	+	-	+	+	+	+	-	-	-	+	+	+
	IIIc	-	+	-	+	-	+	+	-	-	+	+	+	+
3	alpha	+	+	+	+	-	+	+	-	-	-	+	+	+
	IIIb	-	-	-	+	+	+	+	+	+	+	+	+	+
	IIIc	-	+	-	+	+	+	+	+	+	-	+	+	+
	soluble	-	+	-	+	+	+	+	+	+	+	+	+	+
4	isoform 1+3	+	+	+	+	+	+	+	+	+	+	+	+	+
	isoform 2-soluble	-	-	-	-	+	+	+	-	+	-	+	+	+
	ezzatz soluble	+	+	+	+	-	-	+	-	-	+	-	+	+

Isoforms consisting of all three IgG-domains, transmembrane domain and tyrosine kinase domain of FGFR-1 and FGFR-4 were expressed in all cell lines and primary melanocytes, whereas FGFR-2 and FGFR-3 isoforms were absent in some of the melanoma cell lines. This indicates the great importance of FGFR-1 and FGFR-4. What`s more, all alternative splice variants of FGFR-1 investigated in this study, were detected in 100% of our cell lines. Considering isoforms of FGFR-2 and FGFR-3, the expression pattern seems to be quite heterogeneous. What might be surprising is, that all FGFR isoforms investigated, were also detected in our primary melanocytes, apart from the FGFR-2 beta isoform. This indicates the possibility, that the FGFR-2 beta isoform, might be a potent regulator for melanocytic to melanoma transition, although this has to be clarified in further experiments. At this point one has to consider, that primary melanocytes used as reference in all expression experiments are of embryonal and not adult origin. In general, all splice variants seem to be expressed in low amount and in a heterogeneous manner, considering all malignant cell lines as well as non-transformed melanocytes.

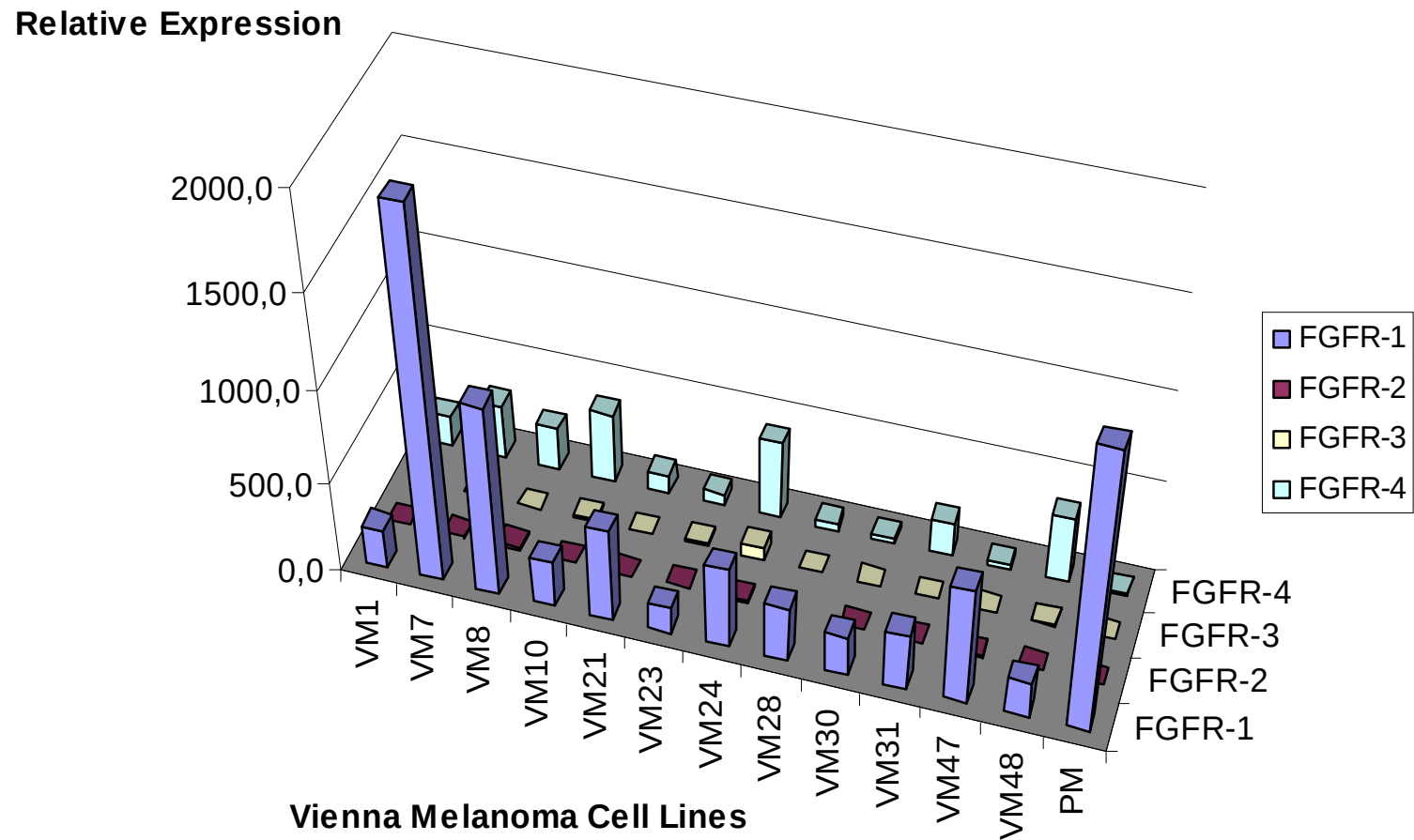


Figure 4.1: Relative expression of FGF-Receptors in all 12 VM Cell Lines and Primary Melanocytes (PM), determined by quantitative Reverse Transcriptase Polymerase Chain Reaction ("Real Time" qRT-PCR). Values shown in the figure were calculated using the formula: $2^{-(dCT)} \times 100\,000$. Determined CT-values were normalized to 18S-rRNA expression.

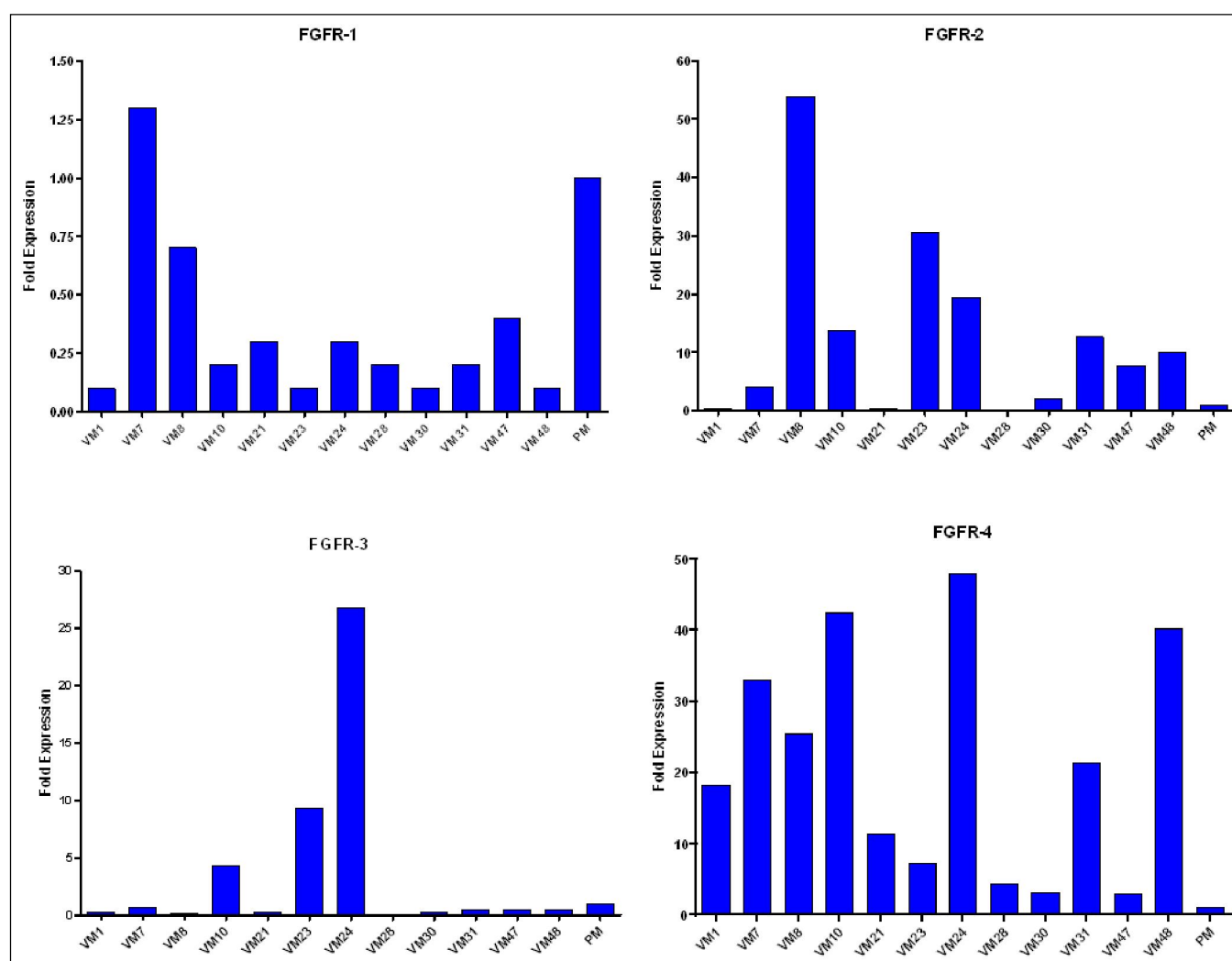


Figure 4.2: FGF-Receptor expression in comparison to Primary Melanocytes (PM). Analysis was performed by quantitative RT-PCR and values were calculated by the formula: $2^{-(ddCT)}$. For each receptor, the expression level in primary melanocytes was set as 1.

Expression analysis by qRT-PCR revealed, that FGFR-1 and FGFR-4 are highly expressed in nearly all malignant melanoma cell lines, compared to FGFR-2 and FGFR-3. Considering the FGFR expression in non-transformed melanocytes, it is shown, that only FGFR-1 is highly expressed. Taking non-transformed melanocytes as reference, the expression of FGFR-4 and FGFR-2 seems to be highly up-regulated in nearly all malignant melanoma cell lines, while high expression of FGFR-1 is maintained and expression of FGFR-3 stays low (Figure 4.1 and 4.2). In general, results from quantitative analysis correlated well with band intensities, observed by standard PCR. Expression pattern of a given FGF-receptor is heterogeneous among all investigated melanoma cell lines and seems not to be specific for a specific tumor stage or metastasis type (Figure 4.3).

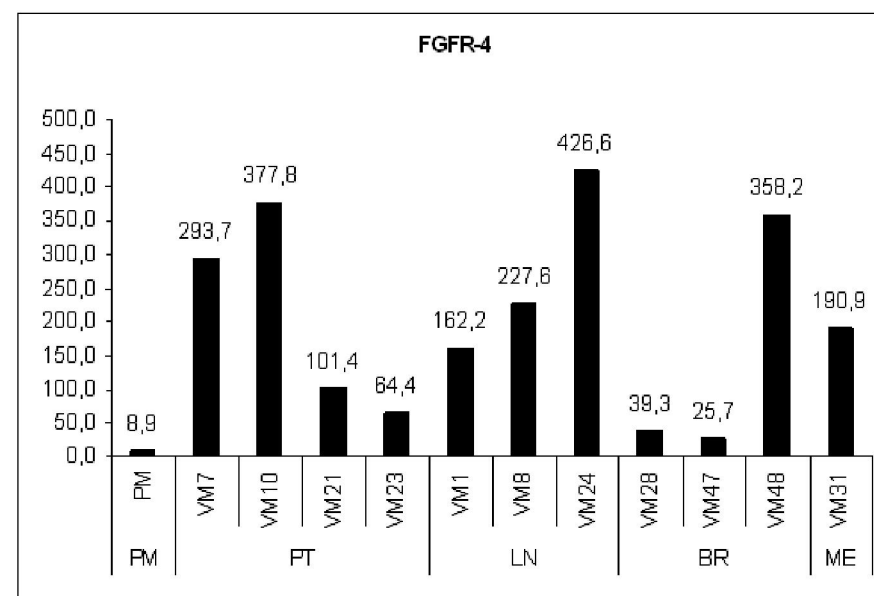
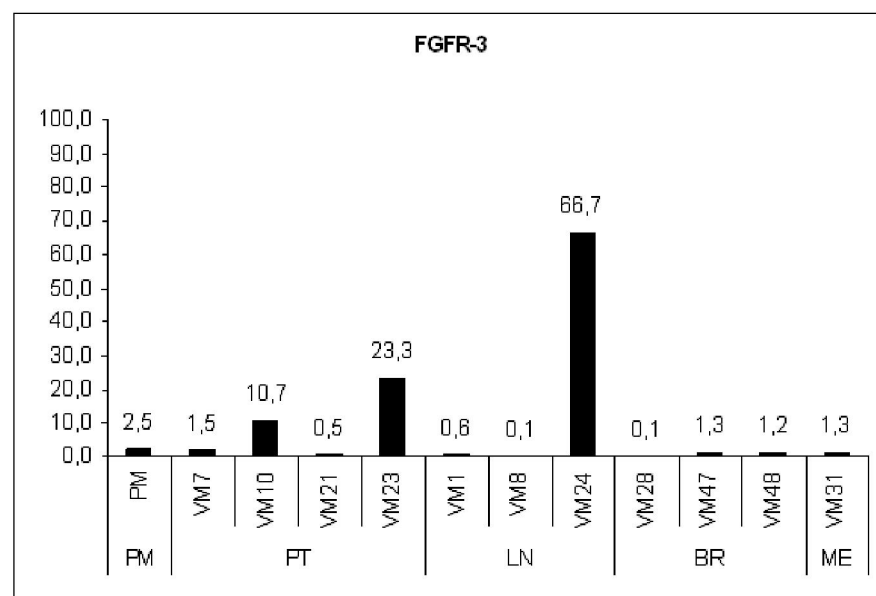
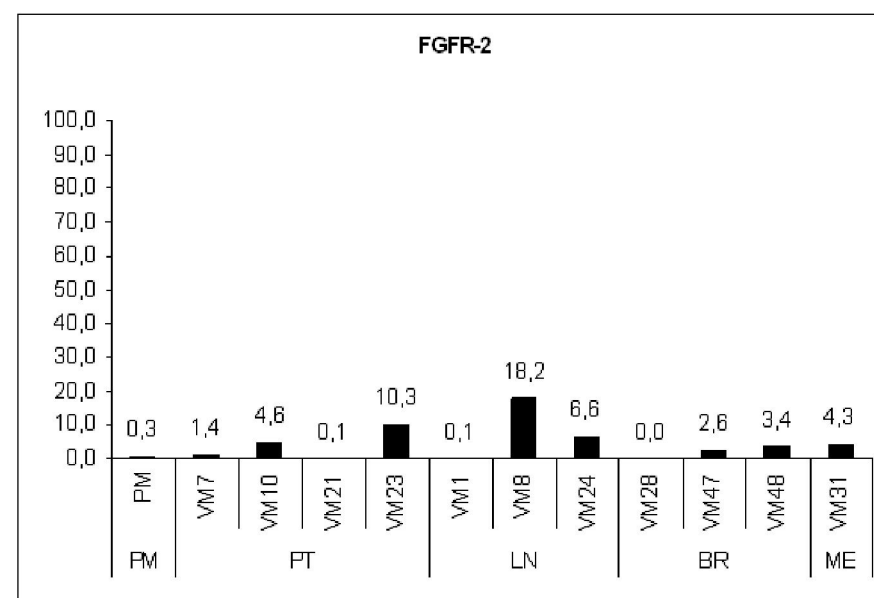
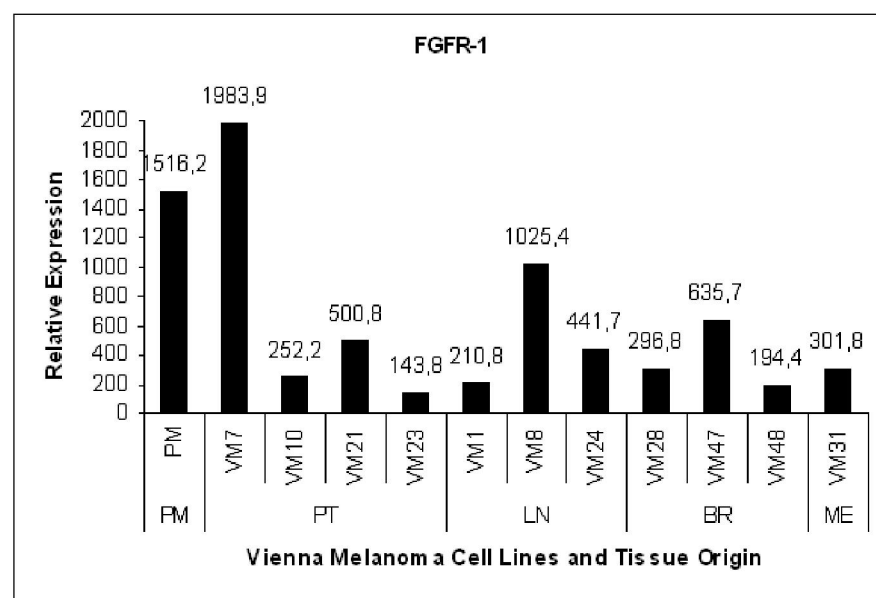


Figure 4.3: Relative expression of FGF-receptors analysed by “Real Time” qRT-PCR in melanoma cell lines grouped by tumor stage (primary tumour versus metastasis) and organ localization of metastases . PM: Primary Melanocytes; PT: Primary Tumour; LN: Lymph Node Metastases; BR: Brain Metastases; ME: Malignant Effusions.

4.2 Expression Analysis of Fibroblast Growth Factors

FGF-1 and FGF-2 were found to be expressed in nearly all cell lines including primary melanocytes. FGF-5 was shown to be expressed in the majority, and FGF-18 in all cell lines. These two factors were recently reported as oncogenic in different tumour tissues, therefore expression levels were analysed by qRT-PCR. FGF homologue factors (FGF11-14), were found to be expressed in nearly all cells. However, it was recently shown, that these are not able to bind FGFR, despite their structural homology (Table 4.2).

Table 4.2: FGF expression analysis, of all 22 FGFs in twelve different melanoma cell lines and primary melanocytes. FGF-15 is the mouse ortholog of FGF-19, therefore is not shown in the table.

	VM 1	VM 7	VM 8	VM 10	VM 21	VM 23	VM 24	VM 28	VM 30	VM 31	VM 47	VM 48	
	FTSL/A	GTBS	GUBS/A	JMUM	RHTP	RKTJ	SHTJ	TMFI	WCRE	WLTJ	HOST	KAKA	PM
FGF1	+	+	+	+	-	-	+	+	+	+	+	+	+
FGF2	+	+	+	+	+	+	+	+	+	+	+	-	+
FGF3	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF4	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF5	+	+	+	-	-	+	+	-	+	+	+	+	-
FGF6	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF7	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF8	-	-	-	+	-	-	+	-	-	-	-	+	-
FGF9	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF10	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF11	+	+	+	+	+	+	+	+	+	+	+	+	+
FGF12	-	+	-	+	+	+	+	-	+	+	+	+	-
FGF13	+	+	+	+	+	+	+	+	+	+	+	+	+
FGF14	+	+	+	-	-	+	+	-	+	+	-	-	+
FGF16	+	+	+	-	+	+	-	+	-	+	-	-	+
FGF17	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF18	+	+	+	+	+	+	+	+	+	+	+	+	+
FGF19	-	-	-	-	-	-	-	-	-	-	-	-	-
FGF20	-	+	-	+	-	-	-	-	-	-	-	+	-
FGF21	+	+	-	-	+	+	-	-	-	+	-	-	+
FGF22	+	+	+	+	-	+	+	-	-	-	+	+	+
FGF23	+	+	-	+	-	-	+	-	-	-	-	+	-

Quantitative expression analysis revealed, that FGF-2 is highly up-regulated in all melanoma cell lines (100%) investigated, the long isoform as well as the short isoform of FGF-5 was up-regulated in the majority (90%) and the short isoform was found to be 4 times less expressed, compared to the long version, in all cell lines. Expression of FGF-18 was up-regulated in around 40%, while expression of FGF-17 and FG-F8 stayed low (Figure 4.4). In general, these results correlate with those obtained by standard PCR.

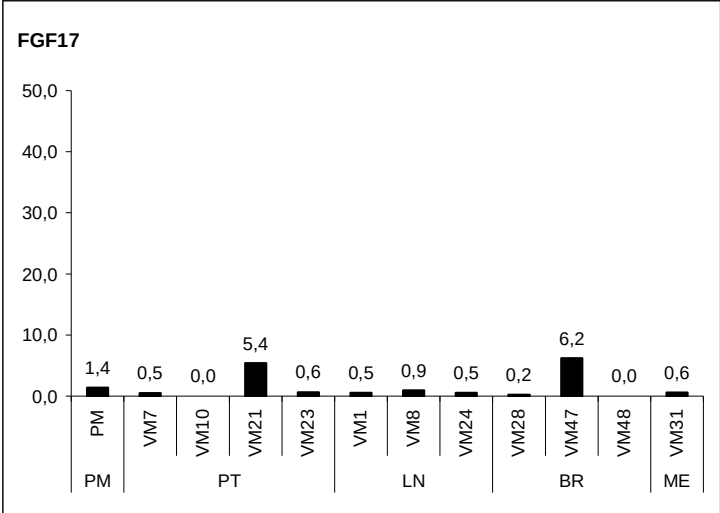
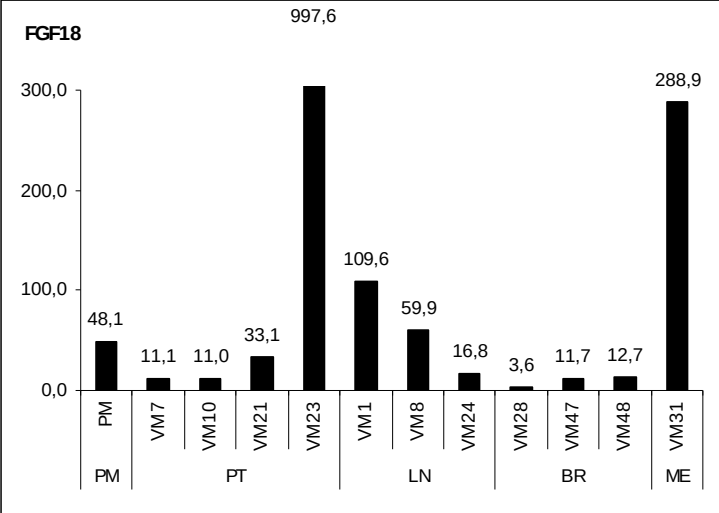
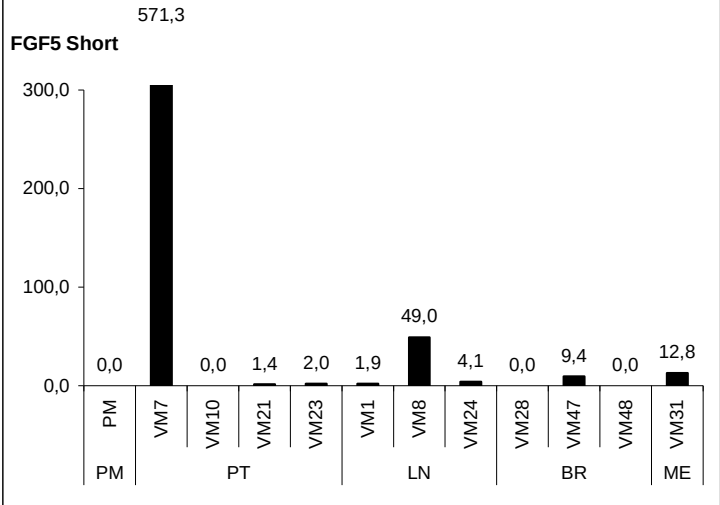
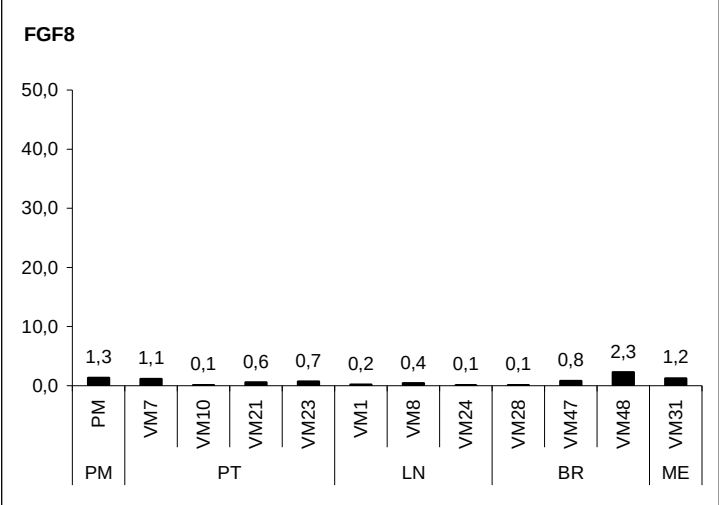
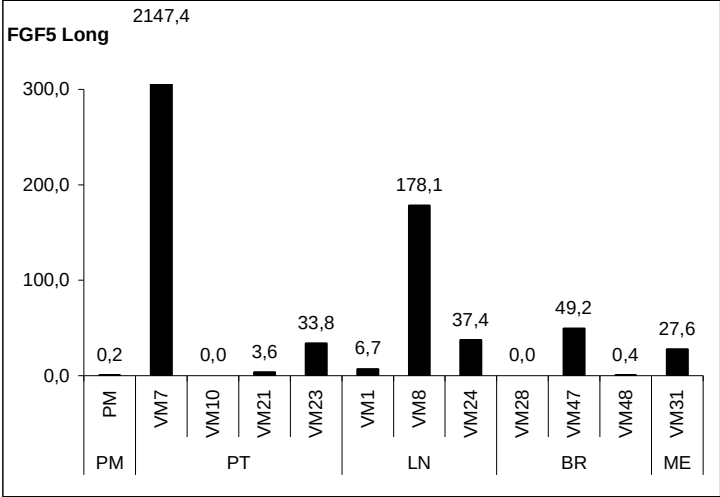
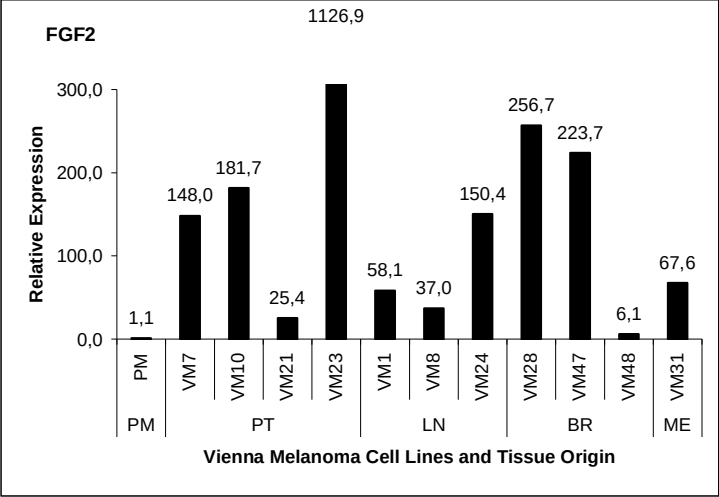


Figure 4.4: Quantitative expression analysis (qRT-PCR) of selected FGFs. Cell lines derived from metastases were stratified according to their tissue origin. Relative values were calculated by the formula: $2^{-(\Delta\Delta CT)} \times 100\,000$. Thus primary melanocytes were used for comparison but were not used as calibrator.

4.3 Analysis of Cellular Effects by Blockade of Different FGFRs by Dominant Negative Receptor Constructs

Four melanoma cell lines were infected with adenoviruses bearing different dominant-negative FGFR constructs. Considering dnFGFR1 adenoviruses, the sequence coding for the kinase domain was replaced by GFP, in case of dnFGFR4 it was replaced by CFP. For the kinase-dead FGFR3 (kdFGFR3) construct, a mutation was introduced leading to a non-functional kinase domain. As controls, adenoviruses expressing GFP, instead of dnFGFR, were used. All viruses were available in the FGF research cooperation of the ICR from previous investigations.

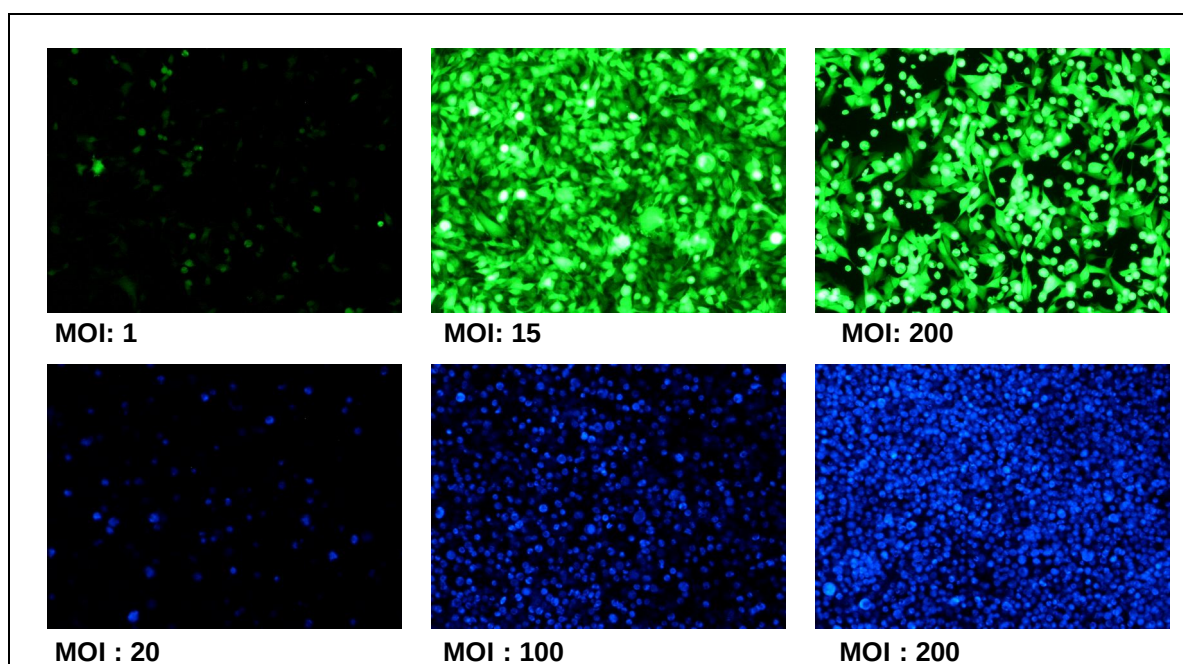


Figure 4.5: Fluorescence microscopy of melanoma cells (FTSL-A; VM1), five days after transduction with adenoviruses containing GFP and dnFGFR4-CFP constructs, at different MOIs.

The most difficult part was to find appropriate MOIs (Multiplicity Of Infection) for the transduction procedure. This was optimized by infection of cell lines at different MOIs and subsequent analysis by microscopy (Figure 4.5 and 4.6), monitoring fluorescence and toxicity, as well as by Western-Blot (Figure 4.7). The aim was to choose appropriate MOI values leading to equivalent episomal protein expression of all adenoviral constructs. Expression of dnFGFRs and GFP was visible already 24h after infection. Cell toxicity of adenoviruses was tested up to an MOI of 60.

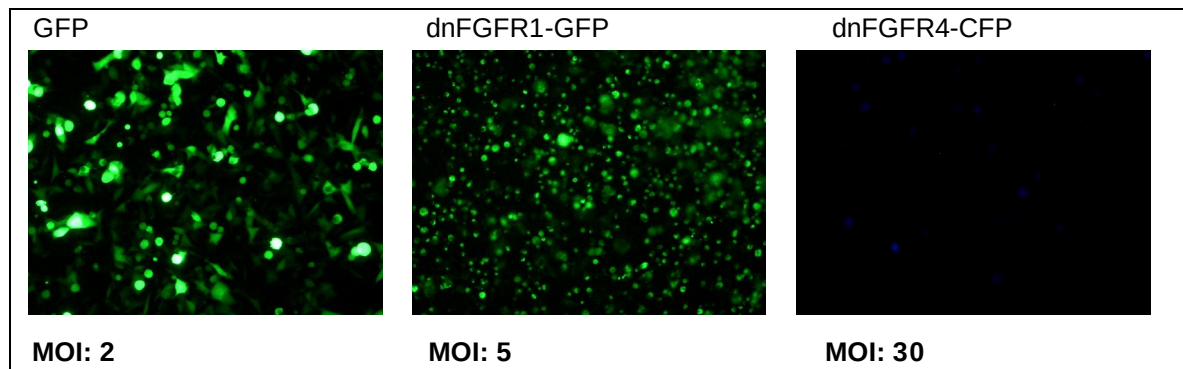


Figure 4.6: Microscopy of melanoma cells (SHTJ; VM24), three days after adenoviral infection. From left to right: Transduced with GFP at MOI: 2; dnFGFR1-GFP at MOI: 5; dnFGFR4-CFP at MOI: 30.

All cells visibly expressing dnFGFR1-GFP and dnFGFR4-CFP were small, round shaped and seemed to undergo apoptosis (Figure 4.6 and 4.8). This gave a first impression of the importance of the FGFR signalling pathway for melanoma cell survival.

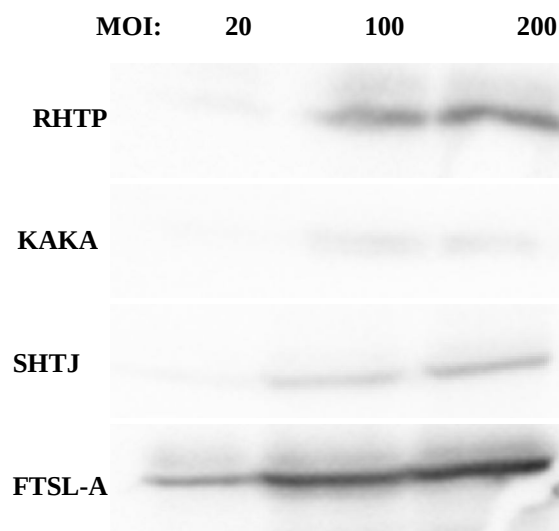


Figure 4.7: Western Blot analysis of four different cell lines transduced with adenoviruses containing the dnFGFR4-CFP construct. Cells were incubated five days, proteins were isolated and dnFGFR4-CFP expression was analysed using anti-GFP antibodies. Protein concentrations were equalized prior to loading to 20 µg.

Ectopic expression of dnFGFRs seems to be cell line dependent (Figure 4.7 and 4.8), with highest expression achieved in FTSL-A (VM1) and lowest in KAKA (VM48). Results from Western Blot analysis, were also confirmed by microscopy. Our objective was to achieve equal expression of all dnFGFRs including GFP control by working with appropriate MOIs. However, this required higher MOIs for dnFGFR adenoviral infections, compared to the GFP control.

This leaves the possibility of cell toxic effects caused by the virus itself. It was shown however, that transduction of cells with adenoviruses containing GFP constructs, did not show visible cell toxic effects up to MOI: 5. At an MOI of 30 or higher, cell proliferation was reduced around 15%, compared to MOI: 2 (Figure 4.10 and 4.11).

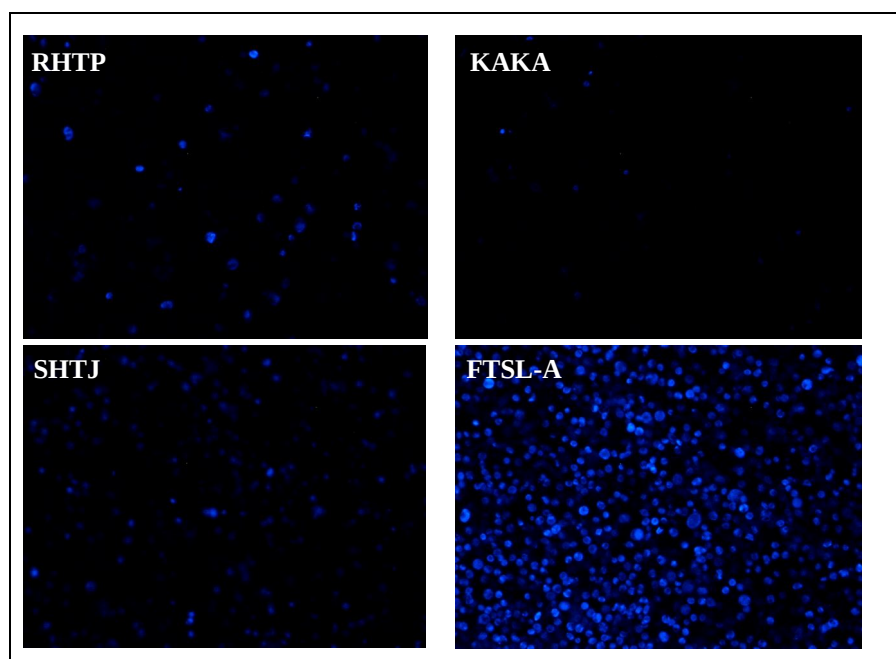


Figure 4.8: Microscopy of four melanoma cell lines transduced with adenovirus containing dnFGFR4-CFP constructs at MOI: 100. It is obvious, that expression is dependent on the cell line. These results also correlate with Western Blot analysis shown above.

The intensity of fluorescence, observed during microscopy, highly correlated with protein expression analyzed by immune-detection (Figure 4.7, 4.8 and 4.9). This implies, that dnFGFR1-GFP is less expressed compared to GFP, although cells were infected with an MOI of 5 considering dnFGFR1-GFP and an MOI of 2 considering GFP viruses (Figure 4.9). This is also the case for dnFGFR4-CFP expression. Although cells were infected with an MOI of 30 the fluorescence is low, and at protein level expression is even below the limit of detection, in three of four cell lines.

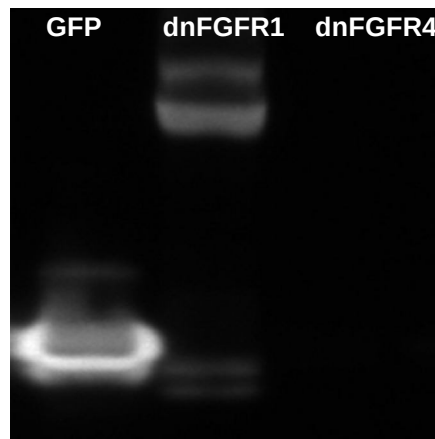


Figure 4.9: The melanoma cell line FTSL-A was infected by adenoviruses containing dnFGFR and GFP constructs. After five days of incubation expression levels were analysed by Western Blot.

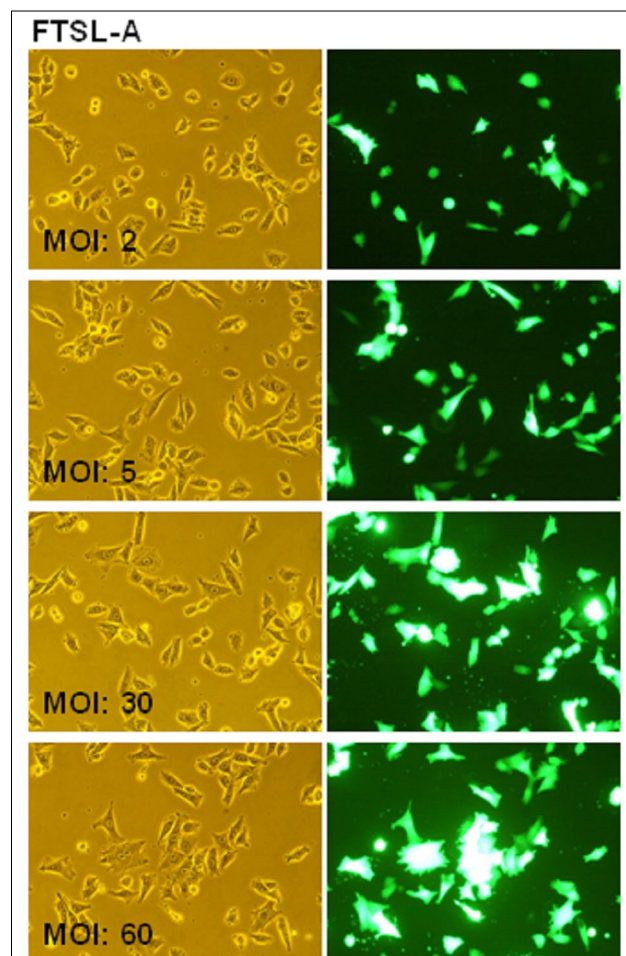


Figure 4.10: Microscopy of GFP adenoviral transduced FTSL-A cells, after 48 hours incubation time. Cells were transfected at different MOI, after 24 hours 10^5 cells were seeded into a 6-well plate. Pictures were taken after 48 hours incubation time.

Finally, we decided to take MOIs ranging from 2 to 30. GFP control adenoviruses were used at MOI: 2, dnFGFR1-GFP adenoviral constructs at MOI: 5, and kdFGFR3 and dnFGFR4-CFP constructs at MOI: 30. It was shown, that protein expression of dnFGFR1-GFP, kdFGFR3 and dnFGFR4-CFP was far below GFP control, although much higher MOIs were applied. What has to be taken into consideration is, that viral induced cell toxic effects reduced cell proliferation about 15% at MOIs of 30. The MOI of 5, applied for dnFGFR1-GFP adenoviruses, did not show cell toxic effects (Figure 4.10 and 4.11), when used for GFP transduction.

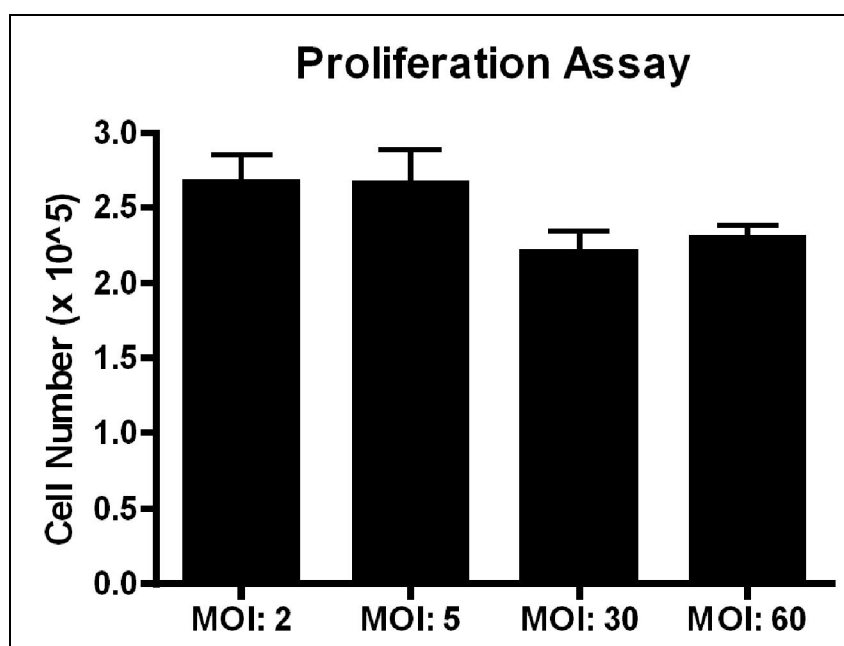


Figure 4.11: Proliferation assay of GFP adenovirus transduced FTSL-A (VM1) melanoma cells, after 48 hours incubation time. Cells were transfected at different MOI, after 24 hours 10^5 cells were seeded into a 6-well plate. Cell number was determined by Casy Cell Counter after 48 hours. Experiment was done in triplicates, and monitored by microscopy.

4.3.1 Viability Assays

Cell lines were infected with adenoviruses containing dnFGFR constructs and GFP control construct, incubated overnight, and seeded at low cell densities the following day. Cell proliferation was measured after two (48 h) and five days (120 h) using spectroscopic and staining methods. Cells were stained with Höchst and propidium-iodide solution and subsequently counted under the microscope. In addition, cell concentration was measured using the Casy Cell Counter.

Used MOIs, for adenoviral infections, were: MOI: 2 for GFP, MOI: 5 for dnFGFR1-GFP and MOI: 30 for dnFGFR4-CFP as well as for kdFGFR3 viruses.

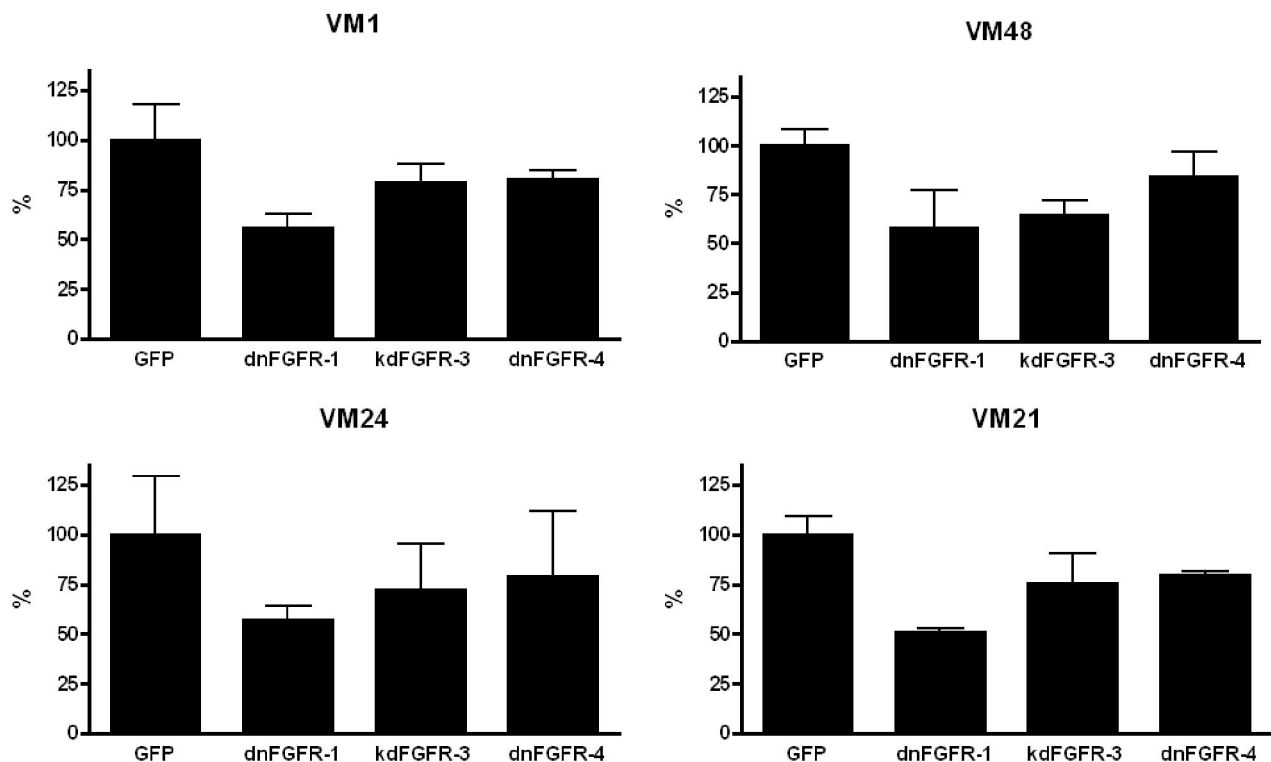


Figure 4.12: Proliferation of melanoma cell lines (FTSL-A/VM1; KAKA/VM48; SHTJ/VM24; RHTP/VM21) infected with adenoviruses containing dnFGFR constructs and GFP control construct. Percent of total cells, taking the GFP control as reference. Cells were incubated 48 hours.

Results clearly show the impact of the FGFR-1 mediated FGF-signalling pathway on cell proliferation (Figure 4.12 and 4.15), and apoptosis (Figure 4.13 and 4.14). Although the dnFGFR1-GFP protein was shown to be less expressed, it leads to a decrease of cell proliferation of around 50%, compared to the GFP control. What's more, inhibition of the FGFR-4 and FGFR-3 mediated pathway, also decreased cell proliferation around 20-30%, although fluorescence of the dnFGFR4-CFP protein was barely visible and both proteins could not be detected by Western Blot analysis, thus are suggested to be expressed in low amounts.

However, cells transfected with kdFGFR3 and dnFGFR4-CFP viral constructs were transduced at MOIs of 30. This high MOI was shown to be anti-proliferative even when only GFP was transfected, thus suggesting reduced proliferation of around 15% by the virus itself. Therefore, it is suggested that anti-proliferative effects of kdFGFR3 and dnFGFR4-GFP are much lower than 20-30%. MOIs of 5 did not affect cell proliferation when transfected with GFP viruses (Figure 4.11), thus around 50% reduction in proliferation is indeed caused solely by the dnFGFR1-GFP protein and, presumably its down-regulation of the FGFR-1 signalling pathway (Figure 4.15).

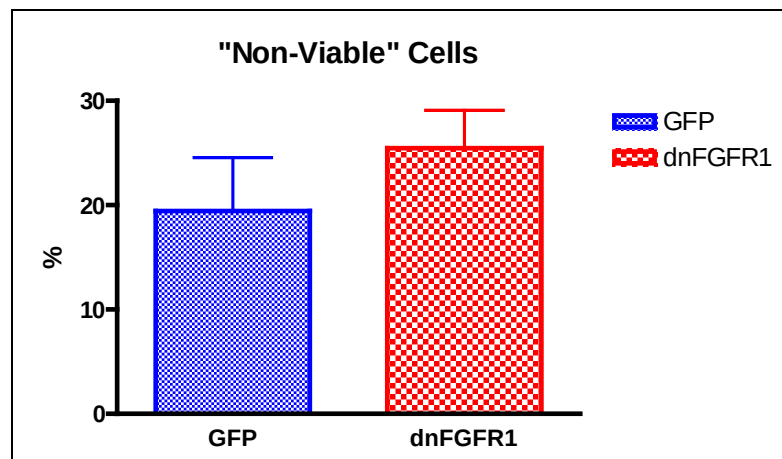


Figure 4.13: Percentages of “non-viable” cells, apoptotic or necrotic, as determined by the Casy Cell Counter. The average of all four melanoma cell lines tested is shown.

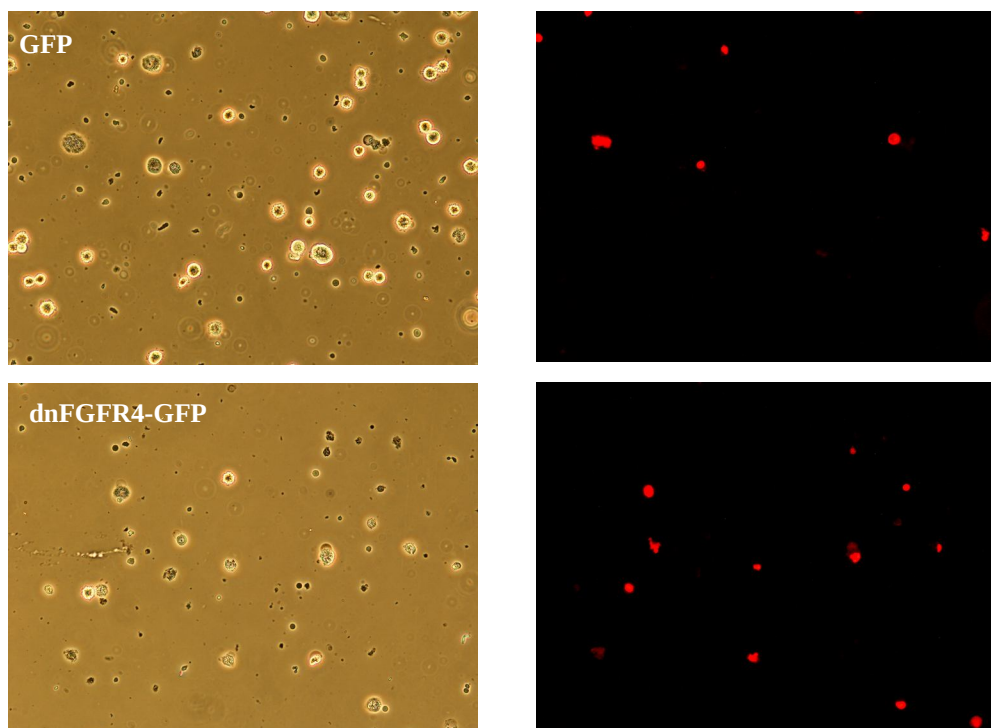


Figure 4.14: Example of melanoma cell line (KAKA; VM48) stained with propidium iodide. Cells were infected with adenoviruses containing dnFGFR constructs, incubated for two and five days, trypsinized and stained. Left pictures show total cells, viable and dead or apoptotic cells (“non-viable”), as well as cell fragments. Stained cells, shown on the right side, were counted as dead or apoptotic. These cells still possess a membrane, thus their cytoplasm is enclosed, but the cell membrane has become porous, therefore is no longer a barrier for the staining solution (red spots).

Cell number can be reduced in two ways, either by inducing apoptosis or by decreasing cell division. Results suggest, that by inhibition of the FGF-signalling pathway, cell number is likely to be reduced by both options. Considering inhibition of the FGFR-1 mediated pathway, around 6% more dead cells were found, in contrast to the GFP control. Thus, apoptosis seems to increase, when the FGF signalling pathway is inhibited. However, it is unlikely, that the observed impact on cell proliferation is only achieved by induction of apoptosis.

The amount of cells, detected as necrotic or apoptotic by the Casy Cell Counter (Figure 4.13), was verified by propidium-iodide staining (Figure 4.14). All red stained cells were counted as “non-viable” or dead and compared to the total number of cells.

Propidium-iodide is an intercalating agent and a fluorescent molecule that can be used to stain DNA. When excited by 488 nm of laser light, it can be detected with 562-588 nm band pass filter. It can be used to differentiate necrotic, apoptotic and viable, thus normal cells. It binds to DNA by intercalating between the bases with little or no sequence preference and once the dye is bound to nucleic acid, its fluorescence is enhanced 20-30 fold, fluorescence excitation maximum is shifted around 30-40 nm to the red and fluorescence emission maximum is shifted around 15 nm to the blue. Propidium-iodide is membrane impermeant and generally excluded from viable cells, thus is commonly used for identifying dead cells among a given population (Moore A, 2002).

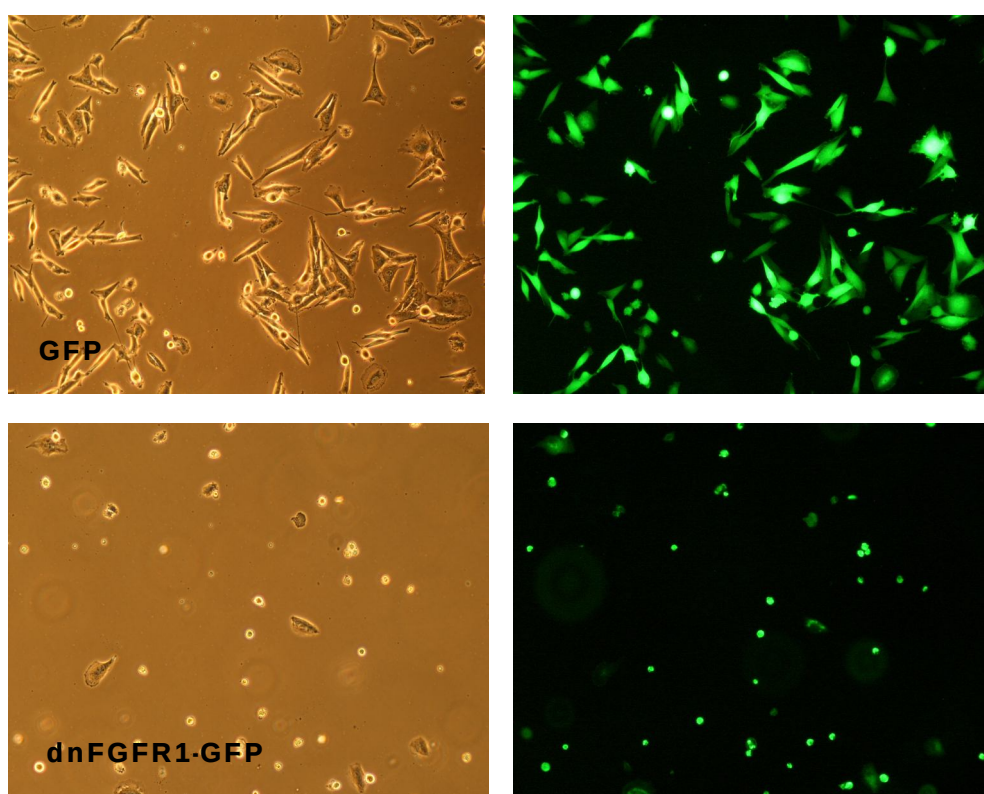


Figure 4.15: Microscopy of VM1 cells, infected with adenoviruses containing either GFP or GFP tagged dnFGFR1 constructs, after 48 hours.

Beside propidium-iodide staining and fluorescence microscopy (Figure 4.14 and 4.15), induction of apoptosis by FGFR-1 inhibition was also verified by Western Blot analysis (Figure 4.16).

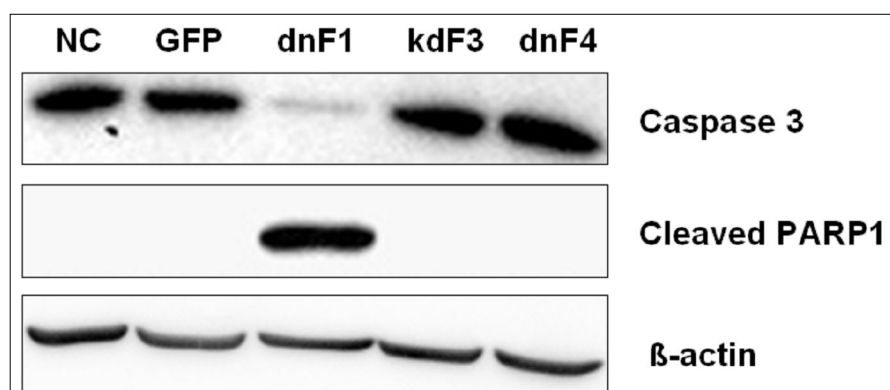


Figure 4.16: Induction of apoptosis in FTSL-A (VM1) cells transduced with GFP, dnFGFR1-GFP, kdFGFR3, dnFGFR4 adenoviruses, analysed by Western Blot. NC was an untransfected control. Likewise treated cells were used for in vivo growth by xenotransplantation experiment in SCID mice. Cells were re-seeded and proteins were isolated around one week after viral transduction..

PARP1 (Poly ADP-Ribose Polymerase 1) is a chromatin-associated enzyme and modifies various nuclear proteins by poly-ADP-ribosylation. The modification is dependent on DNA and is involved in the regulation of various important cellular processes such as differentiation, proliferation, tumour transformation and molecular events involved in DNA repair. It has been shown, that PARP1 is cleaved when apoptosis is induced, thus the protein is also used as apoptotic marker (Ha HC, 2000).

The caspase 3 enzyme is a member of the cysteine-aspartic acid protease (caspase) family. Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. Caspases exist as inactive pro-enzymes that undergo proteolytic processing at conserved aspartic residues to produce two subunits, large and small, that dimerize to form the active enzyme. The protein cleaves and activates caspases 6, 7 and 9, and is itself processed by caspases 8, 9 and 10 (Suzuki Y, 2001). As shown in (Figure 4.16) cleaved PARP1 and reduction of unprocessed caspase 3 are seen in dnFGFR1 transduced VM1 cells indicating induction of apoptosis.

4.3.2 Clonogenic Assays

Melanoma cells were tested for their ability to survive and form clones, when seeded at very low density. After desired clone sizes were reached, depending on specific clonogenicity of the cell line, cells were stained with crystal-violet. Numbers of clones were determined by counting the clone number above a specific threshold level, e.g.: all clones larger than 1mm in diameter.

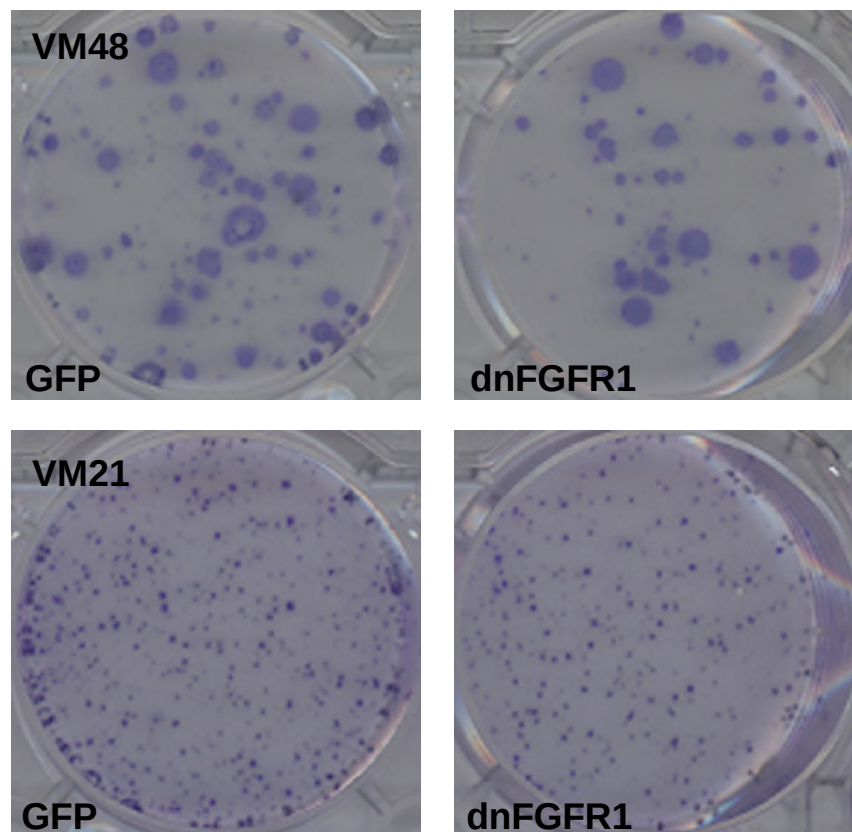


Figure 4.17: Example of two melanoma cell lines (VM48 and VM21) transduced with adenoviruses containing GFP or dnFGFR1-GFP constructs. Cells were infected, incubated for 24 hours, then seeded at low densities (3000 cells/well). Two independent experiment were performed.

Results showed a reduction in clonogenicity of around 30-50%, when melanoma cells were infected with dnFGFR1-GFP viruses, compared to those cells transduced with GFP adenoviruses. Thus, it is suggested that inhibition of the FGFR-1 signalling pathway affects also the ability to form clones, presumably due to reduced stimulation of growth and survival by surrounding cells (Figure 4.17 and 4.18).

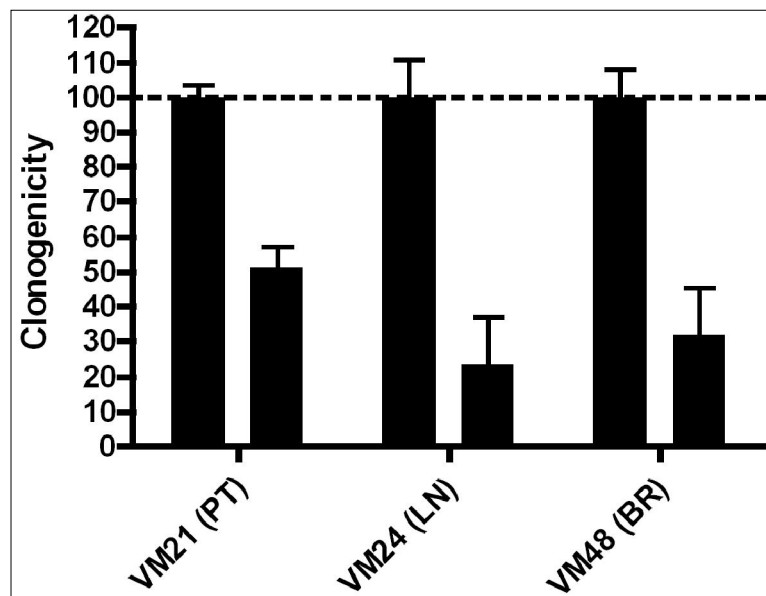


Figure 4.18: Results of Clonogenicity Assay, showing three different melanoma cell lines GFP versus dnFGFR1-GFP infected. Clonogenicity of GFP transduced melanoma cells were set as 100%. One cell line tested (VM1) did not form clones, when seeded at low density.

4.3.3 Soft Agar Assay

Melanoma cell lines were tested for their anchorage independent growth in three-dimensional culture. Cells were seeded in 10% RPMI medium including agar. The formation of clones was monitored and the number of clones with a diameter above a specific threshold ($> 20 \mu\text{m}$) was calculated. Cells were incubated around one month, but adenoviruses are suggested to be diluted out already after three weeks, thus fluorescence was reduced significantly. Therefore, small clones were not taken into consideration. Results showed significant reduction of anchorage independent clonogenicity. Thus, the ability to form clones in soft agar was reduced around 90% in three of four melanoma cell lines infected with dnFGFR1-GFP bearing adenoviral constructs (Figure 4.19 and 4.20).

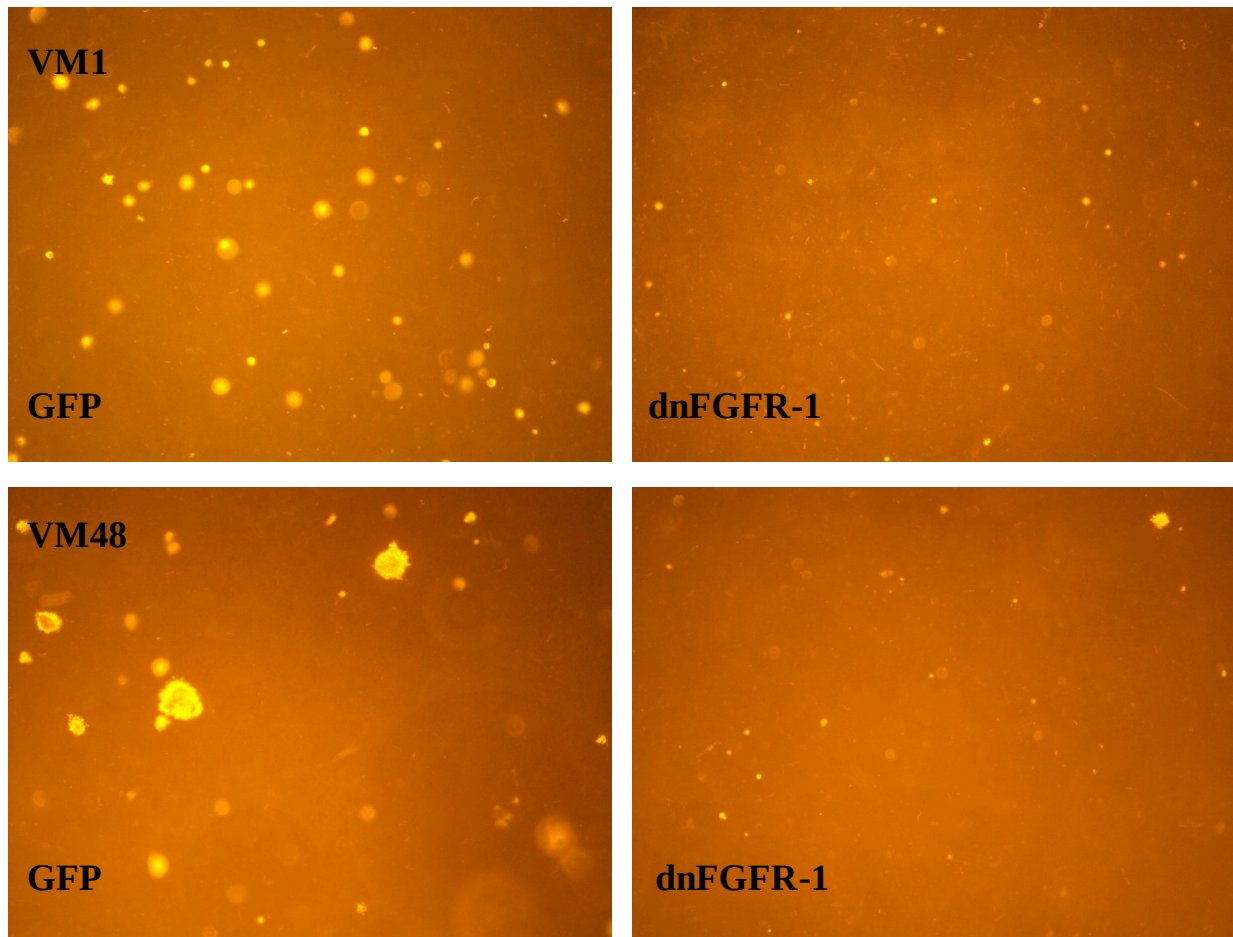


Figure 4.19: Example of two melanoma cell lines (VM1 and VM48) tested for anchorage independent clonogenicity in soft agar. Cells were infected with adenoviruses bearing GFP or dnFGFR1-GFP constructs, before seeding. Experiment was done once for all four cell lines, four different areas per well were counted.

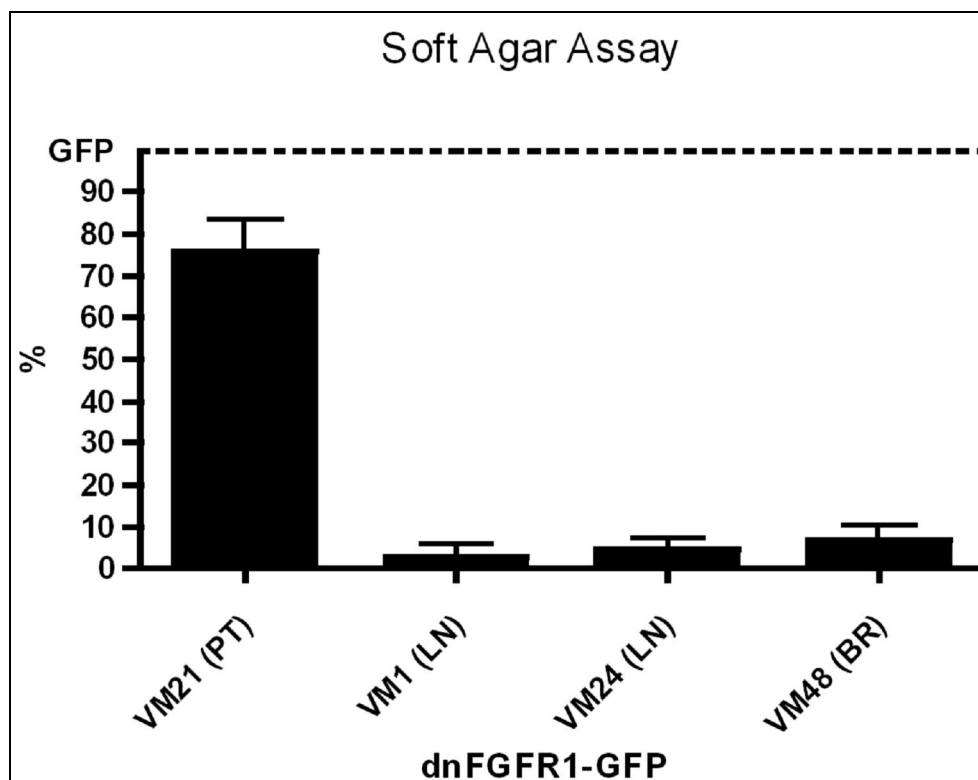


Figure 4.20: Results of soft agar assay of all four cell lines tested. Cells were infected with adenoviruses (GFP versus dnFGFR1-GFP). 30 000 cells were seeded. Melanoma cells infected with GFP adenoviruses were taken as reference and set as 100%.

4.3.4 In Vivo Tumour Growth

To test FGFR-1 inhibition of melanoma cell lines under in vivo conditions, cells were infected with adenoviruses containing GFP versus dnFGFR1-GFP constructs and subsequently injected subcutaneously into SCID mice. Tumour growth was monitored over several months. The tumour volume was calculated according to the formula $((d_1^2 \times d_2)/2 = \text{tumour size in mm}^3$; d_1 ...diameter of tumour in mm; $d_1 < d_2$). Mice were sacrificed when tumours had reached a certain size ($>4000 \text{ mm}^3$) or when mice showed signs of morbidity (Figure 4.21).

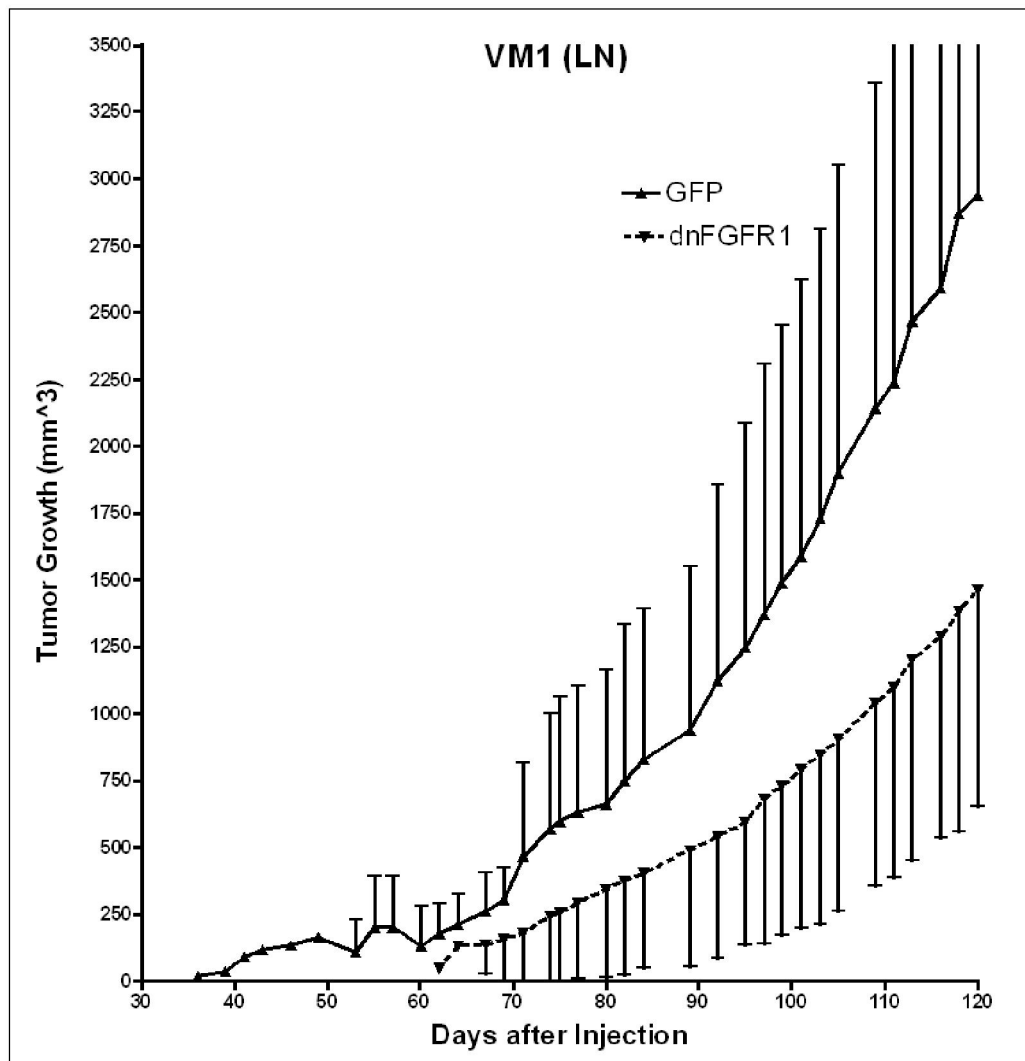


Figure 4.21: Tumour growth of melanoma cell line VM1 infected by adenoviruses containing GFP versus dnFGFR1-GFP constructs. Following infection, one million cells (in 50 μ l serum free medium) were injected subcutaneously into SCID mice.

Tumour growth was reduced to around 50% when the FGFR-1 signalling pathway was down-regulated by dnFGFR1-GFP adenoviral infection (Figure 4.21). VM1 melanoma cells infected with kdFGFR3 or dnFGFR4-CFP adenoviral constructs did not show a reduction in tumour growth. Increased apoptosis, thus programmed cell death, was shown by Western Blot analysis (Figure 4.16) in cells transduced with dnFGFR1-GFP constructs, compared to GFP virus infected controls. Overall survival of SCID mice was also increased, when dnFGFR1-GFP infected melanoma cells were injected, compared to GFP control (Figure 4.22).

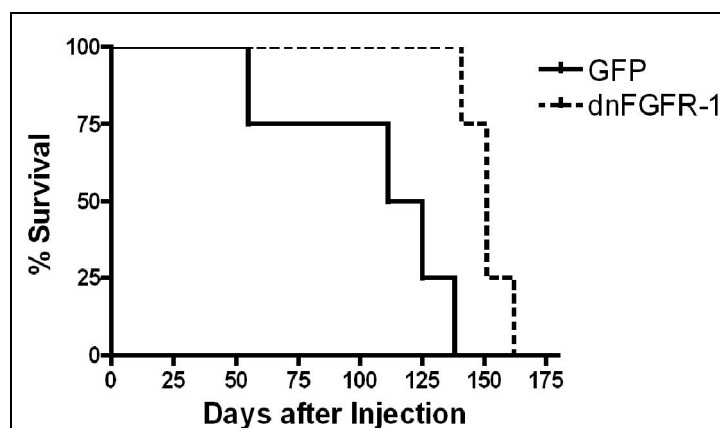


Figure 4.22: Percent survival of SCID mice, when melanoma cells transduced with GFP or dnFGFR1-GFP constructs were injected.

4.4 Cell Biologic Effects of FGF Stimulation

Basic fibroblast growth factor (bFGF or FGF-2) is known to be produced by nearly all melanomas in vitro and in vivo but not by normal adult melanocytes, which require exogenous FGF-2 for growth. This has been verified by different studies, including this one, where all investigated melanoma cell lines showed high FGF-2 expression levels. The following experiments showed, that despite endogenous FGF expression, exogenous FGF-2 not only increases cell proliferation, but also stimulates cell migration and invasion.

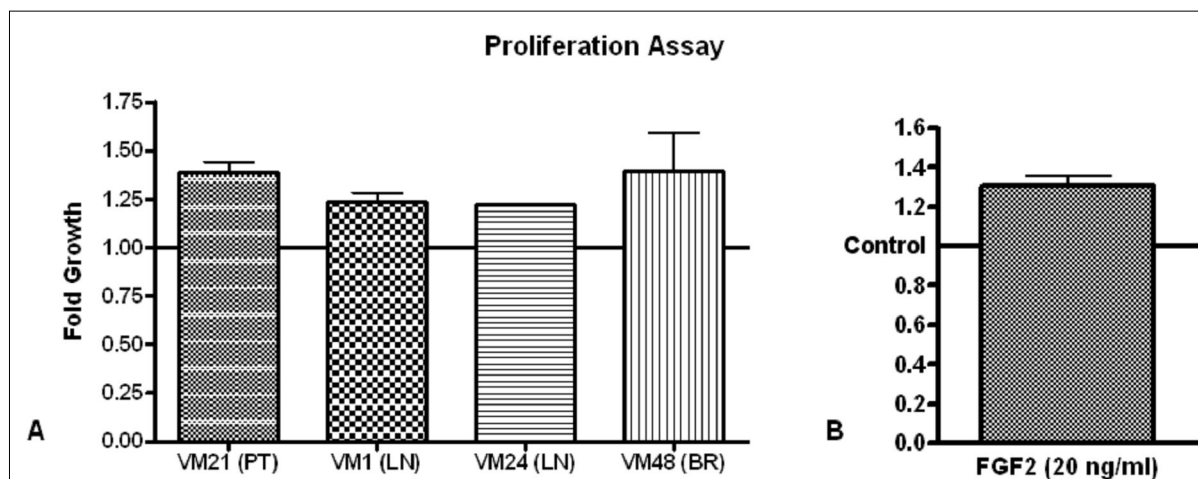


Figure 4.23: Four different VM cell lines were incubated with FGF-2 (20 ng/ml). 6000 cells were seeded in medium with 10% FCS. The following day, FGF-2 was added at 2x concentration in serum free medium, yielding a medium with 5% FCS. Cells were incubated for six days. Controls contained no FGFs. **A:** FGF-2 stimulated growth in all four cell lines tested **B:** Average growth stimulation of VM cell lines, compared to controls containing no FGF-2. Two independent experiments were done in duplicates.

All four melanoma cell lines incubated with 20 ng/ml FGF-2 proliferated around 30% more rapidly than those not stimulated (Figure 4.23). Considering the strong autocrine FGF-2 expression of those cell lines, showing up to 150 x higher expression levels than primary melanocytes (*PM: 1,1; VM21: 25,4; VM1: 58,1; VM24: 150,4; VM48: 6,1 Relative Expression*), these stimulatory effects are quite strong. Those two cell lines showing the lowest FGF-2 expression, namely VM48 and VM21, could be more strongly stimulated for proliferation than the other two. Proliferation of VM48 cells was increased by 50%, despite having 6 x higher FGF-2 expression than primary melanocytes.

Those two melanoma cell lines, having the highest and lowest FGF-2 expression (VM24 and VM48), were subjected to migration assays. Only those cells, able to migrate through the transwell chamber and settle on the bottom of the well, were taken into consideration. Three other cell lines were not further investigated, because cells did not migrate properly. Migration was stimulated by FGF-2 and FGF-5, reported as an oncogenic factor in human glioblastoma (Allerstorfer, Sonvilla et al., 2008), at physiological (20 ng/ml) and supra-physiological (each 100 ng/ml) concentrations (Figure 4.24).

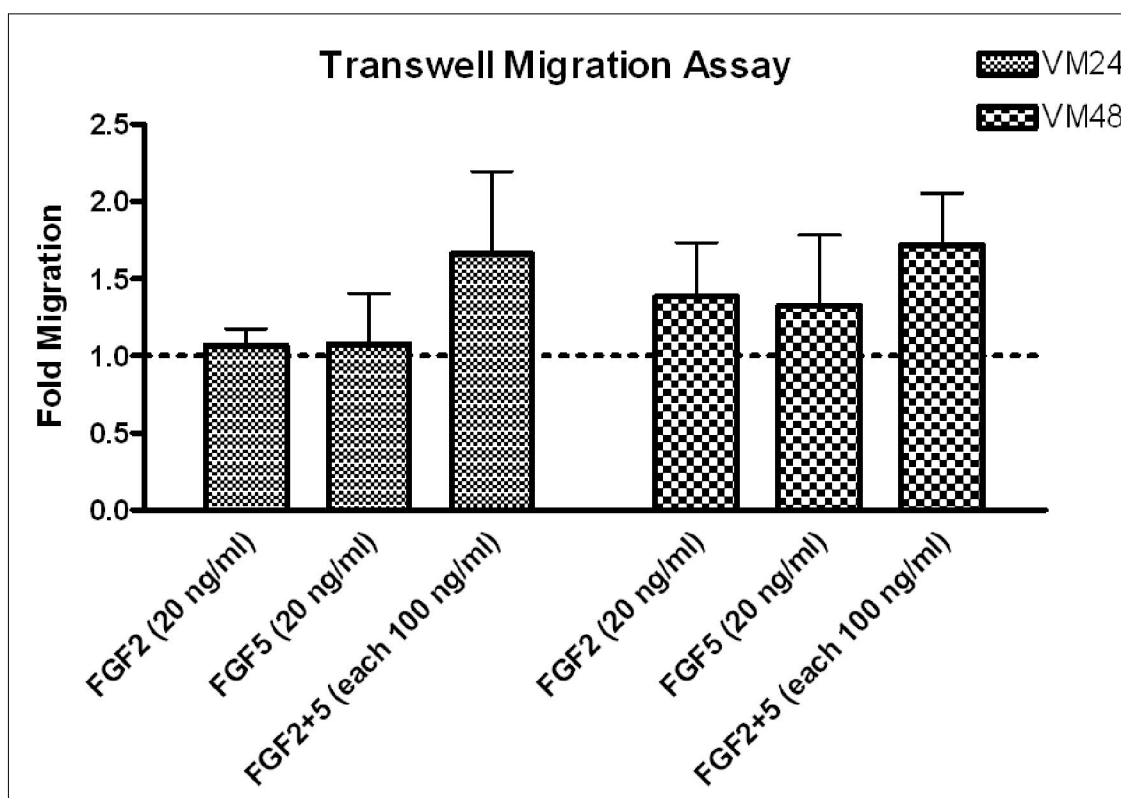


Figure 4.24: Two melanoma cell lines were tested for migration through a porous membrane, when stimulated by different concentrations of FGF-2, compared to non-stimulated cells. 40 000 cells were seeded into a transwell chamber in RPMI containing 4% FBS and incubated for 72 hours. Cells which migrated through the porers of the membrane and dropped to the bottom of the well were stained by crystal violet and analysed by Lucia Software (Nikon). Two independent experiments were done in duplicates.

FGF-5 expression was not detected (FGF-5 short) or very weakly expressed (FGF-5 long) in primary melanocytes, but was up-regulated in many melanoma cell lines. The long isoform of FGF-5 was also up-regulated in VM24 cells (37,4) used in this migration assay, but was low in VM48 melanoma cells (0,4), as well as in primary melanocytes (0,2). Thus, the VM24 cell line showed 187 x higher FGF-5 expression compared to primary melanocytes.

Surprisingly, both FGFs showed nearly the same stimulatory effects on migration (Figure 4.24 and 4.25). The cell line expressing high levels of FGF-2 and FGF-5, thereby stimulating itself in an autocrine manner, showed less stimulatory effects than the VM48 cell line having low FGF-2 and FGF-5 expression levels. In comparison, the VM24 cell line increased migration by around 20-30% and the VM48 even by up to 50%. Besides, migration could be further stimulated at higher FGF concentrations (100 ng/ml each), and resulted in around 2 x higher motility.

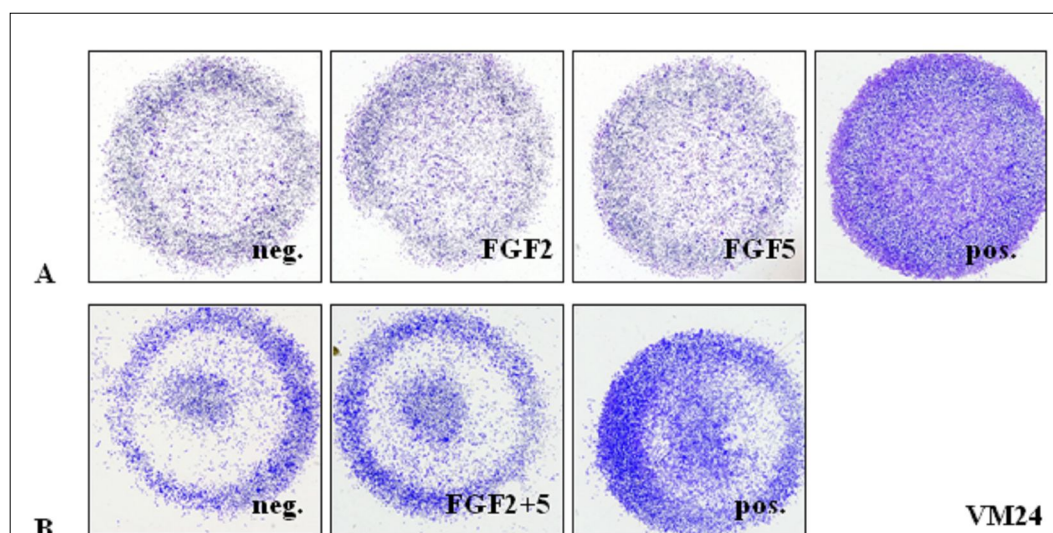


Figure 4.25: Migration ability was tested by Transwell Chamber Assays, for five VM cell lines (VM1; VM48; VM24; VM21 and VM7), at different serum and FGF concentrations. Cells migrated for 72 hours. Negative controls (neg.) and media including FGFs contained only 4% serum, while positive controls (pos.) contained 10% FCS. FGF concentrations: **A**, 20 ng/ml; **B**, each 100 ng/ml. One representative experiment with VM24 cells is shown.

4.5 Effects of FGF Stimulation and Small Molecule Inhibition on FGFR Downstream Signalling

The aim of this part of the study was (A) to stimulate FGFR signalling by FGF-2 for identification of downstream regulators in melanoma, and (B) to suppress FGFR signalling by small-molecule FGFR inhibitors. FGFR signalling is complex, but main signalling is suggested to run over the MAPK-pathway and Akt-pathway, thus we focused on those two signalling cascades. The FGFR1-specific inhibitor PD166866 (Panek, Lu et al., 1998) was generously supplied by Pfizer Global Research and Development and the pan-FGFR inhibitor SU5402 (Mohammadi, McMahon et al., 1997) was obtained from Calbiochem.

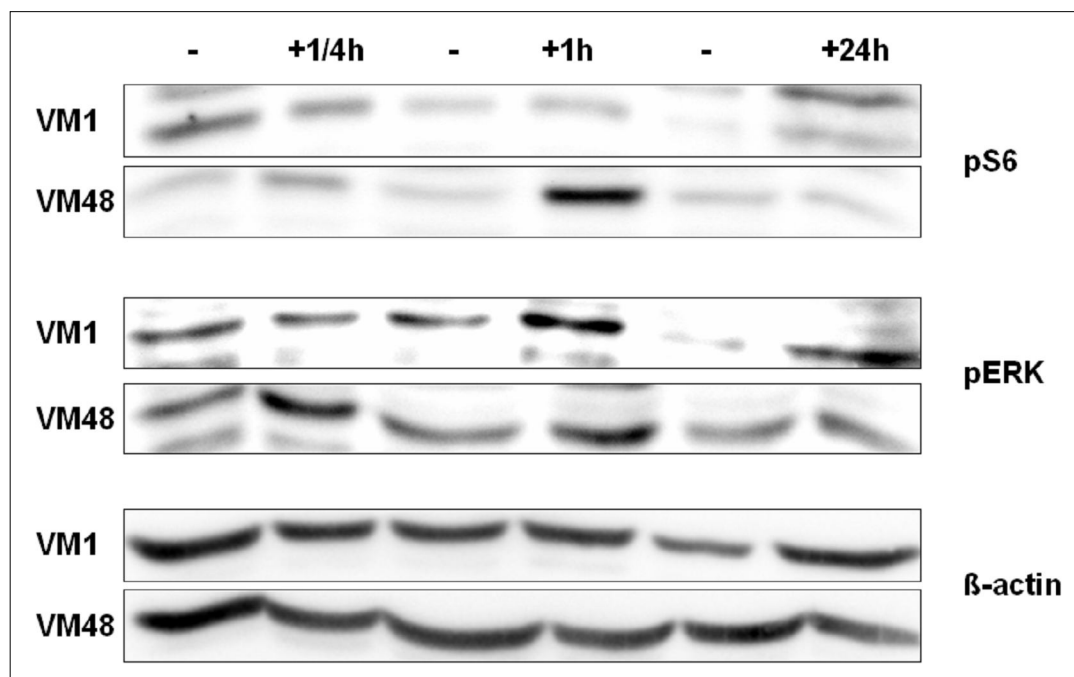


Figure 4.26: Two melanoma cell lines were stimulated with FGF-2 and FGF-5 (each 50 ng/ml) for different durations of time (15 min, 60 min and 24 hours). Cells were starved for 24 hours under serum free conditions, before FGFs were added. Proteins were isolated by scraping cells into ice cold PBS and subsequent centrifugation and lysis by addition of lysis buffer (LBII).

Stimulation of FGFR downstream signalling was found after FGF-2/FGF-5 incubation for different time periods (Figure 4.26). Both melanoma cell lines investigated, displayed autocrine FGF stimulation mainly by FGF-2 up-regulation, shown by qRT-PCR expression analysis. When treated with a combination of exogenous FGF-2 and FGF-5, the VM1 cell line showed increased S6 phosphorylation after 24 hours, while VM48 cells displayed increased phosphorylation after 1 hour. Considering endogenous FGF-2 and FGF5 expression levels, the VM1 cell line had a 50 fold up-regulation of FGF-2 and a 30 fold up-regulation of FGF-5, compared to primary melanocytes. VM48 cells had only a 5 fold up-regulation of FGF-2, FGF-5 was not detected. Nearly the same result was obtained for ERK1/2 phosphorylation. While phosphorylation of ERK1/2 took place in VM48 cells already after one hour, in the VM1 cell line phosphorylation seems to occur at a later stage.

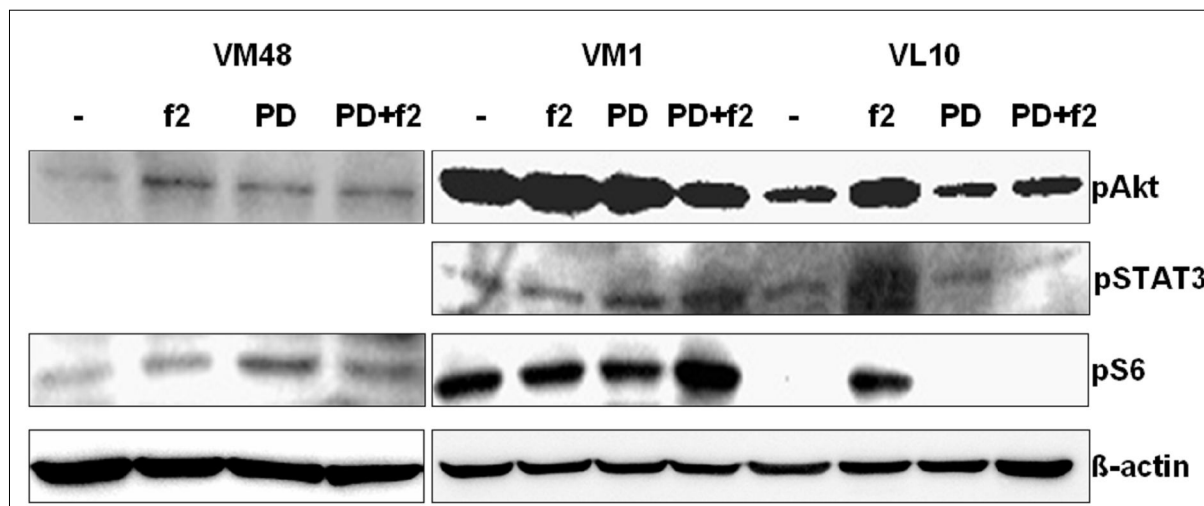


Figure 4.27: Effects of FGF-2 (f2, 20 ng/ml) and “Small Molecule Inhibitor” PD166866 (5 μ M) on FGFR downstream signalling. Cells were starved for 48 hours in serum free medium. PD166866 was added one hour and FGF-2 15 minutes before harvest. Proteins were isolated by scraping cells into ice cold PBS, then according to standard protocol.

FGFR stimulation by FGF-2 for a short time period of 15 minutes, suggested to be sufficient for activation of phosphorylation cascades, showed very weak to no stimulation in both melanoma cell lines, considering the phosphorylation of Akt and S6 and STAT3 (Figure 4.27).

The VL10 cell line was used as positive control, because FGFR downstream signalling was described already in this cell line (Fischer, Taylor et al., 2008). The VM48 cell line, having low endogenous FGF-2 expression compared to VM1 cells, was more suitable for FGF stimulation again. Down-regulation of FGFR signalling by FGFR-1 small-molecule inhibitor PD166866 was not achieved, as far as VM48 cells are concerned, even a weak stimulation was observed. However, prior incubation of PD166866 prevented subsequent stimulation by FGF-2 incubation, at least in VM48 cells.

Further experiments, focusing on incubation with small-molecule inhibitors PD166866 and SU5402 for different periods of time (2h and 24h), showed rather unexpected results. Instead of inhibiting signalling, the opposite was observed, namely stimulation of MAPK- and Akt- signalling pathways (Figure 4.28 and 4.29).

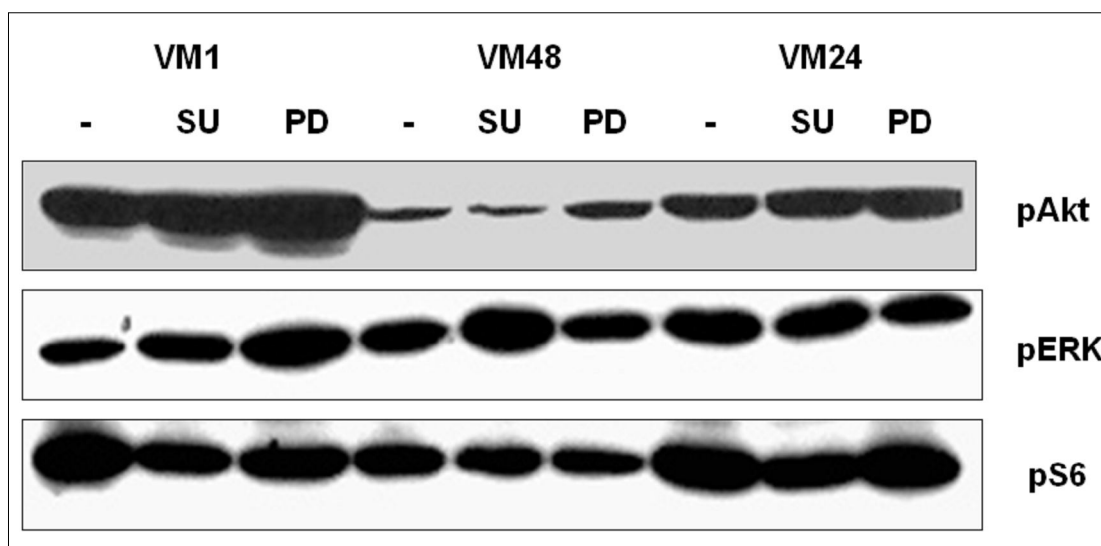


Figure 4.28: Three melanoma cell lines were incubated for 2 hours with “Small Molecule Inhibitors” SU5402 (20 μ M) and PD166866 (20 μ M). Cells were incubated in 5% RPMI. Proteins were isolated by scraping cells into ice cold PBS, then according to standard protocol.

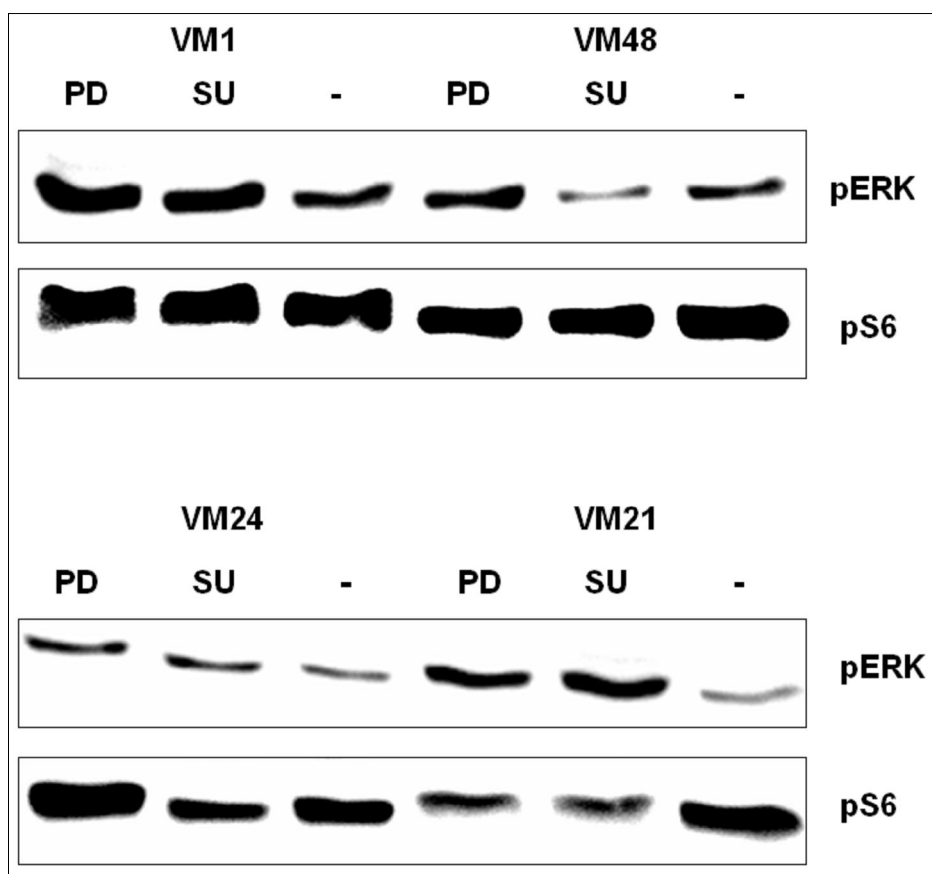


Figure 4.29: Four melanoma cell lines were incubated for 24 hours with “Small Molecule Inhibitors” SU5402 (20 μ M) and PD166866 (20 μ M), in 5% RPMI. Proteins were isolated by cell trypsinization, not by scraping into PBS, the rest was done according to standard protocol.

4.6 Investigation of the Cell Biologic Significance of the FGFR-4 Arg/Gly Polymorphism

Recently, a germ line polymorphism in the gene encoding for FGFR-4 was discovered (Bange, Prechtel et al., 2002). This single nucleotide polymorphism (SNP) results in a change of the amino-acid sequence at codon 388 from glycine to arginine (Gly388 to Arg388). It is suggested, by different studies, that the FGFR-4 Arg388 allele positively stimulates cancer progression in a variety of tumour types (Bange, Prechtel et al., 2002; Falvella, Frullanti et al., 2009; Morimoto, Ozaki et al., 2003; Wang, Stockton et al., 2004; Wang, Yu et al., 2008) and is associated with adjuvant therapy resistance in primary breast cancer (Thussbass, Nahrig et al., 2006).

The purpose of this part of the thesis was, to evaluate whether the FGFR-4 Arg388 allele correlated with metastasis in melanoma, and to investigate its effects on proliferation and migration. This was achieved (A) by RFLP (Restriction Fragment Length Polymorphism) analysis of 41 melanoma cell lines from primary tumours and metastases of melanoma and (B) by establishing FGFR-4 Arg388 versus Gly388 expressing cell lines and investigating their migration and proliferation ability.

4.6.1 Investigation of the FGFR-4 Arg/Gly388 Polymorphism by RFLP Analysis

The FGFR-4 Arg/Gly polymorphism was investigated on the RNA level, thus may not necessarily correspond with genomic DNA. The denotations heterozygous or homozygous are related in this context to expression and not genotype. RNA was isolated, cDNA was synthesized and FGFR-4 fragment was amplified by standard RT-PCR. Afterwards, the amplicon was cleaved with restriction enzyme MspI (Fermentas) and fragments were separated by 8.5% PAGE gels (Figure 4.30).

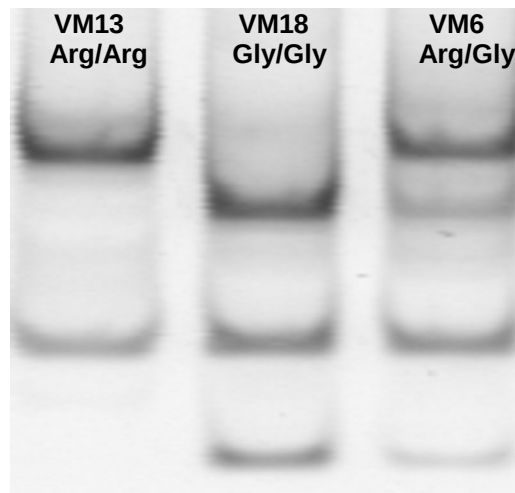


Figure 4.30: Example of RFLP (Restriction Fragment Length Polymorphism) analysis of three different melanoma cell lines, thereby identification of expressed FGFR-4 (Arg/Gly) variants.

Due to the SNP (Single Nucleotide Polymorphism) a second cleavage site is present in the Gly-allele producing three fragments: 85 bp; 50 bp; 30 bp. In contrast, the amplicon of the Arg-allele is cleaved only once generating two fragments of 115 bp and 50 bp.

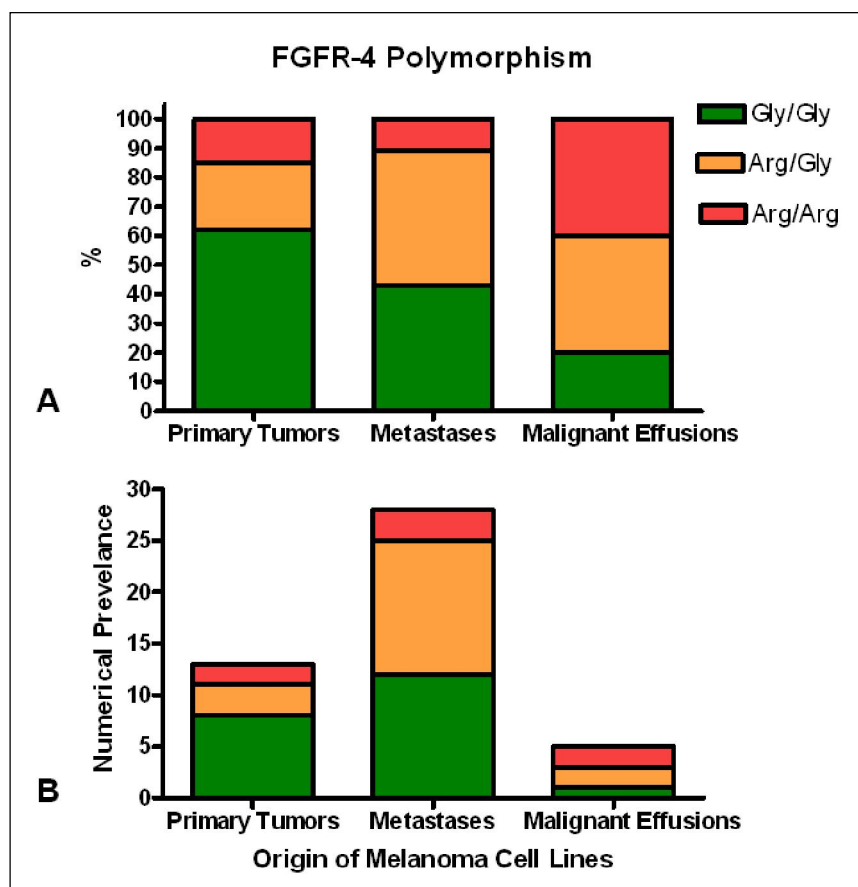


Figure 4.31: Correlation of FGFR-4 "Arg" expression with malignant tumour progression. (A) Percentage of cell lines, of different origins (Primary Tumours; Metastases; Malignant Effusions), expressing specific FGFR-4 variants. (B) Total number of cell lines tested for FGFR-4 Arg/Gly polymorphism.

Co-expression of the “Arg/Gly” allele was found in around 23% of cell lines originating from a primary tumour (Figure 4.31 and Table 4.3), in contrast to 46% of cell lines derived from metastases. Cell lines expressing only the FGFR-4 “Gly” allele were found in 62% of cells originating from primary tumours, but only in around 43% of metastasis derived cells, a reduction of around 19%. Considering cell lines originating from malignant effusions, only one of five cell lines investigated, was found to be “homozygous” for the FGFR-4 “Gly” allele, whereas two of them, were found to be homozygous for the FGFR-4 “Arg” allele. Although only five cell lines could be investigated, the high prevalence of the “Arg” allele in this late stage of melanoma progression is quite impressive. Due to these results, we decided to further investigate the effects of the FGFR-4 “Arg” allele on cell proliferation and cell motility, by generating stable cell lines ectopically expressing the “Gly” and “Arg” alleles.

Table 4.3: Analysis of FGFR-4 Arg/Gly polymorphism by RFLP. List of all 41 melanoma cell lines, including primary melanocytes, VM (Vienna Melanoma) numbers, origin^a and histology^b.

Cell Line	Origin	Histology	Polymorphism	Cell Line	Origin	Histology	Polymorphism
Mela	PM		GG	VM-37 Xfoa	SC	NM	GG
VM-10 Jmum	PT	SSM	GG	VM-18 Pnjc	SC	ALM	GG
VM-19 Rall	PT	SSM	GG	VM-34 Xbne	SC	Unknown	GG
VM-30 Wcre	PT	SSM	GA	VM-35 Xdbc	SC	Unknown	GA
VM-36 Xewl	PT	SSM	GG	VM-9 Gyk	BN	SSM	GG
VM-44 Xapg	PT	SSM	GG	VM-22 Rima	BN	NM	GG
VM-2 Gcrf	PT	NM	AA	VM-46 Huma	BR	NM	AA
VM-7 Gtbs	PT	NM	GG	VM-47 Host	BR	NM	GG
VM-21 Rhtp	PT	NM	GG	VM-48 Kaka	BR	NM	GA
VM-23 Rktj	PT	NM	GG	VM-50 Hoin	BR	NM	GG
VM-25 Tpck	PT	NM	GA	VM-28 Tmfi	BR	Unknown	GA
VM-32 Wpza	PT	NM	GG	VM-41 Ydfr	BR	Unknown	GA
VM-33 Wubi	PT	NM	GA	VM-31 Wltj	ME	NM	GG
VM-38 Xiba	PT	NM	AA	VM-11 Jtst	ME	NM	GA
VM-5 Grbd	LN	SSM	GA	VM-13 Krfr	ME	NM	AA
VM-1 Ftst/a	LN	SSM	GG	VM-16 Myah	ME	Unknown	GA
VM-20 Rgoh	LN	NM	GA	VM-3 Gesp	ME	Unknown	AA
VM-4 Glj	LN	NM	GA				
VM-6 Gsta	LN	NM	GA				
VM-8 Gubs/a	LN	NM	GG				
VM-12 Jwmw	LN	NM	GA				
VM-14 Lcwc	LN	NM	GA				
VM-15 Mjzj	LN	Unknown	GA				
VM-17 Nra	LN	Unknown	GG				
VM-24 Shtj	LN	Unknown	GG				

^aEstablishment of cell lines from primary tumours (PT), lymph node (LN), subcutaneous (SC), bone (BN), brain (BR) metastases, malignant effusions (ME) of malignant melanoma and primary melanocytes (PT), from which cDNA was used as control and was obtained from primary culture only. ^bHistology of the primary tumour: ALM, acral lentiginous melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma.

4.6.2 Generation of FGFR-4 Arg388 versus Gly388 Expressing Cell Lines and Investigation of Migration and Proliferation

Two melanoma background cell lines, namely VM27 (GTBS) and VM24 (SHTJ), were chosen for the generation of stably expressing FGFR-4 Arg/Gly388 cells. Both cell lines displayed low FGFR-4 expression levels and were Gly388 homozygous, according to RFLP and qRT-PCR analysis. Cell lines were lipofected with plasmids coding for FGFR-4 Arg388 or FGFR-4 Gly388, regulated by a constitutively active promoter region, and with an empty control plasmid (pcDNA3). Vectors as well as control DNA coded for an antibiotic resistance gene (G418), making the selection procedure possible. After lipofection and selection, four clones (A-D) per approach were picked, resulting in a total of 24 cell fractions. Subsequently, cDNA was generated from all cell lines, which were then analysed by RFLP, qRT-PCR and Immuno-histochemistry (Figure 4.32 and 4.33). However, only two FGFR-4 Arg388 cell lines (SHTJ) were identified as positive and subjected for proliferation and migration assays. Transfected GTBS cells did not deliver any Arg388 positive cell lines.

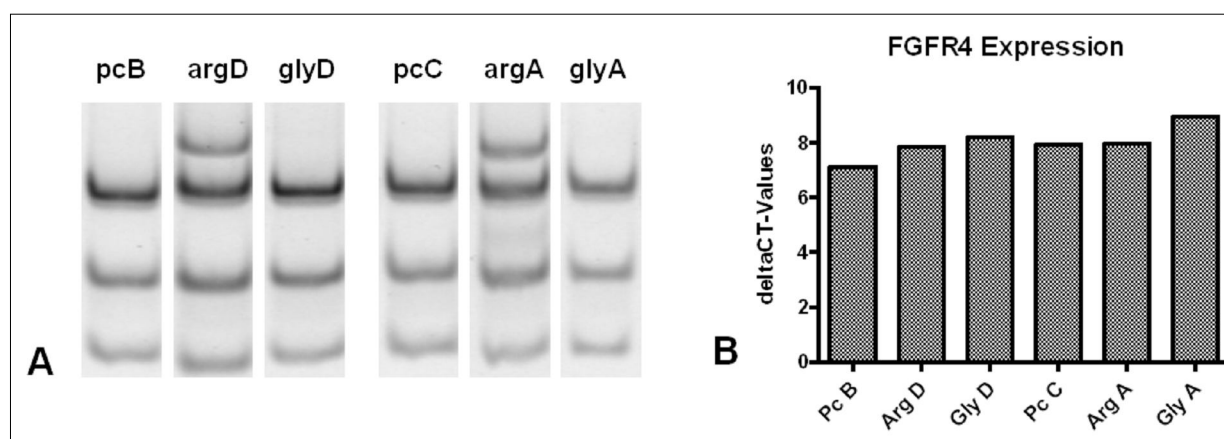


Figure 4.32: RFLP (A) and qRT-PCR (B) analysis of FGFR-4 Arg/Gly388 transfected GTBS and SHTJ cells. Only SHTJ melanoma cells subjected for further analysis are shown. The abbreviations pc/arg/gly imply transfected DNA (pc: pcDNA3 control; arg: FGFR-4 Arg388 vector and gly: FGFR-4 Gly388 vector), while A/B/C/D imply picked clones.

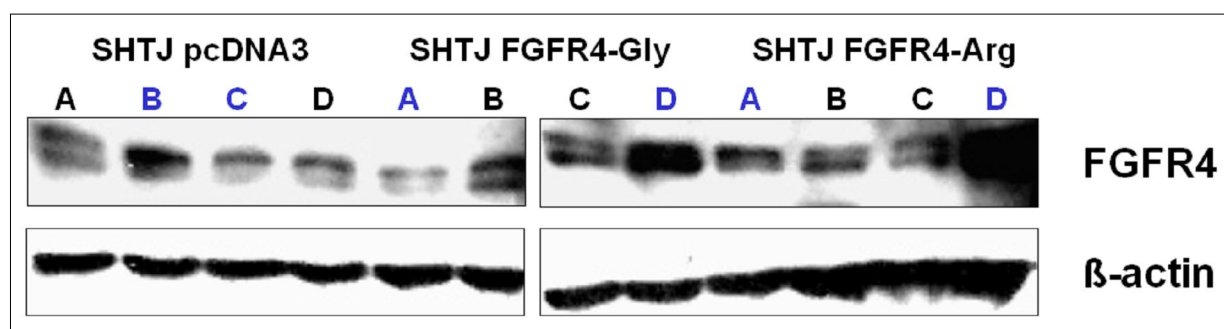


Figure 4.33: Western Blot analysis of FGFR-4 Arg/Gly388 transfected GTBS and SHTJ cells. Only SHTJ melanoma cell lines are shown. Abbreviations A-D imply clone label, blue coloured cell fractions were subjected to further cell biological analysis.

For investigation of cell biological effects, such as proliferation and migration, transfected cell fractions were stratified according to FGFR-4 expression levels, analysed by qRT-PCR and Western blot (Figure 4.32 and 4.33). Thus, SHTJ melanoma cell lines pcB/argD/glyD and pcC/argA/glyA were compared among each other. According to Western Blot analysis, cell lines pcB/argD/glyD seem to have slightly more FGFR-4 expression than the others. However, qRT-PCR analysis showed no differences in FGFR-4 expression levels. Surprisingly, FGFR-4 expression levels of transfected FGFR-4 Arg/Gly388 transfected cells were not significantly higher than pcDNA3 controls. Even if exogenous FGFR-4 expression is low, it must be sufficient to induce a visible shift in the RFLP analysis (Figure 4.32, A).

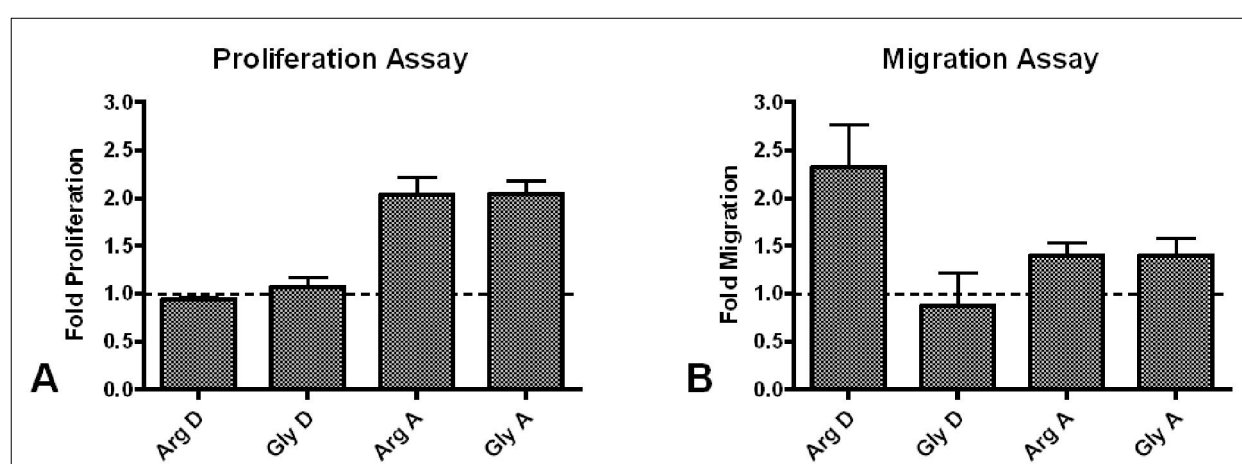


Figure 4.34: Stably expressing FGFR-4 Arg/Gly388 melanoma cells were tested for proliferatory and migratory ability. (A) Proliferation Assay, 12 000 cells were seeded and incubated for 6 days in 5% RPMI, increase in cell number was measured by Casy Cell Counter (Schärfe). (B) Migration Assay, 40 000 cells were seeded into a Transwell Chamber and incubated in 4% RPMI for 72 hours, cells were stained by crystal violet afterwards. Control pcDNA3 was used as reference and set as 1. Two independent experiments in duplicates were done at least.

Two cell lines, lipofected with FGFR-4 Arg/Gly388 (ArgA and GlyA), had a two-fold increased proliferation rate, compared to those cells transfected with control pcDNA3 (pcC). However, the other two cell lines (ArgD and GlyD) showed no significant difference to their control cell line (pcB). Considering cell migration, ArgD cells were 2.5 times more motile than GlyD cells, although having the same proliferative ability. ArgA and GlyA cell lines had also a 1.5 x increased migratory activity than the control, but this effect might be an indirect one caused by the increased proliferation of these two clones (Figure 4.34 and 4.35).

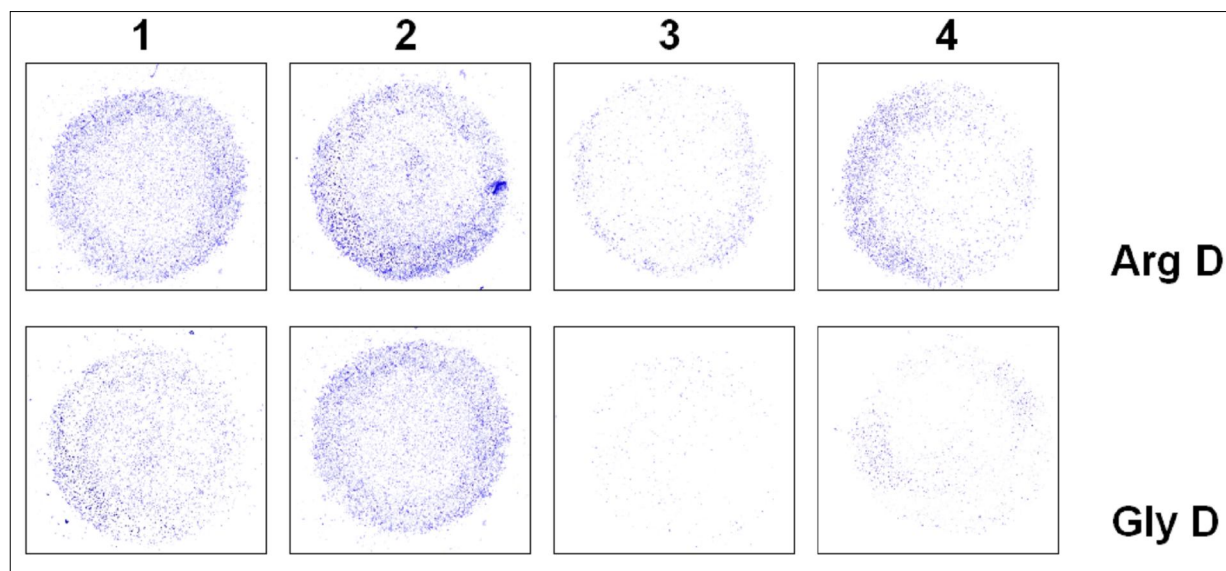


Figure 4.35: Migration assay, example of two cell lines, namely ArgD versus GlyD. Two independent experiments were done in duplicates (1-4). 40 000 cells were seeded into a Transwell Chamber and incubated in 4% RPMI for 72 hours, cells that migrated through the membrane and dropped to the bottom of the well were stained by crystal violet afterwards.

4.7 Generation of Cell Lines for Inducible Expression of Dominant-Negative FGFRs (dnFGFRs) - Using the Advanced Tet-Off System

Two melanoma cell lines (KAKA;VM48 and FTSL-A; VM1), that showed high lipofection efficiency (>30%), were chosen for the generation of inducible expression clones with the Advanced Tet-Off System (Clontech).

In summary, the system consists of a small (22 kDa) transcriptional regulator protein TetR (Tet Repressor), also referred as rTA-Advanced (transcriptional activating fusion protein) in the modified advanced system, and a TRE (Tet-Response Element) sequence prior to a gene of interest. TetR blocks transcription of genes, by binding to TRE sequences, only in presence of doxocyclin, a derivate of tetracyclin. A more detailed description of the system is given in Clontech manuals (Tet-Off Advanced Inducible Gene Expression System User Manual, Clontech). Melanoma cell lines were transfected with a plasmid containing the TetR gene driven by a constitutive promoter and an antibiotic resistance gene coding for G418 resistance. Lipofection and selection was done according to the standard protocol. After clones were picked and expanded, cells were functionally tested for TetR expression.

For that purpose, cells were transiently transfected with a plasmid encoding an EGFP reporter gene coupled to a Tet Response Element (TRE) acting as promoter. Cells from a TetR-expressing clone, were separated into two wells. One sample was incubated with doxocyclin (100 ng/ml) while the other was not. The sample including doxocyclin should prevent reporter gene expression, which in our case would be EGFP, thus no fluorescence should be visible under the microscope (Figure 4.36).

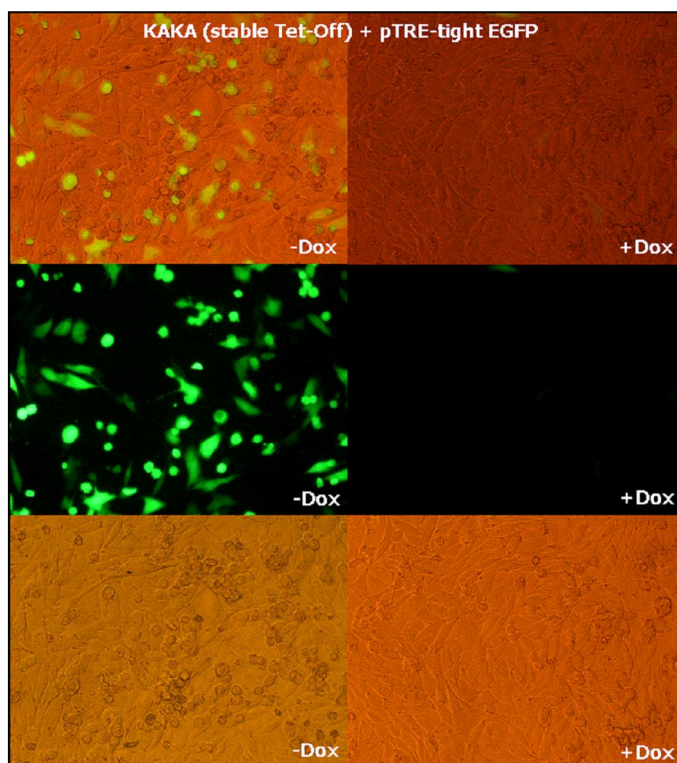


Figure 4.36: Example of one expanded KAKA (VM48) TetR positive clone, tested functionally by suppressing EGFP expression due to doxocyclin supplementation. Putative TetR expressing KAKA cells were lipofected with the plasmid coding for the response element and the EGFP gene. Therefore, GFP expression can be only considered as transient. Pictures were taken 48 hours after addition of doxocyclin. On the left side, the sample without doxocyclin and on the right side including doxocyclin.

Suppression of EGFP fluorescence in the TetR transfected stable clones by the addition of doxocyclin proves functional expression of the TetR protein. Fluorescence of transfected cells corresponded with standard lipofection efficiency in this cell line of about 30%, thus cells can be seen as stable TetR expressing. In our case, four out of six picked clones, were identified as TetR positive (Kaka TetR 1/2/3 and 6).

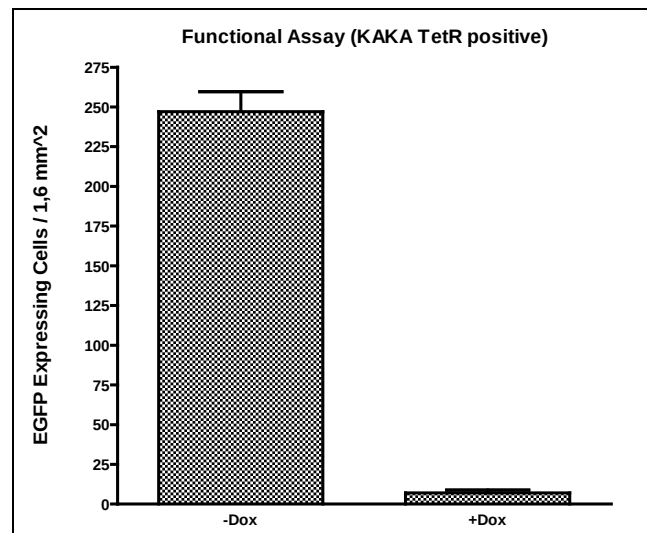


Figure 4.37: Quantification of fluorescent TetR positive melanoma cells (KAKA; VM48), with and without doxocyclin (100 ng/ml). Cells were counted under the microscopy.

Fluorescent cells were counted under the microscopy (Figure 4.37), within a defined space, and compared with the sample including doxocyclin. Quantification revealed a reduction of fluorescent cells, in the sample with doxocyclin, of around 98%. However, this does not implement, that expression of the reporter gene (EGFP) is zero in non fluorescent cells, there might be a low and invisible expression level. Therefore, expression levels of EGFP should be determined by qRT-PCR and Western Blot analysis. For further and more accurate quantification of fluorescent cells, one could perform FACS (Fluorescence Activated Cell Sorter) analysis.

For generation of stable, inducible expression of a gene of interest (GoI; EGFP and dnFGFRs), one has to integrate the GoI, linked to TRE sequence, into the genome. Thus, a second transfection and selection procedure was necessary. We decided to use the KAKA TetR-2 cell line as background strain. Because the TRE vectors used, did not possess a selection marker, they had to be co-lipofected with a selection plasmid. In our case, the plasmid for selection contained the puromycin resistance gene with a constitutive promoter.

However, only KAKA TetR-2 TRE-EGFP clones could be generated. When cells were transfected with dnFGFRs, no clones were formed at all. This might happen due to leakiness of the TRE-tight system causing low expression of the gene of interest. Combined with suggested cell toxicity of dnFGFRs this could lead to cell death of all positive cells.

In case of KAKA TetR TRE-EGFP clones, cells were expanded and functionally tested again. The second round of lipofection and selection was done without G418, to conserve cells, but with doxocyclin to suppress expression of dnFGFRs (P/S, Puromycin, Doxocyclin). Therefore, the KAKA TetR-2 background strain was always kept in doxocyclin (P/S, G418, Doxocyclin).

For functional testing, the first step was to remove the doxocyclin and look for EGFP expression. Only three clones, out of eight, showed EGFP expression (Figure 4.38). Surprisingly not all cells were expressing the EGFP protein, but only a small subpopulation of about 1-3%. The first suggestion was, that the used cell line KAKA TetR2 was contaminated with the normal background cell line (KAKA, VM48), and this subpopulation might have overgrown TetR cells. Therefore, the G418 was immediately resupplemented. The expanded, “positive”, cells were monitored by microscopy for several days, but no change in subpopulations could be observed.

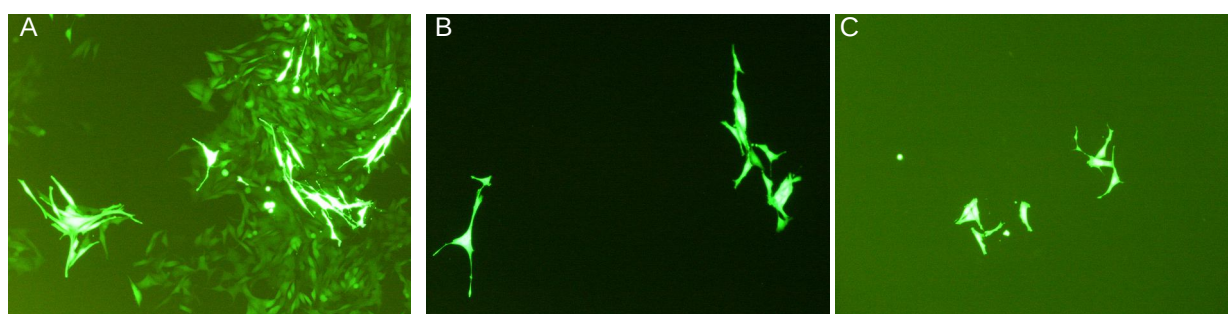


Figure 4.38: KAKA TetR-2 TRE-EGFP “positive clones (A-C), in medium without doxocyclin, after 3 weeks. Plates were about 90% confluent. Only a subpopulation of around 1-3% were fluorescent. When doxocyclin was added, EGFP expression was silenced. Only in clone A background cells were also fluorescent, but were only weakly expressing EGFP and not inducible, thus could not be silenced by adding doxocyclin. Cells of clone B were expanded and subsequently sorted (FACS), twice.

Finally, we decided to sort out the fluorescent subpopulation by FACS. However, this might be even more difficult for KAKA TetR-2 TRE-dnFGFR cells, because one has to turn on expression of putative cell toxic dnFGFRs. Besides, only dnFGFR1 and dnFGFR4 are coupled to fluorescent proteins, making FACS possible.

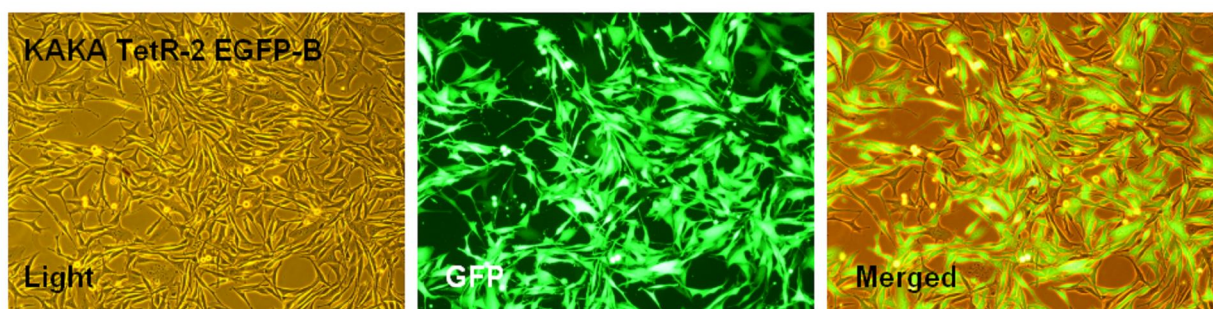


Figure 4.39: Expanded KAKA TetR-2 EGFP-B clone, after two rounds of sorting (FACS) and subsequent expansion.

Following this, the new established cell line KAKA TetR-2 EGFP-B (Figure 4.39) was functionally tested by addition (Figure 4.40 and 4.41) and removal (Figure 4.42 and 4.43) of doxocyclin. The culture was split into two wells, one was incubated with doxocyclin and the other one without.

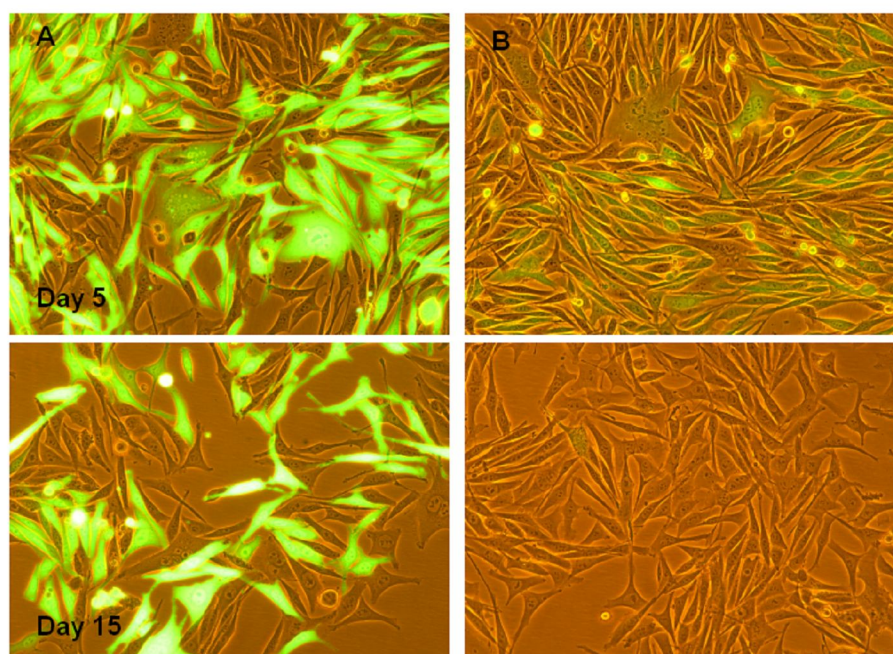


Figure 4.40: Expression of GFP was inhibited by addition of doxocyclin to cell culture medium. One cell fraction was incubated without doxocyclin (A), while the other fraction was incubated in a medium including doxocyclin (B).

Visible GFP expression was completely lost after around two weeks, monitored by microscopy. Additionally, GFP expression intensity was measured by FACS analysis, 24 days after addition of doxocyclin. Original cell line (KAKA) was used as control, and displayed no fluorescence (FL1-H value $< 10^1$). KAKA TetR-2 TRE-EGFP-B culture including doxocyclin (+DOX) had a small second population of cells showing low fluorescence intensity (FL1-H value $> 10^1$ but $< 10^2$), compared to control cell line.

This clearly shows the extent of leakiness. The KAKA TetR-2 TRE-EGFP-B culture incubated without doxocyclin (-DOX) can be classified into three cell populations, namely a small, non-emitting population, a large population showing average fluorescence intensity, and another small population with strong fluorescence, even beyond the limit of detection (LoD). It is believed, that expression of the gene of interest, in this case GFP, was immediately shut down, when doxocyclin was added. However, because of long persistence of the GFP protein, the fluorescence was visible for a longer period (~2 weeks).

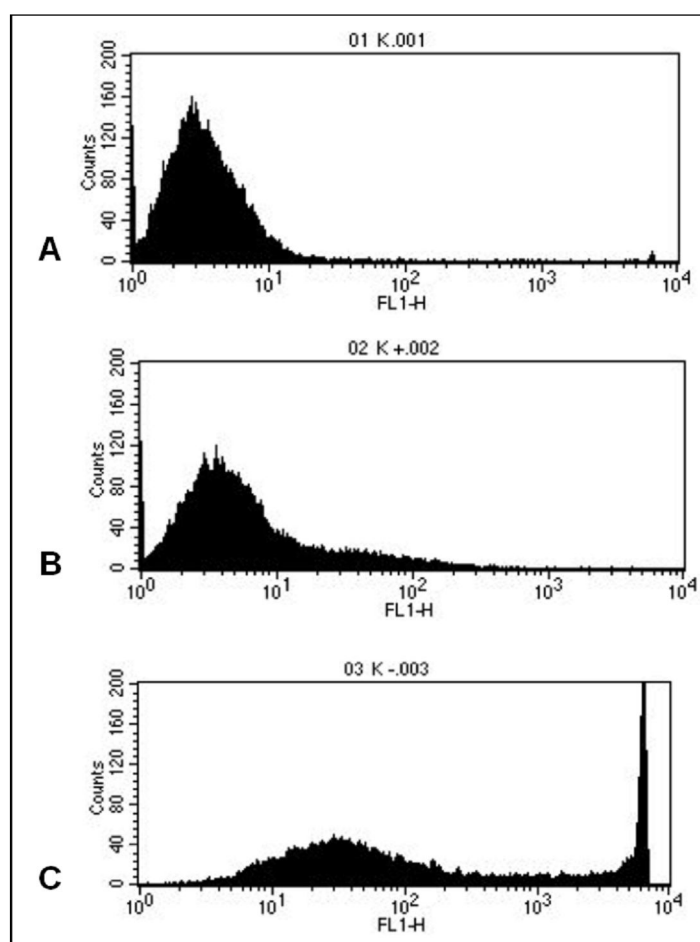


Figure 4.41: Results of FACS analysis, after 24 days. (A) KAKA background melanoma cell line, (B) KAKA TetR-2 TRE-EGFP-B cells incubated with doxocyclin, and (C) KAKA TetR-2 TRE-EGFP-B cell fraction incubated without doxocyclin.

After GFP expression was shut down, it was turned on again by withdrawing doxocyclin. Cells were washed before doxocyclin free medium was added. Re-activation of gene expression was monitored by microscopy, and FACS analysis at the end of the experiment. At around one week after doxocyclin deprivation, GFP expression reached visible maximum again.

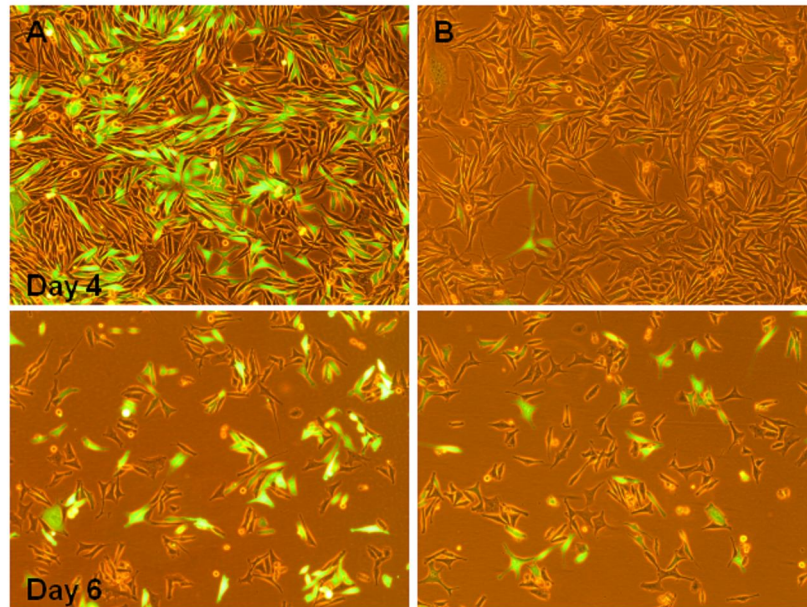


Figure 4.42: Activation of GFP expression monitored by microscopy. One cell fraction (A) was always incubated without doxocyclin, while in the other fraction (B), doxocyclin was withdrawn at day 0, thus GFP expression was activated.

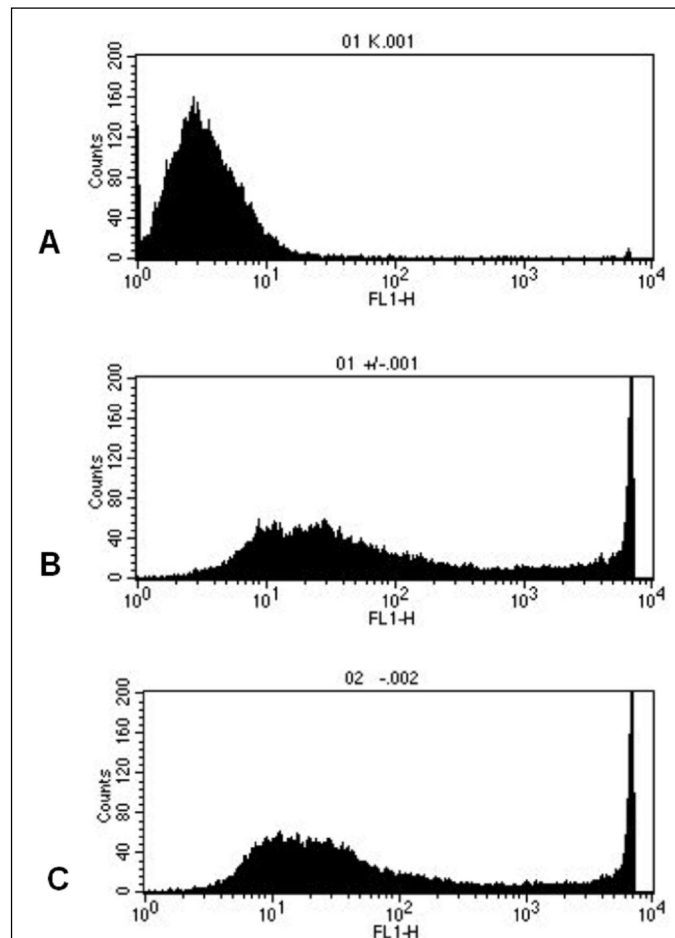


Figure 4.43: FACS analysis verified re-activation of GFP expression, done two weeks after doxocyclin deprivation. (A) KAKA background melanoma cell line, (B) KAKA TetR-2 TRE-EGFP-B cells doxocyclin deprived, and (C) KAKA TetR-2 TRE-EGFP-B cell fraction incubated always without doxocyclin serving as reference.

5 Discussion

5.1 Expression Analysis of FGFs and FGF-Receptors

The fibroblast growth factor (FGF) signalling network plays a ubiquitous role in normal cell growth, survival, differentiation, and angiogenesis, but has also been implicated in tumour development. Recent studies have shown that FGF can act synergistically with vascular endothelial growth factor (VEGF) to amplify tumour angiogenesis. In addition, through inducing tumour cell survival, FGF has the potential to induce chemotherapy resistance highlighting that chemotherapy may be more effective when used in combination with FGF inhibitor therapy. Furthermore, FGFRs have variable activity in promoting angiogenesis, with the FGFR-1 subgroup being associated with tumour progression and the FGFR-2 subgroup being associated with either early tumour development or decreased tumour progression (Korc & Friesel, 2009).

Considering the status of research on FGF and FGFR expression in cancer, over-expression of FGFR-1 by gene amplification frequently occurs in breast cancer (Jacquemier, Adelaide et al., 1994), while over-expression through translocation was reported for FGFR-3 in multiple myeloma (Chesi, Nardini et al., 1997). An activating point mutation of FGFR-3 is frequently found in bladder and cervix carcinoma (Cappellen, De Oliveira et al., 1999), whereas a constitutively activated form of FGFR-4 was found in pituitary tumours (Ezzat, Zheng et al., 2002). Recently, glioblastoma and NSCLC have been added to the list of malignancies containing mutations in the FGFR molecules (Rand, Huang et al., 2005; Zhao, Liu et al., 2005). Apart from changes in receptor expression and function, over-expression of FGF ligands has been reported for a great variety of human tumours. Elevated expression of FGF-2 was found in body fluids of patients with cancers of colon, liver, bladder, head and neck, skin, lung, kidney, brain and soft tissue (Brunner, Metz et al., 1994; Nguyen, Grossi et al., 1994; Nguyen, Steinberg et al., 1994; Nguyen, Watanabe et al., 1994). But also FGF-3, FGF-4, and FGF-8, which are normally not expressed to high levels in adult tissues, have been found over-expressed in a number of human neoplasms, for instance in Kaposi's sarcoma, and in carcinomas of the breast, prostate, ovary and esophagus (Kiuru-Kuhlefelt, Sarlomo-Rikala et al., 2000; Valve, Nevalainen et al., 2001; Wagata, Ishizaki et al., 1991; Zammit, Coope et al., 2002).

In melanoma, several reports have established an oncogenic role of FGF-2 and FGFR-1 (Becker, Lee et al., 1992; Nesbit, Nesbit et al., 1999; Ozen, Medrano et al., 2004) and a correlation between FGFR-4 protein expression and survival of melanoma patients has been described (Streit, Mestel et al., 2006; Wang & Becker, 1997). It is known that normal melanocytes do not express FGF-2 (Reed, McNutt et al., 1994; Scott, Stoler et al., 1991), while all melanomas produce FGF-2 and expression levels seem to increase during melanoma progression (al-Alousi, Barnhill et al., 1996; al-Alousi, Carlson et al., 1996; LaVail, Faktorovich et al., 1991). An autocrine growth stimulatory role for FGF-2 in melanoma has been clearly established through inhibition of FGF-2 activity by intracellular injection of blocking antibodies (Halaban, Kwon et al., 1988; Halaban, Langdon et al., 1988a; Halaban, Langdon et al., 1988b) or by suppression of synthesis with antisense oligodeoxynucleotides (Becker, Meier et al., 1989).

Inhibition of expression of the FGFR-1, inhibits proliferation in vitro (Becker, Lee et al., 1992) and in vivo (Wang & Becker, 1997). The presence of dominant negative receptors (Yayon, Ma et al., 1997) and the use of aromatic anionic compounds such as Suramin, that sequester FGF-2 (Davol, Beitz et al., 1995), have further delineated the autocrine role of FGF-2 in melanoma progression. FGF-2 is mitogenic not only for melanocytes, but also for fibroblasts and endothelial cells. Thus, it is known that melanoma-derived FGF-2 has potential paracrine functions in angiogenesis and stromal formation (Nesbit, Nesbit et al., 1999).

In our study we showed, that FGFR-1 and FGFR-4 are highly expressed in nearly all melanoma cell lines, while expression levels of FGFR-2 and FGFR-3 were found to be significantly lower, thus seem to have less importance in melanoma development. Taking non-transformed melanocytes as reference, the expression of FGFR-4 and FGFR-2 seems to be up-regulated in nearly all melanoma cell lines, while expression of FGFR-1 is maintained on a high level and FGFR-3 stays low. Due to the fact that those primary melanocytes used in this study as reference are of embryonic origin, and because of the known importance of FGFRs during embryonic development, high FGFR-1 expression levels in embryonic melanocytes does not ensure high expression in adult “non-transformed” melanocytes. In general, expression levels of all FGFRs and isoforms were heterogeneous. Truncated FGFR isoforms, especially those lacking the TM-domain, were not detected in around half of melanoma cell lines and are suggested to be expressed on a low level.

It was shown recently, that around 10% of melanoma tumours harbour mutations in the FGFR-2 gene. These novel mutations include three truncating mutations occurring at evolutionary conserved residues. Mutations result in receptor loss of function through several distinct mechanisms, including loss of ligand binding affinity, impaired receptor dimerization, destabilization of the extracellular domains, and reduced kinase activity. This study suggests FGFR-2 to even oppose melanoma development and correlates with our findings, namely its low-level expression in melanoma cells. Therefore, FGFR-2 was not subjected to further investigations considering tumour promoting effects. Thus, experiments investigating cancer promoting effects of FGFRs focused on FGFR-1 and FGFR-4, because of significantly higher expression levels. Expression of FGF ligands showed that FGF-2 was found to be highly up-regulated in all melanoma cell lines (100%) investigated and FGF-5 was up-regulated in the majority (90%). Expression of FGF-18 was found to be up-regulated in 40%, while expression of FGF-17 and FGF-8 stayed low in all melanoma cell lines. Similar to FGF-Receptors, ligands were also expressed in a heterogeneous manner considering investigated cell lines.

Results showing high FGF-2 expression levels in all melanoma cell lines, agree with other studies published in recent years. Its importance in tumour angiogenesis, beside the more popular VEGF, might also apply for melanoma. Targeting both the FGF and VEGF pathways may be more efficient in suppressing melanoma angiogenesis, than targeting VEGF alone. Besides, around 90% of melanoma cell lines investigated showed FGF-5 up-regulation and about 40% FGF-18 up-regulation. In case of FGF-18, expression was also high in primary melanocytes, in contrast to very low FGF-5 expression. Oncogenic roles of both factors have been described in other tumour types already, namely in glioblastoma and colorectal tumours (Allerstorfer, Sonvilla et al., 2008) and may also have an important role in melanoma development. FGF-5 was originally identified by a screening approach for transforming oncogenes (Zhan, Bates et al., 1988) and subsequently characterized as a regulator of the hair growth cycle (Hebert, Rosenquist et al., 1994; Taniguchi, Harada et al., 2003). Moreover, FGF-5 affects astroglial properties in vitro (Reuss, Hertel et al., 2000), and shows neurotrophic activity in vivo (Ozawa, Suzuki et al., 1998). While murine FGF-5 is widely expressed during embryogenesis, it is suggested to be restricted to the central nervous system in adult mice (Goldfarb, Bates et al., 1991; Haub, Drucker et al., 1990; Haub & Goldfarb, 1991). Considering its oncogenic role, it was shown recently, that apoptosis induction of glioblastoma (GBM) cell lines by prolonged serum starvation was significantly prevented by FGF-5.

siRNA-mediated FGF-5 down-modulation reduced GBM cell proliferation, while recombinant FGF-5 increased this parameter preferentially in cell lines with low endogenous expression levels. Moreover, tumor cell migration was distinctly stimulated by FGF-5 but attenuated by FGF-5 siRNA (Allerstorfer, Sonvilla et al., 2008). Due to its known importance in the central nervous system and the neuroectodermal origin of melanocytes, the oncogenic role of FGF-5 in melanoma might be expected. Because of the high expression levels in melanoma, FGF-5 was also tested for its ability to stimulate melanoma cell migration. Migration assays showed, that FGF-5 reached the same stimulatory effects on migration like FGF-2, namely up to a 50% increase. Considering FGF-18, its expression was shown to be up-regulated in 34/38 colorectal tumours and is progressively enhanced during colon carcinogenesis reaching very high levels in carcinoma. Moreover, it is suggested that FGF-18 affects both tumour cells and tumor microenvironment in a pro-tumourigenic and pro-metastatic way (Sonvilla, Allerstorfer et al., 2008). Our expression data suggests the probability of a similar role of FGF-18 in a melanoma subpopulation.

5.2 Inhibition of FGFR Signalling by Dominant Negative Receptor Constructs

The purpose of this study was to evaluate FGF-Receptors as putative therapy targets in melanoma. Experiments were done in vitro as well as in vivo by prior adenoviral transduction of melanoma cell lines, thereby generating transient dominant-negative FGF-Receptor expression. Adenoviruses contained dominant-negative receptor constructs under a constitutively active promoter region. Transient, but extensive, expression of non-functional FGFRs was suggested to down regulate FGFR mediated signalling via dimerization with the endogenous receptors, thus act in a dominant negative fashion.

A difficult part was to find appropriate MOIs (Multiplicity Of Infection) for the transduction procedure. The aim was to choose appropriate MOI values leading to equivalent episomal protein expression of all adenoviral constructs. Investigations showed that MOIs up to 5 had no effect on cell proliferation, but an MOI of 30 reduced proliferation around 15%.

However, the differences in episomal protein expression between the diverse adenoviral constructs were enormous, when equal MOIs were used. Therefore, we decided to raise MOIs for dnFGFR1-GFP (MOI: 5), kdFGFR3 (MOI: 30), dnFGFR4-CFP (MOI:30) compared to our GFP control (MOI: 2). Reduced proliferation, caused by high MOI values, of kdFGFR3 and dnFGFR4-CFP infected cells had to be considered afterwards. Protein expression levels of dnFGFR constructs were still significantly lower than those of GFP control. The highest gap was found between kdFGFR3, dnFGFR4-CFP and GFP control. Expression of dnFGFR4-CFP was visibly lower when monitored by fluorescence microscopy. Considering analysis by immune-blotting, protein expression of kdFGFR3 (data not shown) and dnFGFR4-CFP was not detected with similar exposure time, used for GFP and dnFGFR1-GFP. Expression of dnFGFR1-GFP was also found to be reduced, compared to GFP infected cells, but reduction was not as strong as for kdFGFR3 and dnFGFR4-CFP expression.

Results of the proliferation or viability assay clearly showed the impact of the FGFR-1 mediated FGF-signalling pathway on cell proliferation and apoptosis. Although the dnFGFR1-GFP protein was shown to be less expressed, it leads to a decrease of cell proliferation of around 50%, compared to the GFP control. What's more, inhibition of the FGFR-4 and FGFR-3 mediated pathway, also decreased cell proliferation around 20-30%, although fluorescence of the dnFGFR4-CFP protein was barely visible and both proteins were not detected by Western blot analysis using similar exposure time, thus are suggested to be expressed in low amounts. However, cells transfected with kdFGFR3 and dnFGFR4-CFP viral constructs were transduced at MOIs of 30. This was shown to be anti-proliferative already by the GFP transfected, thus reduced proliferation around 15% by the virus itself. Therefore, it is suggested that anti-proliferative effects of kdFGFR3 and dnFGFR4-GFP transfected cells are much lower than 20-30%. MOIs of 5 did not affect cell proliferation when transfected with GFP viruses, thus around 50% reduction in proliferation is indeed caused solely by the dnFGFR1-GFP protein and its down-regulation of the FGFR-1 signalling pathway.

That all four cell lines investigated showed nearly the same reduction in proliferation, might seem surprising. Expression of dnFGFRs was suggested to be cell line specific. What could explain the high correlation between those cell lines is that inhibition of the FGF-signalling cascade becomes saturated, thus fully blocked, at lower expression levels already.

Alternatively, one could argue, that decreased proliferation is caused solely by the effects of viral infection but not by expression of dnFGFR1. However, virus caused cell toxic effects were tested up to MOIs of 60 by transfection with GFP control viruses. As already discussed no cell toxic effects were visible at MOI: 5 suggesting that anti-proliferative effects are solely caused by the expression of dnFGFR1 indeed.

Furthermore, it was shown that inhibition of the FGFR-1 pathway in melanoma cells leads to apoptosis. This was shown either by propidium-iodide staining of dead cell subpopulations, by fluorescence microscopy as well as by immune-blotting. Caspase-3 and PARP1 were both cleaved in dnFGFR1-GFP-infected cells. However, it remains an open question whether anti-proliferative effects are mainly induced by apoptosis, or also by inhibition of growth stimulatory effects. Thus, cell number can be reduced in two ways, either by inducing apoptosis or by decreasing cell division.

Considering clonogenic assays, melanoma cells were tested for their ability to survive and form clones, when seeded at very low density. Reduction in clonogenicity was found to be approximately 30-50%, when melanoma cells were infected with dnFGFR1-GFP viruses compared to cells transduced with GFP adenoviruses. Because of significant reduction of surrounding cells, thus able to stimulate growth and proliferation, it is suggested that the FGFR-1 signalling pathway affects also cell-cell contact-independent growth and the ability to form clones.

Following this, a similar assay was performed seeding cells in a three-dimensional environment, where cells cannot attach to a surface thereby testing anchorage independent clonogenicity. This soft agar assay is suggested to resemble some aspects of in vivo growth conditions. The assay revealed, that reduction of clonogenicity was even strengthened, when cells were forced to grow in an anchorage independent way. To verify those results in vivo, tumour growth was tested by a xenotransplantation experiment in SCID mice. Melanoma cells were transduced with adenoviruses containing dnFGFR or GFP constructs and were then injected subcutaneously. Results highly correlated with proliferation and clonogenic assays, reducing tumour growth to around 50%. Besides, survival of SCID mice was also shown to be enhanced, when xenotransplanted melanoma cells were infected with dnFGFR1-GFP instead of GFP control viruses. However, tumour growth was not affected when cells were infected with kdFGFR3 or dnFGFR4-CFP viruses.

5.3 Cell Biologic Effects of FGF Stimulation

Two fibroblast growth factors, namely FGF-2 and FGF-5 were tested for oncogenic effects on melanoma cell lines. The better investigated factor FGF-2 was tested for its ability to stimulate proliferation and migration of melanoma cells, while FGF-5 was tested only for influencing migration and invasion. Both factors, investigated in this study, were shown to be highly up-regulated in all or most melanoma cell lines.

FGF-2 is a ubiquitous cellular mitogen that is not readily secreted due to the lack of a signal peptide sequence (Florkiewicz & Sommer, 1989). Expression of FGF-2 was shown to be up-regulated in many human tumours including melanoma. Whereas normal human melanocytes do not express FGF-2 (Reed, McNutt et al., 1994; Scott, Stoler et al., 1991), some nevi express it (Ahmed, Ueda et al., 1997; Mancianti, Gyorfi et al., 1993; Ueda, Funasaka et al., 1994) and virtually all melanomas produce FGF-2, with expression levels increasing during tumour progression (al-Alousi, Barnhill et al., 1996; al-Alousi, Carlson et al., 1996; Albino, Davis et al., 1991). An autocrine growth stimulatory role for FGF-2 in melanoma has been clearly established through inhibition of FGF-2 activity by intracellular injection of blocking antibodies (Halaban, Kwon et al., 1988) or by suppression of synthesis with antisense oligodeoxynucleotides (Becker, Meier et al., 1989), FGF-2 is mitogenic not only for melanocytes but also for fibroblasts and endothelial cells. Thus, melanoma derived FGF-2 has potential paracrine functions in angiogenesis and stroma formation (Nesbit, Nesbit et al., 1999). Transient expression of FGF-2 in melanocytes, achieved by adenoviral transduction and vector based transfection, showed increased proliferation and survival, when injected into human skin grafted to mice (Nesbit, Nesbit et al., 1999).

Considering our study, it was shown, that FGF-2 expression was indeed highly up-regulated in all melanoma cell lines investigated. More than half of these cell lines, namely six out of eleven, showed 100 x increased expression levels compared to primary melanocytes. Proliferation was shown to be stimulated up to around 20% in cell lines known to have high FGF-2 expression levels, and even up to 40% in cell lines with low endogenous expression. Stimulatory effects on migration ranged from around 20% to 50%, also depending on endogenous expression levels. Expression analysis showed FGF-5 up-regulation in around 90% of investigated melanoma cell lines, indicating its importance in melanoma.

In our study, FGF-5 reached the same stimulatory effects on migration like FGF-2, namely up to a 50% increase. These results highly correlate with a recent study, that investigated FGF-5 influence on migration of glioblastoma cell lines (Allerstorfer, Sonvilla et al., 2008).

5.4 Effects of FGF Stimulation and Small Molecule Inhibition on the FGFR Downstream Signalling

Several new targeted drugs against growth factors and/or their receptors have been developed successfully during the last years. The anti-angiogenic vascular growth factor (VEGF) antibody bevacizumab and the epidermal growth factor receptor (EGFR) small-molecule inhibitor erlotinib, have both been approved for different cancer types. Patients with the BCR-ABL translocation can be treated with imatinib-mesylate and those with amplification of the oncogene ERBB2, also known as HER2, with trastuzumab or lapatinib. New technologies give deeper insights in changes that occur within individual tumours and how these changes differ between individual tumours. With assays to detect these aberrations that are now used to stratify patients for treatment, these data sets are now transforming the practice of cancer medicine, as is shown by the success of therapies that target distinct molecular events (Chin & Gray, 2008).

However, many of these targeted therapy approaches, showed activity in patient subgroups only, suggesting that cancer cells can evade anticancer effects by activating alternative growth and survival pathways. Evidence has accumulated that members of the FGF family together with their four FGFRs might be a further valuable target in cancer therapy (Fischer, Taylor et al., 2008). The objective of this study was, to show that FGFR downstream signalling can be stimulated by FGFs, thus to find downstream activators in melanoma, and that the FGFR signalling pathway can be down-regulated by addition of small-molecule inhibitors targeting FGFRs specifically. Probably because of the fact, that all melanoma cell lines investigated showed up-regulation of FGF-2, additional stimulation of the pathway by addition of exogenous FGF-2 was low. However, the cell line with lower up-regulation of endogenous FGF-2 clearly showed increased phosphorylation of Akt, ERK1/2 and S6. It was also shown, that FGF-2 stimulation of FGFRs can be prevented by prior incubation with small-molecule inhibitor PD166866. In general, all these effects were lower than initially expected, but may be explained by high autocrine stimulation of FGF-2.

Inhibition of FGFR downstream signalling, by incubation with small-molecule inhibitors, was not shown. It seemed, that those FGFR inhibitors used, even stimulated FGFR downstream signalling instead of inhibiting. In vitro proliferation assays, not shown in this thesis, showed that cells incubated with PD166866 and SU5402 displayed reduced growth, and incubation with other clinical approved inhibitors, targeting other key players, showed additive and in some cases synergistic effects. FGFR downstream regulators, affected by PD166866 and SU5402 inhibition, need to be identified. Other putative signalling pathways, that are known to be activated by FGFRs, include PLC γ , PKC, Src and Shc pathways.

5.5 Cell Biologic Significance of the FGFR-4 Arg/Gly Polymorphism

In the human FGFR-4 gene, a coding polymorphism in exon 9 results in an amino acid change, namely from glycine to arginine (Gly388Arg), in the transmembrane domain of the receptor. The FGFR-4 Arg388 allele may predispose cancer patients to disease progression, based on the reported significant association between FGFR-4 genotype and tumour aggressiveness or survival in different cancer types (Bange, Prechtel et al., 2002; Falvella, Frullanti et al., 2009; Ma, Tsuchiya et al., 2008; Morimoto, Ozaki et al., 2003; Wang, Stockton et al., 2004; Wang, Yu et al., 2008). However, this association was not confirmed in all studies (Becker, Nieters et al., 2003; Jezequel, Campion et al., 2004). Considering prostate and lung cancer development and progression, recent studies seem to agree on the oncogenic role of the FGFR-4 Arg388 allele (Falvella, Frullanti et al., 2009; Ma, Tsuchiya et al., 2008; Wang, Yu et al., 2008), but not so in other tissue types.

Another study investigating the effects of the FGFR-4 Arg388 allele suggests an association with resistance to adjuvant therapy in primary breast cancer. In node-positive patients, FGFR-4 Arg388 was significantly associated with poor disease-free survival and overall survival and seems to be attributable to relatively poor therapy response. Patients carrying the Arg388 allele showed only about half as much benefit from adjuvant systemic therapy as wild type carriers (Thussbas, Nahrig et al., 2006). Considering the status of research in melanoma, the FGFR-4 Arg388 allele correlated with tumour thickness. However, only FGFR-4 protein expression correlated with survival of patients (Streit, Mestel et al., 2006).

Our results suggest, that the Arg388 allele has a significant promoting influence on melanoma progression, which is achieved not by stimulating growth, but by stimulating migration and invasion. Increased migration ability would also explain high prevalence of the allele in metastatic samples and samples of malignant effusions, indicating late clinical stage. Because of improvements in early tumour diagnostics and melanoma resection, it was not possible to analyse more samples of late stage malignant effusions. These findings also correlate with a study (Bange, Prechtel et al., 2002), which investigated migratory effects of the Arg388 allele on several mammary carcinoma cell lines. It was shown there, that mammary tumour cells expressing FGFR-4 Arg388 exhibited increased motility relative to cells expressing the FGFR-4 Gly388 isotype. What's more, the Arg388 allele was associated with early lymph node metastasis and advanced tumour-node-metastasis of colon cancer patients and a significantly reduced disease-free survival time in breast cancer patients.

5.6 The Advanced Tet-Off System in Melanoma Cell Lines

Two melanoma cell lines, showing high lipofection efficiency (>30%), were chosen for the generation of inducible dnFGFR expression. This was done by using the Advanced Tet-Off System (Clontech), described by the company as reliable and tight, and was already briefly explained in chapter 3 (Results). It was shown however, that the system is still leaky, at least as far as melanoma cell lines are concerned. Generation of stably TetR expressing cell lines was achieved, but subsequent transfection with TRE-dnFGFR plasmids yielded no clones, apart from EGFP controls. It was shown, by using FACS analysis, that the system still expressed EGFP when actually silenced by doxocyclin. However, the fact that no TetR TRE-dnFGFR clones were obtained, further strengthens the acceptance of FGFRs as being cell toxic. This observation could be made also, when melanoma cell lines were transfected with plasmids expressing dnFGFR constructs from constitutively active promoters. Stable GFP controls were obtained from several cell lines, but not a single clone expressing dnFGFRs.

The system itself is actually very expedient, especially to avoid the MOI discussion when cells are infected by adenoviruses to allow transient expression of the gene of interest. However, the leakiness of the system makes it only useful for genes less cell toxic or those stimulating cell growth and viability instead. For instance, it would be reasonable to further investigate the FGFR-4 Arg/Gly388 polymorphism, suggested to stimulate growth and migration.

6 Appendix

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6.3 Abbreviations

APS - Ammonium Persulfate
BSA - Bovine Serum Albumin
CFP - Cyan Fluorescent Protein
DEPC - Diethylpyrocarbonate
DMSO - Dimethylsulfoxid
DOX - Doxocyclin
DTT - Dithiothreitol
ECM - Extra Cellular Matrix
EDTA - Ethylendiaminetetraacetic Acid
EGF - Epithelial Growth Factor
EGFR - Epithelial Growth Factor Receptors
ERK - Extracellular Signalregulated Kinase
EtOH - Ethanol
FBS - Fetal Bovine Serum
FGF - Fibroblast Growth Factor
FGF-BP - FGF Binding Protein
FGFR - Fibroblast Growth Factor Receptors
FRS2 - Fibroblast Growth Factor Receptor Substrate 2
GFP/EGFP - Green Fluorescent Protein/Enhanced Green Fluorescent Protein
GOI - Gene Of Interest
HBS - Heparin Binding Site
HLGAG - Heparin-like Glycosaminoglycan
HRP - Horseradish Peroxidase
ICH - Immunohistochemistry
IRES - Internal Ribosomal Entry Site
MAPK - Mitogen Activated Protein Kinase
MeOH - Methanol
MOI - Multiplicity of Infection
o/n - Overnight
P/S - Penicillin/Streptomycin
PAGE - Polyacrylamide Gelelectrophoresis
PBS - Phosphate Buffered Saline
PFA - Paraformaldehyde
PVDF - Polyvinylidene fluoride
Rb - Retinoblastoma
ROS - Reactive Oxygen Species
RPMI - Roswell Park Memorial Institute
RT - Room Temperature
SCID - Severe Combined Immunodeficiency
SDS - Sodium Dodecyl Sulfate
STET - Sodiumchloride Tris EDTA Triton X-100 Buffer Solution
T/E - Trypsin/EDTA
TBE - Tris/Borate/EDTA
TBS - Tris Buffered Saline

TE - Tris EDTA
TEMED - N,N,N',N'-Tetramethylethylenediamine
Tris - Trishydroxymethyl Aminomethane

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6.5 Curriculum Vitae

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Austrian Citizen

Date of Birth: 04.07.1984



EDUCATION

Since 10/2003

University of Vienna, *Molecular Biology*

- Specialization: Molecular Medicine, Immunology/Microbiology and Genetics
- Additional Education: ***Business in Life Sciences*** (Processes and Methods in the Life Science Industry; Business in Biotech; Presentation Techniques)

**05/2003-10/2002
2002-1998**

Military Service

Austrian Grammar School

“Militärrealgymnasium - MilRG” in Wiener Neustadt, graduated with top grades (“Guter Erfolg”)

**1998-1994
1994-1990**

Secondary School in Wiener Neustadt

Primary School in Aspang

UNIVERSITY EDUCATION (Summary)

Molecular Medicine Principles of Virology Molecular Pathology Molecular Medicine II Project Planning in Molecular Medicine Specific Hormonal Aspects of Women Molecular Medicine Seminars Presentation Techniques in Science Microbiology and Immunology Introduction to Microbiology Immunology and Cellular Microbiology A+B Applied Microbiology Immunology of Diseases of the CNS Infections and Immune-Defence of Hosts Molecular Microbiology A+B	Cellbiology Anatomy of Animals Introduction to Molecular Cellbiology Developmental Biology Cellbiology of Animal Cells Methods in Cellbiology Quality Control of Proteins Cell-Type Specific Features of the CNS Advanced Neuronal Cellbiology Neurobiology Seminars in Cellbiology Anthropology in Molecular Biology Structure and Function of Plants Challenges in Molecular Biology Business in Life Sciences Business in Biotech Processes and Methods in Life Science Industry Presentation Techniques	Biochemistry Chemistry of Physics I+II Chemistry of Analytics I+II+III Chemistry of Organics A+B Biochemistry in Molecular Biology Scientific Theories I+II Mathematics and Statistics in Molecular Biology Structural Biology I+II Genetics Introduction to Genetics Cell- and Developmental Genetics Project Planning in Genetics Molecular Biotechnology Gene Expression Molecular Genetics A+B RNA Profiling
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WORK / RESEARCH EXPERIENCE

Since 11/2008 **Medical University of Vienna,
Department of Medicine I; Institute of Cancer Research
(Group: Michael Grusch and Walter Berger)**

Projects: *Evaluation of fibroblast growth factor receptors as therapeutic targets in melanoma;
Investigating the role of activins and follistatins in liver physiology and cancer*

Methods: Expression Analysis; MTT-Assays (Small Molecule Inhibitors); Soft Agar Assays; Xenotransplantation Experiments in SCID Mice; Adenoviral Transduction Experiments (dnFGFR constructs); Transwell-Chamber Migration Assays; Angiogenesis (Tube Formation) Assays; Advanced Tet-Off System; RFLP Analysis; FACS Analysis; Immunohistochemistry

02/2008-01/2008 **Medical University of Vienna,
Department of Medicine I; Institute of Cancer Research
(Group: Brigitte Marian)**

Project: *Investigating the role of peroxisome-proliferator-activated receptors in carcinogenesis and their potential as therapeutic targets*

Methods: RNA-Interference; “Real Time” qRT-PCR; standard RT-PCR; Western Blotting; Luciferase/GFP Reporter Assays; Clonogenic Assays; Electroporation; Lipofection; Adenoviral Transduction

11/2007-10/2007 **Max F. Perutz Laboratories,
Department of Medical Biochemistry
(Group: Karl Kuchler)**

Project: *Identification of therapeutic targets of the opportunistic human pathogen *Candida glabrata**

Methods: Site-Specific Homologue Recombination (Generation of “Knock-Out”-strains); Yeast Colony PCR; Chitosan and Zymolyase Sensitivity Assays; Growth Inhibition Assays; Southern-Blotting; Chemiluminescent Detection of Reactive Oxygen Species (Macrophage Interaction); Light Microscopy

08/2003-05/2003 **Reckitt Benckiser Austria GmbH
Quality Control** (Chemical Analysis; Optical and Mechanical Inspection; Sampling Point)

02/2006-10/2004 **Intersport Austria GmbH
Sales** (Consulting for bicycle and skis)

2008-2003 **Part time work to help finance studies**
- **Barkeeper**, Wiener Neustadt
- **Promotion** for Young Enterprises Media GmbH
- **Public pool attendant**, Aspang
(Supervision; Chlorine- and pH-value determination)

PUBLICATIONS and PRESENTATIONS

- Kreidl E, Öztürk D, **Metzner T**, Berger W, Grusch M. **Activins and Follistatins: Emerging Roles in Liver Physiology and Cancer**. World Journal of Hepatology. 2009, Oct. 15; 1(1): 17-27.
- **Metzner T**, Held G, Peter-Vörösmarty B, Spiegl-Kreinecker S, Micksche M, Berger W, Grusch M. **Inhibition of Fibroblast Growth Factor Receptor-mediated Signals Impairs Malignant Growth and Enhances Chemosensitivity of Human Melanoma Cells**. In Preparation.
- **Metzner T**, Berger W, Micksche M, Grusch M. **FGF-Rezeptor Antagonisten: Eine neue Therapie-Chance beim Melanom?** Krebs-Hilfe Journal. Heft 3:2009. Seite 34-35.
- Grusch M, Petz M, **Metzner T**, Öztürk D, Schneller D, Mikulits W. **The Crosstalk of Ras with the TGF- β Family during Carcinoma Progression and its Implication for Targeted Cancer Therapy**. Invited Review submitted to Current Cancer Drug Targets.
- **7th World Congress on Melanoma and 5th Congress of the European Association of Dermato-Oncology**, May 12-16, 2009; Poster-Presentation; Evaluation of Fibroblast Growth Factor Receptors as Therapeutic Targets in Melanoma (topic: Targeted Therapies, page: 58, 388)
- **“Academic Medicine-Does It Pay Off?”FWF Discussion**, June 17, 2009; Poster-Presentation; Targeted Inhibition of Fibroblast Growth Factor Receptors (FGFRs) – A New Treatment for Melanoma?

SPECIAL SKILLS

Foreign Languages	English	-	fluently spoken and written
	Spanish	-	final school examination level
Computer Skills	MS Office, GraphPad Prism, Clone Manager, Adobe Photoshop, Image Quant, ImageJ		
Driving Licence	A, B		

INTERESTS

- Travelling, foreign countries and cultures
- Sports
(Triathlon, *European Cups 2006-2008*;
Modern Pentathlon, *participation at Youth European- and World Championships 1996-2001*)